Development of a polychromatic flow cytometry panel for the evaluation of HIV-specific T cell responses

by

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LIST OF ABBREVIATIONS

AIDS	Acquired Immune Deficiency Syndrome
ARVs	Antiretroviral drugs
APC	Antigen presenting cells
APC	Allophycocyanin
APC-H7	Allophycocyanin-H7
CCR5	Chemokine receptor 5
CCR7	Chemokine receptor 7
CD	Cluster of differentiation
CFSE	Carboxyfluorescein succinimidyl ester
CMV	Cytomegalovirus
CTL	Cytotoxic T lymphocyte
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
EBV	Epstein Barr virus
ELISA	Enzyme-linked immunosorbent assay
ELISPOT	Enzyme-linked immunospot
FBS	Foetal Bovine Serum
FITC	Fluorescein isothyocyanate
FMO	Fluorescent Minus One
FSC	Forward scatter
FSC-H	Forward scatter-height
Gag	Group specific antigen
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HAART	Highly active antiretroviral treatment
HIV-1	Human Immunodeficiency type 1
HLA	Human leukocyte antigen
h	hour
HSV	Herpes simplex virus
ICS	Intracellular cytokine staining

IFN-γ	Interferon gamma
IL	Interleukin
LCMV	Lymphocytic Choriomeningitis Virus
LTNP	Long-term nonprogressor
MHC	Major Histocomaptibility Complex
MFI	Median fluorescent intensity
min	minutes
MIP-1β	Macrophage inflammatory protein 1 beta
Nef	Negative factor
NICD	National Institute for Communicable Diseases
PE	Phycoerythrin
PerCP-Cy5.5	Peridininchlorophyll protein-Cy5.5
PBMCs	Peripheral Blood Mononuclear Cells
PBS	Phosphate buffer saline
PD-1	Programmed death-1
PMA	Phorbol myristate acetate
PMT	Photomultiplier tube
Qdot	Quantum dot
RT	Room temperature
SATVI	South African Tuberculosis Vaccine Initiative
SEB	Stapylococcal enterotoxin B
SFU	Spot forming units
SHIV	Simian Human Immunodeficiency Virus
SIV	Simian Immunodeficiency Virus
SLC	Secondary lymphoid tissue chemokine
SSC	Side scatter
Tat	Transactivator of transcription
TCR	T cell receptor
TNF-α	Tumor necrosis factor alpha
Vivid	Violet-fluorescent reactive dye
Vpr	Viral protein R

WPBTS	Western province blood transfusion services
µl	Microlitre
^{0}C	Degrees Celsius

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ABSTRACT

Investigating T cell responses in HIV infection has revealed several correlates of viral control, but their importance is not fully understood. Further studies to understand the relationship between HIV and the immune system are warranted. The advent of polychromatic flow cytometry has allowed for in depth analysis of T cell functions and phenotypes in HIV infection, including the measurement of T cells that can produce multiple immune molecules simultaneously. The aim of this study was to develop a polychromatic flow cytometry panel to measure multiple functional markers, and optimise a stimulation and staining protocol for use in the laboratory. A flow cytometry panel measuring IFN-γ, IL-2, TNF-α and the degranulation marker CD107a was established. It was found that a stimulation time of 16 h and the use of both monensin and BFA protein transport inhibitors were required for optimal detection of the four functions. HIV-specific T cell responses were investigated in 20 chronically-infected individuals and 7 individuals with acute infection that were followed longitudinally over the first year of infection. HIV-specific $CD8^+$ T cell responses were detectable in chronic infection, with $CD8^+$ responses greater in magnitude and frequency than $CD4^+$ responses. HIV-specific $CD8^+$ T cells predominantly produced IFN-γ and degranulated, with a near complete absence of IL-2 and TNF-α production in chronic infection. In contrast, HIV-specific $CD4^+$ T cells showed a broader cytokine repertoire, producing IFN-γ, TNF-α and IL-2, but rarely degranulating. There were poor polyfunctional $CD8^+$ and $CD4^+$ T cell responses in chronic infection. In the acute cohort, a decline in polyfunctionality over the first year of infection was observed, consistent with observations from the chronic cohort. There was a greater magnitude of $CD4^+$ T cell responses in acute infection compared to chronic infection, and a greater magnitude of $CD8^+$ T cell responses in chronic infection compared to acute infection. In summary, it appears that HIV-specific T cell polyfunctional responses are lost early in infection, and this is most striking in the $CD4^+$ T cell compartment. These results emphasize the importance of acute and longitudinal HIV studies to understand better the relationship between HIV and the immune system, which will hopefully assist with the development of an effective HIV vaccine.

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INTRODUCTION

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INTRODUCTION

It has been more than 25 years since the human immunodeficiency virus (HIV) was discovered, yet there is still much that is unknown about the interactions between the virus and the human immune system. This interaction is important to understand for applying the knowledge to vaccine development, since vaccination may be the only hope for long-term control of the epidemic (as reviewed in Letvin, 2005). The unknown immunological factors involved in viral control impact both on effective vaccine development as well as vaccine evaluation.

The interaction between HIV and the immune system has been extensively studied in order to determine which immune features correlate with HIV control. There are a number of immunological techniques available and as technology advances, more techniques are becoming accessible to researchers, thereby increasing our knowledge about HIV and the immune system. This review will discuss some of the immunological techniques used to measure immune responses to HIV and features of cellular immunity that have been implicated in the control of HIV.

1.1 Techniques to measure T cell responses

A wide variety of immunological methods have been applied to measure T cell responses in HIV infection. These techniques are reviewed briefly in this section and both the advantages and limitations of the assays are considered.

The enzyme-linked immunospot (ELISPOT) assay has been widely applied to measure the frequencies of HIV-specific T cells (Edwards *et al*, 2002; Addo *et al*., 2003; Kaufmann *et al*., 2004, Masemola, *et al*., 2004, Zuniga *et al*., 2006; Geldmacher *et al*., 2007a, 2007b; Gray *et al*., 2008). It is a sensitive and robust technique for the quantification of IFN-γ and has a high throughput as many peptides, or pools of peptides, can be screened at once and specific reactive peptides can be identified. A disadvantage of the technique is that the phenotype of the responding

cell is unknown and the assay measures only a single function. Dual ELISPOTs that can measure IL-2 and IFN-γ simultaneously have been developed (Boulet *et al*., 2007), although these are not in common use.

Flow cytometry, similar to the ELISPOT assay, has been used to quantify HIV-specific T cells (Betts *et al*., 2001, Ramduth *et al*., 2005). The power of flow cytometry is the fact that specific T cell phenotypic subsets can be identified in addition to measuring cytokine production. With the advent of polychromatic flow cytometry, allowing more than four markers to be measured, up to 17 markers can be evaluated (Perfetto *et al*., 2004), depending on the configuration and lasers of the cytometer utilised to acquire samples. Polychromatic flow cytometry has been used to quantify combinations of cytokines expressed from single cells (Betts *et al*., 2006), as well as identifying complex differentiation phenotypes of T cells (Duvall *et al*., 2008). One limitation of flow cytometry is that a large number of cells are required to measure combinations of cytokines reliably.

Immune assays have been developed to measure cell functions such as proliferation and cytotoxicity (as reviewed by Hickling, 1998). The advantage of these techniques is that rather than measuring surrogates of immunity, such as IFN-γ or CD107a, they measure functions of cells directly. Traditional assays used radioactive materials, such as the ^{51}Cr-release assay, measuring cytotoxicity, and the ^{3}H incorporation assay, measuring proliferation. Proliferation can also be assessed by flow cytometry using fluorescent dyes such as carboxyfluorescein succinimidyl ester (CFSE) (Younes *et al*., 2003) and oregon green. The nuclear protein Ki67 has also been used as an indication of cell division (Starr *et al*., 2002).

For measuring cytotoxicity, several flow cytometry-based assays have been developed. Cytotoxic T lymphocytes (CTLs) are capable of mediating apoptosis of infected cells via cytotoxic molecules (as reviewed by Suni *et al*., 2005) and the Fas/Fas ligand-killing pathway (Bossi and Griffths 1999). These cytotoxic molecules can then be used as surrogate markers for the assessment of cytotoxicity. CTLs have lytic granules containing perforin and granzymes, which induce apoptosis (as reviewed in Krenksy and Clayberger, 2005). Besides these molecules, the lytic

granules also consist of granulysin, which is involved in lysis of cells. During cytolysis, the granule membrane fuses with the cell membrane and releases perforin and granzymes into target cells (Figure 1.1). Within the inner membrane of the granule are the proteins CD107 and CD63, and during the fusion process, CD107 is transiently expressed on the cell surface. CD107 is used as a marker for degranulation and hence an indirect measure of cytotoxic activity (Betts *et al*., 2003). The lysosomal membrane protein, CD63 (LAMP-3), is also expressed on the cell surface during the degranulation process (Peters *et al*., 1991). However, the expression of CD63 was either negative or low compared to CD107a and CD107b in SEB-stimulated $CD8^+$ T cells, and the background for CD63 was greater compared to CD107a and CD107b (Betts *et al*., 2003). Thus, it appears that CD107a and CD107b are more reliable markers for degranulation compared to CD63. CD107a, perforin and granzymes have been used to measure cytotoxic activity in HIV infection (Appay *et al*., 2000).

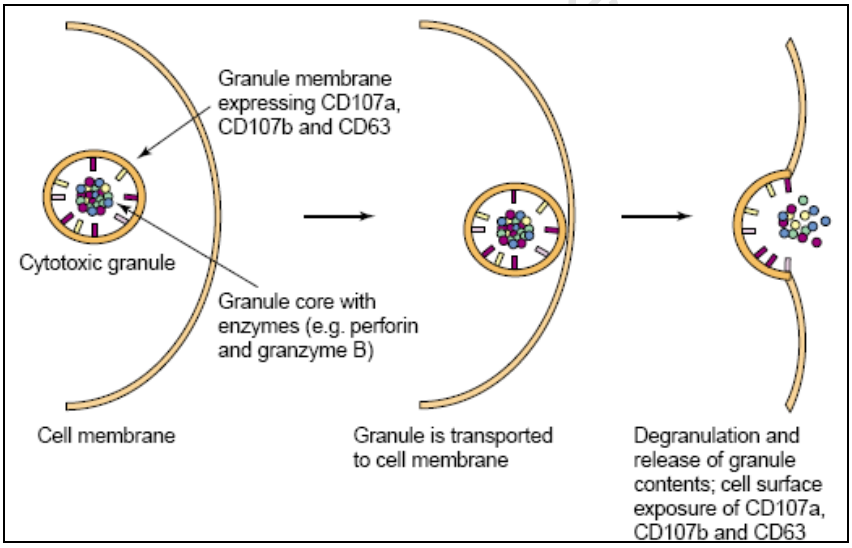

Figure 1.1: The cytolytic molecules expressed during degranulation (taken from Suni *et al*., 2005).

Another recent technique that has now become more widely available is the measurement of a vast array of analytes by cytokine bead array (CBA) and the Luminex system (duPont *et al*., 2005). Traditionally, soluble analytes secreted from stimulated cells were measured using enzyme-linked immunosorbent assays (ELISAs). Although this method is well established, it requires a larger sample

volume and single analytes are measured (Aziz *et al*., 1999). The Luminex system, however, allows for a greater throughput using a small sample volume, by using antibody-conjugated beads that are specific for each analyte in the Luminex system, and a secondary enzyme conjugated to a fluorophore, which fluoresces at different intensities to distinguish between the analytes. The samples are read on a bio-plex suspension array reader. Standards are also run in conjunction with samples so analytes of interest can be quantified. The advantage of this technique is that up to 42 analytes (Millipore, Multiplex Kit) can be measured per sample, and may include those rarely measured in other assays, such as Eotaxin, FGF-2 (Fibroblast Growth Fatcor-2), IP-10 (Interferon-Inducible Protein), MCP-1 (Monocyte Chemotactic Protein-1), and VEGF (Vascular Endothelial Growth Factor). The Luminex system has been used to evaluate twenty analytes in lymph nodes of HIV-infected individuals (Biancotto *et al*., 2007). A limitation of this procedure is that the analytes quantified may be produced by any cell type in the population, i.e. not necessarily T cells, thus the disadvantage is that the phenotype of the responding cells can not be identified.

The viral inhibition assay is another technique which measures the ability of CTLs to inhibit viral replication (Kimberlin *et al*., 1995). It is an even more direct measure of antiviral function than the cytotoxicity assay. Briefly, cells infected with viral particles are incubated with effector cells, and the viral RNA is quantitated from the supernatant. It has been observed that epitope-specific $CD8^+$ T cell clones from SIV elite controllers were able to suppress SIV replication, and this suppression was not always linked to the production of cytokines (IL-2, TNF-α and IFN-γ) that are commonly measured (Chung *et al*., 2007). Therefore, measuring these cytokines may not reveal antiviral functioning, thus emphasizing the importance of using assays that measure functions directly.

A number of factors in immunological assays may influence assay outcome. These include sample choice (peripheral blood mononuclear cells or other tissues), cryopreserved materials, and type of stimuli. Human T cell studies are performed *in vitro* in defined experimental conditions and results obtained might not accurately reflect the complex immune interactions occurring in the human body.

Measurement of HIV-specific T cell responses have been extensively studied using peripheral blood mononuclear cells (PBMCs). PBMCs can easily be extracted from blood samples and obtaining blood samples from participants is less invasive than many other tissues. Thus, PBMCs allow for the characterisation of circulating T cells, but may not be an accurate description of what is happening in other compartments of the body that may be important in HIV pathogenesis, such as the gut (Brenchley *et al*., 2004). PBMCs may be cryopreserved, and multiple samples can then be thawed and assayed simultaneously. However, cryopreservation can lead to loss of $CD4^+$ T cell responses with samples cyropreserved for more than a year (Owen *et al*., 2007). Therefore, investigating $CD4^+$ T cell responses from samples frozen for more than a year may underestimate responses.

In the HIV immunology field, synthetic overlapping peptides are commonly used as antigen to stimulate T cells. Overlapping peptides of 15-18 amino acids are generally used, with each peptide overlapping the adjacent one by 10-12 amino acids, to ensure that no epitopes are lost. Peptides of longer lengths tended to detect more $CD4^+$ T cell responses, but this was not significantly different compared to shorter peptides (Draenert *et al*., 2003). It is also important to note that these peptides are synthesized primarily based on consensus sequences. A study by Altfeld *et al* (2003) showed that using autologous peptides increased the likelihood of detecting responses by 29 %, and this was especially true for more variable proteins such as Tat and Vpr (Altfeld *et al*., 2003). Thus, it is likely that consensus peptides may underestimate the breadth and total magnitude of responses to HIV.

In summary, since the discovery of HIV in the early 1980's, there have been many advances in understanding the immune response to HIV. This knowledge has been greatly enhanced with new technologies being developed as well as improvements on existing assays.

1.2 The characteristics of HIV-specific T cell responses

Studies have investigated the nature of HIV-specific T cell responses that may play a role in viral control, by determining phenotypic and functional characteristics of these cells, as well as their specificity, frequency and breadth. HIV-specific T cell responses

do persist in HIV infection, however immunity is either insufficient or impaired, leading to AIDS in the majority of cases if left untreated. It is however relevant to investigate T cell functioning in HIV infection due to two significant early findings. Firstly, depletion of $CD8^+$ T cells in SIV-infected macaques during acute infection resulted in uncontrolled viremia and rapid progression of disease (Schmitz *et al*., 1999) and secondly, the initial decline in viremia in acute infection is associated with a concomitant increase in T cell responses (Koup *et al*., 1994). What we know about the immune T cell features that do (and do not) correlate with viral control will be the focus of this review.

1.2.1 The magnitude and breadth of IFN-γ HIV-specific T cell responses

Initial studies to elucidate correlates of viral control focused on defining the magnitude and breadth of IFN-γ T cell responses. IFN-γ is a T_H 1 cytokine that is key for cell-mediated immunity (as reviewed in Schroder *et al*., 2004). It is an antiviral cytokine that induces a number of anti-viral proteins (such as Mx, dsRNA protein kinase, ribonucleic acid nuclease, and nitric oxide), which in turn inhibit viral replication, degrade single stranded RNA, and inhibit protein synthesis (as reviewed in Chesler *et al*., 2002). IFN-γ has been implicated in the control of viral infections, such as herpes simplex virus (HSV), measles virus, vesticular stomatitis virus and LCMV (Lymphocytic Choriomeningitis virus; Cantin *et al*., 1999; Chesler *et al*., 2002). Due to the vital role it plays in controlling these viral infections, its role in HIV infection has been investigated.

Characterising HIV-specific T cell responses using the IFN-γ ELISPOT assay has been the focus of numerous studies (Edwards *et al*, 2002; Addo *et al*., 2003; Kaufmann *et al*., 2004, Masemola, *et al*., 2004, Zuniga *et al*., 2006; Geldmacher *et al*., 2007a, 2007b; Gray *et al*., 2008). HIV-specific T cell responses have also been evaluated by intracellular cytokine staining (ICS) and flow cytometry (Betts *et al*., 2001; Boaz *et al*., 2002; Ramduth *et al*., 2005). HIV-specific T cells can accumulate to high numbers in chronic infection, ranging from 280 to 25,860 spot-forming cells (SFC)/10^6 (median = 4245 SFC/10^6), where each spot represents an HIV-specific T cell (Addo *et al*., 2003). HIV-specific T cells can recognize a range of epitopes, from

different HIV proteins in a whole genome analysis, with an average of about 15 epitopes (range of 2 to 42 epitopes; Addo *et al*., 2003).

Flow cytometry studies have revealed that both $CD4^+$ and $CD8^+$ T cells are capable of producing IFN-γ in response to HIV proteins (Betts *et al*., 2001; Ramduth *et al*., 2005; Boaz *et al*., 2002), and that the magnitudes of HIV-specific $CD4^+$ and $CD8^+$ T cells positively correlated (Ramduth *et al*., 2005). However, the magnitude and frequency of HIV-specific $CD8^+$ T cells were greater compared to HIV-specific $CD4^+$ T cells (Betts *et al*., 2001; Ramduth *et al*., 2005). Whole HIV genome analysis illustrated that all HIV proteins are targeted, although Gag and Nef are the most commonly recognized for both T cell compartments (Betts *et al*., 2001; Ramduth *et al*., 2005, Addo *et al*., 2003; Kaufmann *et al*., 2004; Zuniga *et al*., 2006; Masemola *et al*., 2003).

Correlations of viral load and $CD4^+$ counts with the magnitude and breadth of IFN-γ T cell responses have given conflicting results. No correlation was observed between the magnitude (Addo *et al*., 2003; Kaufmann *et al*., 2004, Ramduth *et al*., 2005) and breadth (Addo *et al*., 2003; Kaufmann *et al*., 2004; Masemola *et al*., 2004) of total HIV-specific T cell responses in both $CD8^+$ and $CD4^+$ T cells and viral load, when the entire HIV genome was analysed. In contrast, a positive correlation was found between the total HIV-specific $CD8^+$ T cell frequency and viral control, which was determined by flow cytometry, in which the IFN-γ responses were evaluated in untreated HIV-infected individuals (Betts *et al*., 2001). The authors concluded that this was a result of viral load driving T cell responses. Similarly, a positive correlation between total magnitude of HIV-specific $CD8^+$ T cell responses and viral load was also observed in untreated HIV-infected individuals by an IFN-γ ELISPOT assay (Masemola *et al.,* 2004). Thus, magnitude or breadth of total HIV-specific T cells does not appear to control viral replication, but merely reflect the high antigen burden.

Important insights have been revealed when examining the specificity of T cell responses. Studies have shown the importance of Gag-specific T cell IFN-γ responses in the control of viral load in chronic infection. A greater breadth of Gag-specific T cell IFN-γ responses inversely associated with decreasing viremia (Kiepiela *et al*., 2007; Geldmacher *et al*., 2007a). Furthermore the total breadth of Gag-specific T cell

IFN-γ responses positively correlated with $CD4^+$ counts (Edwards *et al*, 2002; Geldmacher *et al*., 2007a). In addition to this, it has been revealed that the preferential targeting of Gag (i.e. when the Gag protein is the dominant T cell target) inversely correlates with viral control (Ramduth *et al*., 2005; Masemola *et al*., 2004; Zuniga *et al*., 2006), in particular Gag protein p24 (Zuniga *et al*., 2006). Thus, when Gag is the preferred target of $CD8^+$ T cells, as measured by IFN-γ production, there is a significant association with viral control.

HIV-specific T cell responses evaluated in the above-mentioned studies consisted of cross-sectional studies in chronic infection. A recent study followed chronically HIV-infected untreated individuals for nine months and evaluated the dynamics of Gag-specific IFN-γ T cell responses using the ELISPOT assay (Geldmacher *et al*., 2007b). This study revealed that the magnitude and breadth of HIV-specific T cell responses was influenced by time post infection, with longer time of infection correlating with a loss in Gag responses. Furthermore, high baseline viral loads and sustained viral replication led to a loss of responses. Thus, previous cross-sectional studies indicating that Gag responses are important for viral control may merely have reflected the duration of infection and persisting viremia, leading to escape and exhaustion and therefore loss of Gag responses in individuals with higher viral loads. Examining HIV-specific T cell functioning during acute infection can also give valuable insights into the interaction between the immune response and HIV. The magnitude and breadth of early HIV-specific T cell IFN-γ responses, at 3 months post infection, did not correlate with viral load at 12 months post infection (Gray *et al*., 2008). Thus, it was concluded that early HIV-specific T cell IFN-γ responses were also not playing a role in viral control.

In summary, the numerous studies indicate that control of HIV infection cannot be explained merely by measuring HIV-specific $CD4^+$ or $CD8^+$ T cell IFN-γ responses, and that neither the breadth nor magnitude of IFN-γ production alone is associated with viral control, and thus may not be involved in the control of viral replication. An exception may be IFN-γ T cell responses to the Gag protein.

1.2.2 The influence of host genetics in HIV infection

As discussed in previous section, HIV-specific T cell responses were shown to lack the ability to control viral replication and hence clear infection. One reason for the lack of viral control may be because HIV continually mutates and evolves, due to significant pressure from the immune system (Leslie *et al*., 2004), thereby allowing it to evade the immune system (Goulder *et al*., 2001). Certain human leukocyte antigens (HLAs) have been identified to be associated with either good or poor outcome of HIV disease (Table 1.1). However, viral immune escape may be at a fitness cost to the virus, which has been observed in the Gag-epitope TW10 (Leslie *et al*., 2004). Gag is a highly conserved protein and forms the viral capsid. Integrity of the capsid is vital for viral productivity and when certain regions are targeted by epitope responses, viral replication may be negatively influenced, resulting in low viral loads, or viral escape that exacts a fitness cost. There are also HLA alleles in which escape rarely occurs, as in the case of HLA-B27 restricting the Gag epitope, KK10, in which escape resulted only after 9 years of infection, followed by progression to AIDS occurred (Goulder *et al*., 1997).

Table 1.1: HLA Class I and II alleles associated with HIV disease outcome

HLA class	Good outcome	Poor outcome	Reference
Class I	B*14, B*27, B*57, C*08, C*14	B*35, Cw*04	Hendel *et al*., 1999; Carrington *et al*., 1999
	B*58	A*02-Cw*16, A*23-B*14, A*23-Cw*07	Leslie *et al*., 2004; Tang *et al*., 2002; Dorak *et al*., 2003
	A*30, B*39	A*29, B22, B*35, C*16, B44, B35	Tang *et al*., 2002; Hendel *et al*., 1999; Flores-Villanueva *et al*., 2003
Class II	DRB1*13,	-	Itescu *et al*., 1994
	DRB1*11	-	
	DRB1*01	-	MacDonald *et al*., 2000

1.2.3 Interleukin 2 (IL-2) and proliferation of HIV-specific T cells

T cells produce a variety of cytokines and effector molecules besides IFN-γ, such as IL-2, IL-6, MIP-1β and TNF-α, IL-17, IL-15 and perforin, just to name few, which play a role in immune function (as reviewed in Ma *et al*., 2006). IL-2 is one of the primary cytokines that $CD4^+$ T cells secrete upon antigen recognition (Lichterfeld *et al*., 2004). It is a growth factor for antigen-specific cells, and is responsible for their proliferation. In mouse studies, $CD4^+$ T cells are crucial for the development of memory $CD8^+$ T cells (Janssen *et al*., 2003; Shedlock *et al*., 2003; Sun *et al*., 2004), and this has also been observed in HIV infection (Lichterfeld *et al*., 2004).

The ability of $CD4^+$ T cells to secrete IL-2 or both IL-2 and IFN-γ has been shown to inversely correlate with viral control in chronic HIV infection (Harari *et al*., 2004). In these individuals, the frequency of Gag-specific IL-2^+, IL-2^+ IFN-γ^+ and total IL-2^+ secreting $CD4^+$ T cells all inversely correlated with viral control, as detected by ICS and flow cytometry. Individuals that had progressive infection were shown to have a dominant $CD4^+$ IFN-γ^+ response compared to individuals with non-progressive disease, who produced IL-2. Similar results have been obtained in long term non-progressors (LTNPs) (Rosenberg *et al*., 1997; Boaz *et al*., 2002). LTNPs are individuals that control HIV replication in the absence of treatment and are able to sustain normal $CD4^+$ counts and maintain low viral loads for long periods (Rosenberg *et al*., 1997). These studies have shown that Gag-specific $CD4^+$ T cells producing IFN-γ and IL-2 inversely correlated with viral load in LTNPs. The presence of SIV-specific T cells secreting both IFN-γ and IL-2 was also associated with long term control of infection in vaccinated and SHIV-challenged macaques, demonstrating that this type of immune response is also important in viral control in a vaccine setting (Sadagopal *et al*., 2005).

IL-2 can be used as a surrogate marker for proliferation, since a direct correlation between IL-2 secretion and HIV-specific proliferation was observed (Harari *et al*., 2004). The ability of both HIV-specific $CD4^+$ and $CD8^+$ T cells to proliferate has been implicated in the control of viral replication (Rosenberg *et al*., 1997; Kalams *et al*., 1999; Boritz *et al*., 2004; Lichterfeld *et al*., 2004; Day *et al*., 2007). Gag-specific

$CD4^+$ T cell proliferative responses have not only been observed in LTNPs (Rosenberg *et al*., 1997), where viral loads are low to undetectable, but also in chronic HIV infection (Kalams *et a.,* 1999; Boritz *et al*., 2004). These studies illustrated that there was an inverse correlation between viral load and Gag-specific $CD4^+$ T cell proliferative responses. HIV-specific $CD8^+$ T cell proliferative responses have also been shown to inversely correlate with viral load in a large cohort of chronically infected, untreated individuals (Day *et al*., 2007). This highlights the importance of HIV-specific T cell proliferation HIV viral control.

Several investigations have revealed that viremic subjects show proliferative impairment (McNeil *et al*., 2001; Palmer *et al*., 2002; Younes *et al*., 2003; Iyasere *et al*., 2003). Viral load appears to play an important role in immune function and influencing proliferation in a negative manner. HIV-infected, treated individuals had detectable HIV-specific $CD4^+$ T cell proliferation, however, when treatment was interrupted and viral load increased, these HIV-specific proliferative responses decreased, even in the presence of sustained IFN-γ responses (McNeil *et al*., 2001) which was also observed for HIV-specific $CD8^+$ T cells (Younes *et al*., 2003). This loss of proliferation in response to HIV could then be restored when viral replication was controlled again due to treatment (McNeil *et al*., 2001; Palmer *et al*., 2002). Besides antiviral therapy, defects in proliferation can be corrected *in vitro* by the addition of exogenous IL-2 (Iyasere *et al*., 2003) or autologous IL-2 secreting $CD4^+$ T cells (Lichterfeld *et al*., 2004).

Thus, studies have shown the importance of IL-2 production and HIV-specific proliferation of T cells with viral control in HIV infection, however during chronic infection, high viral loads appear to impair the proliferative function of T cells.

1.2.4 Cytotoxic activity of T cells

As discussed earlier, $CD8^+$ T cells have the ability to kill infected cells. Thus, this function in HIV infection has been investigated. Cytotoxic $CD8^+$ T cells have been measured in several studies in HIV infection (Appay *et al*., 2000; Kuerten *et al*., 2008; Migueles *et al*., 2002). It has been shown that HIV-specific $CD8^+$ T cells can produce

perforin and granzymes, however, the cytolytic response appears to be impaired, as HIV-specific $CD8^+$ T cells produce significantly lower levels of perforin in chronic infection compared to CMV-specific $CD8^+$ T cells (Appay *et al*., 2000). This may be due to constant exposure to antigen during chronic infection, leading to impairment in cytotoxic function. In the same study, lower levels of perforin were also found in ARV-naïve individuals who controlled viral load and chronically infected subjects on HAART (highly active antiretroviral therapy) who suppressed viral load, compared to higher Cytomegalovirus (CMV)-specific $CD8^+$ T cell perforin levels (Appay *et al*., 2000). Thus, regardless of viral loads, in HIV-infected individuals $CD8^+$ T cells appear to produce lower levels of perforin, which cannot be restored with HAART. In contrast, HIV-specific $CD8^+$ T cell proliferation in LTNPs was related to greater expression of perforin, compared to progressors (Migueles *et al*., 2002). However, this may have been the result of stimulating these samples with autologous $CD4^+$ T cells, resulting in expansion of cells and an increase in perforin expression. The increase in perforin expression was not observed in HIV-infected progressors in which only a small portion of cells were capable of proliferating. In terms of viral control, cytotoxic function in chronically -infected and untreated individuals was shown to have no correlation with viral load (Day *et al*., 2007). In this study, cytotoxic function was measured by CD107a expression on antigen-stimulated $CD8^+$ T cells, and may not necessarily be an accurate measurement of cytotoxic function as compared to measuring perforin and granzymes.

Interestingly, $CD4^+$ T cell cytotoxicity has been documented in several infections, such as hepatitis B, C and D and *Cryptococcus neoformans* infections (Aslan *et al*., 2006; Zheng *et al*., 2007). Cytotoxic $CD4^+$ T cells were shown to be the major subset for killing *Cryptococcus neoformans* infected cells. In HIV infection, it was observed that $CD4^+$ cytotoxic T cells produce perforin (Appay *et al*., 2002a). However, HIV-specific $CD4^+$ cytotoxicity was impaired in terms of perforin and granulysin expression (Zheng *et al*., 2007). In a single LTNP, perforin-mediated $CD4^+$ T cell cytolysis exerted potent viral suppression (Norris *et al*., 2004). Although there are a limited number of studies, it appears that HIV-specific $CD4^+$ cytotoxicity is perforin mediated but is impaired in HIV infection.

These studies provide insight into cytotoxic function and suggest that it too, like proliferative ability, is impaired in HIV infection. Further studies investigating CTL functioning for both $CD8^+$ and $CD4^+$ T cells are still required for a complete analysis of markers that are expressed during cytotoxicity, such as perforin, granzymes, granulysin, and CD107a.

1.2.5 Polyfunctional T cell responses

T cells are capable of secreting a broad range of cytokines and chemokines. Cells that produce multiple cytokines simultaneously have been termed 'polyfunctional'. Increasingly, immune studies are investigating polyfunctional T cell responses to a variety of diseases, including *Leishmania*, hepatitis C, tuberculosis, CMV, SARS coronavirus (Darrah *et al*., 2007; Ciufredda *et al*., 2008; Day *et al*., 2008; Fuhrmann *et al*., 2008; Arens *et al*., 2008; Li *et al*., 2008), as well as in vaccine evaluation (Darrah *et al*., 2007; Genesca *et al*., 2007; Sun *et al*., 2008; Beveridge *et al*, 2007; Bansal *et al*., 2008). Gradually more evidence is accumulating that illustrates that the presence of these cells is associated with HIV non-progression (Betts *et al*., 2006; Kannanganat *et al*., 2007b; Duvall *et al*., 2008). It is important to note how vital the development of polychromatic flow cytometry is for allowing measurement of polyfunctional T cells.

1.2.5.1 Polyfunctional T cells as a correlate of protection

Recently, T cell polyfunctionality has been implicated in viral control. However it is still not known whether the presence of polyfunctionality is a result of low viremia, or the cause of control of viral replication. A study conducted by Betts *et al.* (2006) evaluated whether polyfunctionality correlated with viral control. Chronically-infected and elite HIV controllers were characterized for their HIV-specific $CD8^+$ T cell responses, by measuring five T cell functions, namely degranulation (CD107a expression), IL-2, IFN-γ, TNF-α and MIP-1β. It was found that the frequency of HIV-specific $CD8^+$ T cells with the greatest amount of polyfunctionality ($CD8^+$ T cells producing all five functions) inversely correlated with viral control in the elite controller group compared to chronically-infected individuals. Furthermore, HIV-

specific $CD4^+$ T cells secreting three cytokines (IL-2, IFN-γ and TNF-α) were shown to correlate inversely with viral control in subjects that controlled infection compared to non-controllers (Kannanganat *et al*., 2007b). This highlights the importance of not only $CD8^+$ but also $CD4^+$ polyfunctional T cells in the control of HIV infection. There is also evidence that suggests that the presence of polyfunctional memory T cells is associated with HIV-2 infection, which is characterised by low viral loads and slow or no progression to AIDS (Duvall *et al*., 2008). It was observed that both HIV-2 specific memory $CD4^+$ and $CD8^+$ T cells were more polyfunctional compared to both HIV-1 specific memory $CD4^+$ and $CD8^+$ T cells. However, it is crucial to note that in the above-mentioned studies, cohorts in which polyfunctionality was associated with HIV nonprogression consisted of individuals that were characterised by low to undetectable viral loads, thus the results obtained could be a consequence of low viral loads rather than a correlate of control.

Two studies have performed longitudinal analyses to evaluate T cell functionality as the cause or consequence of viral load. Chronically-infected individuals that had started ARVs were followed for 3 months (Rehr *et al*., 2008). Initially plasma viral loads were high and $CD8^+$ T cell cytokine production was limited to IFN-γ. However, after 3 months on therapy, plasma viral loads decreased significantly and IL-2 and TNF-α production significantly increased. It was also shown that polyfunctional T cells were present more frequently 3 months after initiation of therapy compared to baseline. Further evidence to support this comes from a study in which individuals that received ARVs and then reduced antigen loads were shown to either partially restore or maintain functionality (Streeck *et al*., 2008). Here, HIV-specific $CD8^+$ T cells had diverse functions, such as the ability to degranulate and produce IL-2, IFN-γ, TNF-α and MIP-1β during primary infection. However, monofunctionality increased after primary infection in untreated HIV infection. This loss in functionality was not observed for CMV, EBV (Epstein Barr virus) and influenza-specific $CD8^+$ T cells in the same individuals. This suggests that the impairment in T cell function may be a consequence of ongoing viral replication from acute to chronic HIV infection.

Vaccine-induced polyfunctional T cells have been shown to correlate with protection in a number of viral, parasitic and bacterial infections (Precopio *et al*., 2007; Darrah *et*

al., 2008). Smallpox has been eradicated due to immunization with vaccinia virus. What made this vaccine so successful? One reason could be the fact that this vaccine induces highly polyfunctional $CD8^+$ T cells which are capable of degranulation and secreting IL-2, IFN-γ, MIP-1β and TNF-α after antigenic stimulation (Precopio *et al*., 2007). Leishmania species are protozoans, which cause skin and mucosal diseases in humans as well as other vertebrates (as reviewed in Melby, 2002)**.** In mouse studies**,** immunization with leishmania polyprotein induced polyfunctional $CD4^+$ T cells that were capable of producing IL-2, IFN-γ and TNF-α simultaneously (Darrah *et al*., 2007). The frequency of the triple-producing $CD4^+$ T cells inversely correlated with lesion size, indicating that these cells were involved in the reduction of lesions, whilst single cytokine producing cells were not involved with protection.

Similarly, a protective role of vaccine-induced polyfunctional T cells has been observed in SHIV (Sun *et al*., 2008) and SIV (Genesca *et al*., 2008) vaccine studies. Rhesus macaques vaccinated with live attenuated SHIV 89.6 and challenged with SIVmac239 who controlled virus replication compared to those that were unable to control viral replication had polyfunctional SIV-specific $CD8^+$ T cells (Genesca *et al*., 2008). Additionally, rhesus macaques vaccinated with various vectors and challenged with pathogenic SHIV-89.6 were shown to have a better disease outcome due to vaccine-induced polyfunctional $CD8^+$ T cell responses compared to non-vaccinated macaques (Sun *et al*., 2008). Thus, vaccine-induced polyfunctional T cells appear to play a role in disease progression. Some candidate HIV vaccines are also capable of eliciting polyfunctional $CD4^+$ and $CD8^+$ T cells, such as a DNA prime/poxvirus boost vaccination strategy (Harari *et al*., 2008) and similarly, an HIV DNA prime/Env protein boost vaccine strategy, that elicited polyfunctional HIV-specific $CD4^+$ T cell responses in seronegative individuals (Bansal *et al*., 2008). While these candidate vaccines appear promising since they elicit polyfunctional T cells, only efficacy studies will reveal whether they do indeed provide protection from HIV infection or better disease outcome.

It therefore appears that polyfunctional T cell responses may be impaired in HIV infection, although in other infections they are present and appear to have a protective

function. Polyfunctional T cells can be induced by vaccination, however their protective function has yet to be determined.

1.2.5.2 Presence of polyfunctional T cells in different compartments

As mentioned earlier, HIV-specific T cell responses have been investigated primarily in blood. There have been few studies that have looked at other compartments in the human body, such as lymphoid or mucosal tissues, due to the obvious difficulty of sampling these sites. Polyfunctional HIV-specific T cells were shown to be present at mucosal sites such as bronchoalveolar lavage (lung tissue) and the rectum in untreated individuals (Brenchley *et al*., 2007; Critchfield *et al*., 2007).

In the first study, it was shown that both HIV-specific $CD4^+$ and $CD8^+$ polyfunctional T cells (cells producing IL-2, IFN-γ and TNF-α) were present at a higher frequency in lung tissue compared to blood, which may be due to less depletion of $CD4^+$ T cells in the lung tissue, even though infection frequencies were similar to blood (Brenchley *et al*., 2007). Therefore, preservation of HIV-specific $CD4^+$ T cells was associated with polyfunctionality. However, in rectal tissue, no differences were seen in Gag-specific polyfunctional $CD8^+$ T cell responses ($CD107a^+IFN\text{-}\gamma^+TNF\text{-}\alpha^+$) compared to blood in chronic infection, even though there was a greater magnitude of $CD8^+$ T cell responses in rectal tissue (Critchfield *et al*., 2007). This work was then extended to investigate two additional markers, IL-2 and MIP-1β, in a chronically infected cohort (Critchfield *et al*., 2008). Here, it was shown that rectal tissue in treated and untreated individuals had polyfunctional Gag-specific $CD8^+$ T cell responses. These responses were also shown to inversely correlate with plasma viral load and positively with blood $CD4^+$ counts. These results indicate the presence of polyfunctional cells at mucosal sites.

1.2.5.3 Polyfunctional T cells are superior effectors

Why might polyfunctional cells be superior effectors? Recent studies have shown that polyfunctional T cells are capable of producing more cytokine per cell than a monofunctional or single-producing cell (Precopio *et al*., 2007; Darrah *et al*., 2007;

Kannanganat *et al*., 2007a; Duvall *et al*., 2008). This was determined by obtaining the median fluorescence intensity (MFI) of the cytokine population, since the fluorescence intensity is related to the amount of cytokine produced within an individual cell. Polyfunctional $CD8^+$ T cell responses induced by vaccinia virus, the smallpox vaccine, secrete more cytokine per cell compared to monofunctional cells (Precopio *et al*., 2007). In this study, the $CD8^+$ IFN-γ MFI of four populations was determined ($four^+$, $three^+$, two^+ and one^+ cytokine producing cells). The MFI of IFN-γ was the highest in the $three^+$ polyfunctional population and lowest in the monofunctional population. This suggests that these are superior effector cells that may be involved in viral control. This result was not observed for the other cytokines, IL-2 and TNF-α, and chemokine, MIP-1β.

In agreement with these results, mice vaccinated with leishmania polyprotein generated polyfunctional $CD4^+$ T cells that were producing more IFN-γ, IL-2 and TNF-α compared to single-cytokine secreting cells (Darrah *et al*., 2007). This was observed in the spleen, lymph node and lungs of these mice. The IFN-γ MFI inversely correlated with lesion size, thus supporting the idea that these polyfunctional cells are indeed superior in terms of function and controlling infection. The ability of polyfunctional $CD4^+$ T cells to secrete more cytokine per cell was also found in healthy individuals that either had vaccinia virus, CMV or influenza responses (Kannanganat *et al*., 2007a). Additionally, HIV-2-specific $CD4^+$ and $CD8^+$ polyfunctional T cells ($four^+$ and $three^+$) were also capable of secreting more IFN-γ and TNF-α compared to monofunctional cells (Duvall *et al*., 2008).

Interestingly, triple and dual-cytokine producing $CD4^+$ T cells from healthy individuals that either had vaccinia virus, CMV or influenza responses showed increased levels of CD40 ligand (CD40L) compared to monofunctional cells (Kannanganat *et al*., 2007a). CD40L is a co-stimulatory molecule for $CD8^+$ T cells (Bennett *et al*., 1998) and B cells (Foy *et al*., 1993). This indicates that multiple cytokine-producing T cells have an enhanced potential for activating $CD8^+$ T and B cells.

These studies demonstrate that polyfunctional T cells are important in protection of certain infections, and are superior in terms of their function as they are more efficient effectors compared to monofunctional T cells, due the fact that they secrete more cytokine per cell and may better activate $CD8^+$ T and B cells.

1.2.6 T cell differentiation in HIV infection

Upon antigen stimulation, T cells proliferate and mature and differentiate into specialised subsets that can be identified by expression of different surface markers, as well as being defined by particular functions that they possess. Thus cellular phenotype is linked to function. Therefore it is relevant to investigate T cell differentiation in HIV infection to determine whether there are particular subsets of cells that are involved in viral control.

1.2.6.1 What are Memory T cells?

Memory T cells are cells that have differentiated and matured to perform their functions and hence are distinct from naïve (and effector) T cells. This differentiated state can be used to identify them based on expression of certain surface markers. Two major memory subsets have been defined (Sallusto *et al*., 1999), but recent work reveals that memory phenotypes are highly complex (as reviewed in Wherry and Ahmed, 2004). Different isoforms of CD45 are expressed when T cells differentiate (Michie *et al*., 1992). Naïve and terminally differentiated effector T cells express $CD45RA^+$ (as reviewed by Esser *et al*., 2003), whereas memory T cells lose the expression of this marker to become $CD45RA^-$ and instead express CD45RO. The two major memory subsets are: central memory (T_{cm}) and effector memory (T_{em}) T cells which occur in both CD4 and CD8 compartments (Sallusto *et al*., 1999). Memory T cells form a unique subset due to the expression of specific markers, chemokines and adhesion molecules, and are responsible for a rapid and enhanced response upon subsequent encounter with antigen.

Central memory cells are the long-lived memory cells and secrete IL-2 (as reviewed by Esser *et al*., 2003). They are found in lymph nodes due the expression of CD62L, which is required for adhesion to high endothelial venules, which then permits cells

expressing the chemokine receptor CCR7 to bind to secondary lymphoid tissue chemokine (SLC) and re-circulate between blood and lymph nodes (Sallusto *et al*., 1999). It is well accepted that T_{cm} may be identified on the basis that they are $CD45RA^{-}$/ $CD62L^{hi}$/$CCR7^{+}$ (Figure 1.2)

According to the linear model of T cell differentiation, at encounter with a respective antigen, T_{cm} differentiate into effector memory T cells (T_{em}) by losing the expression of CCR7 (and therefore the ability to migrate to lymph nodes) and become $CD62L^{lo}$ (Sallusto *et al*., 1999). T_{em} are thus identified as $CD45RA^{-}$/ $CD62L^{lo}$/$CCR7^{-}$ (Sallusto *et al*., 1999; Figure 1.2). Other markers that can be used to distinguish T_{cm} from T_{em} cells are CD28 and CD27 (as reviewed in Esser *et al*., 2003; Hamman *et al*., 1997). CD27 and CD28 are co-stimulatory receptors and the expression of CD28 and then CD27 are lost as T cells differentiate (Appay *et al*., 2002b). Effector and T_{em} cells are short lived and they perform a rapid effector function upon re-infection, and hence are found in peripheral blood sites of inflammation and in tissues.

There are additional T cell markers that have been used to identify and characterise memory T cells, such as CD57 and CD127 (as reviewed in Ma *et al*., 2006). CD127 is a marker for long-term memory since it confers sensitivity to IL-7 and therefore homeostatic survival. CD57 is used to identify senescent T cells, i.e. cells that can no longer divide and are therefore short-lived (Brenchley *et al*., 2002). Telomeres are sequences at the ends of chromosomes, which shorten with each round of cell division (as reviewed in Akbar and Fletcher, 2005; Harley 1990), thus the length of telomeres in combination with phenotypic markers have been used to identify T cell differentiation subsets (Dunne *et al.,* 2002), since the lengths of telomeres decrease as differentiation increases (Figure 1.2).

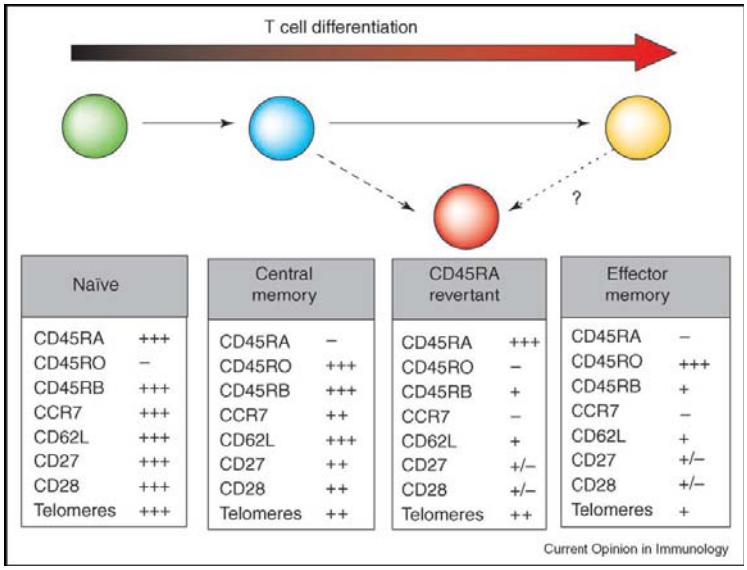

Figure 1.2: T cell differentiation from naïve to memory cells. The markers that are expressed for a particular subset are shown (taken from Akbar and Fletcher, 2005).

1.2.6.2 Current literature on T cell differentiation in HIV infection

In HIV infection, it appears that the immune system is constantly activated, which leads to exhaustion and dysfunction and ultimately progression to AIDS (Papagno *et al*., 2004). This link between exhaustion and disease progression is not clearly understood and studies have attempted to evaluate the differentiation status of T cells, particularly HIV-specific T cells, in HIV-infected individuals. This section will summarise the literature surrounding T cell differentiation in HIV infection.

It was observed that during untreated HIV infection, HIV-specific $CD8^+$ T cells were dominated by the effector memory phenotype ($CD45RA^-CCR7^-$) compared to CMV-specific $CD8^+$ T cells, which were terminally differentiated ($CD45RA^+CCR7^-$) (Champagne *et al*., 2001). This skewing towards an effector memory phenotype has also been observed for HIV-specific $CD4^+$ memory T cells were (Tilton *et al*., 2007). These observations suggest that an incomplete maturation phenotype occurs for HIV-specific T cells that may be dysfunctional. Furthermore, a greater frequency of HIV-specific effector memory $CD8^+$ T was observed in controllers compared to untreated progressors (Addo *et al*., 2007). An inverse correlation was observed between HIV-specific $CD8^+$ T effector memory T cells and viral set point, with a higher viral load

set point associated with the presence of HIV-specific $CD8^+$ T effector memory T cells (Northfield *et al*., 2007). Thus, the presence of these terminally differentiated cells is associated with a better disease outcome.

It appears that the maintenance of T_{cm} may be a correlate of protection in HIV infection, as the percentage of HIV-specific $CD4^+$ T_{cm} cells were greater in HIV controllers compared to viremic individuals or those on treatment, respectively (Potter *et al*., 2008). This finding was also true for total $CD4^+$ T_{cm} cells, where reduced levels of total $CD4^+$ T_{cm} cells were associated with higher viral loads (Ladell *et al*., 2008). Elite controllers were shown to have preserved total $CD4^+$ T_{cm} cells compared to HIV-infected treated individuals (van Grevenynghe *et al*., 2008). Thus, in HIV infection, HIV-specific and total $CD4^+$ T_{cm} cell phenotypes associated with HIV non-progression.

The relevance of SIV-specific and total $CD4^+$ T_{cm} cells has also been investigated in SIV-infected (Ling *et al*., 2007) and vaccinated macaques (Sun *et al*., 2005; Letvin *et al*., 2006, Mattapalli *et al*., 2006; Manrique *et al*., 2008). SIV-specific $CD4^+$ T_{cm} preservation can be induced through vaccination which leads to better survival and slower disease progression (Sun *et al*., 2005; Letvin *et al*., 2006, Mattapalli *et al*., 2006; Letvin *et al*., 2006; Manrique *et al*., 2008). The preservation of SIV-specific $CD8^+$ T_{cm} cells were also shown to be associated with delayed disease progression (Sun *et al*., 2006). Thus, these studies illustrate the importance of preservation of T_{cm} cells.

In addition to memory markers, there are other markers that have been used to investigate T cell differentiation in HIV infection. Total $CD4^+$ T cells that were $CD127^-/CD25^-$ i.e. not long-lived cells, were related to the decline of total $CD4^+$ naive and T_{cm} cells, and $CD127^-/CD25^-$ $CD4^+$ T cells correlated inversely with $CD4^+$ counts (Dunham *et al*., 2007). Similarly, it was observed that HIV-specific $CD8^+$ T cells express lower levels of CD127 compare to Flu, vaccinia and EBV-specific $CD8^+$ T cells (Wherry *et al*., 2006), indicating impairment in HIV-specific $CD8^+$ T cell differentiation. However, CD127 expression on HIV-specific $CD8^+$ T cells can be maintained if individuals are treated early in infection, thus cells surviving long-term are maintained if HIV is treated early (Sabbaj *et al*., 2007).

PD (programmed death)-1 is a receptor and upon binding with one of its ligands (PD-L1 or PD-L2), inhibits T cell activation (Ishida *et al*., 1992). However, in HIV infection, PD-1 has been shown to be upregulated on HIV-specific $CD8^+$ T cells in chronically infected individuals compared to CMV infection (Day *et al*; 2006; Trautman *et al*., 2006), and the PD-1 MFI of total HIV-specific $CD8^+$ T cells positively correlates with viral load. Day *et al.* (2006) also showed that PD-1 MFI of total HIV-specific $CD8^+$ T cells inversely correlates with $CD4^+$ counts, as well as HIV-specific $CD4^+$ T cells. Thus, the increased expression of PD-1 on HIV-specific T cells may render them unresponsive, which may be associated with disease progression. Blocking the PD-1/ligand pathway reversed this 'exhausted' phenotype of HIV-specific $CD8^+$ T cells, so proliferative, cytokine and cytotoxic ability was restored (Day *et al*., 2006; Trautman *et al*., 2006). Similarly, CTLA-4 (cytotoxic T lymphocyte-associated antigen 4), which also functions to regulate T cell activation, has been shown to be upregulated on HIV-specific $CD4^+$ T cells. Likewise, by blocking the CTLA-4 pathway, T cell function was restored in terms of IL-2 production (Kaufmann *et al*., 2007). Thus, these studies illustrate a mechanism in which T cells are dysfunctional in HIV infection, but also very importantly demonstrate that this dysfunction may be reversible and hence provide a mechanism in which T cell exhaustion may be treated in chronic infection.

In HIV infection, it appears that memory T cell differentiation is skewed. What might account for this observed dysfunction? It is becoming apparent that immune activation may drive HIV-specific T cell differentiation and hence dysfunction (Sousa *et al*., 2002; Papagno *et al*., 2004). Thus, HIV-specific $CD8^+$ T cells are pushed to differentiate due to a chronically activated immune system, driven by sustained vial replication.

1.3 Conclusion

Since the discovery of HIV, there are constantly new insights into viral pathogenesis and interactions between HIV and the immune system that are being identified. Several T cell correlates of viral control have been identified, such as the preferential targeting of Gag, proliferative ability, polyfunctionality, and preservation of T_{cm} cells. However, it is difficult to know whether these responses are controlling viral

replication, or merely reflect lower viral loads in individuals controlling viral replication. There are likely to be additional mechanisms, which may be involved in viral control, which have yet to be identified.

1.4 Aims of thesis

Studies have revealed the importance of investigating HIV-specific T cell responses in HIV infection using flow cytometry, and more recently, polychromatic flow cytometry, which allows the investigation of more than four markers. The first aim of this project was to establish a nine-colour polychromatic flow cytometry panel for use in the laboratory. The panel was to include five functional markers, namely IL-2, IFN-γ, TNF-α, MIP-1β and CD107a. These were chosen since the expression of these markers simultaneously has been implicated with viral control. However, this association with viral control is not clear and further investigations are required. Therefore, the second aim of this project was to apply the panel to investigate HIV-specific polyfunctional T cells in chronic and acute HIV infection, where individuals were followed for12 months post infection. Longitudinal investigations may provide different insights compared to cross-sectional studies. This study allowed a comparison between acute and chronic infection, and enabled patterns of polyfunctionality to be determined, adding to the body of data on the function of HIV-specific T cells and their role in HIV infection.

CHAPTER 2
MATERIALS AND METHODS

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CHAPTER 2

MATERIALS AND METHODS

2.1 General laboratory operations and reagents

All procedures with biohazardous material were performed in a Biosafety Class II hood with appropriate protective wear and procedures, including appropriate disinfection and disposal of waste. Table 2.1 lists commonly-used laboratory reagents, their composition and storage requirements. Fetal bovine serum (FBS; Invitrogen) is blood serum that is obtained from foetuses of cows. FBS is used to supplement culture media as it contains important growth factors and proteins, which are required by cells for growth and survival. Prior to use, it was heat inactivated for 30 min at 56 °C in order to denature complement proteins to avoid cell lysis. RPMI (Invitrogen) media contains vitamins and amino acids and it is usually supplemented with 1-20 % FBS. Prior to use, Penicillin/ Streptomycin (Invitrogen; 1 unit/ml/ 1 µg/ml) was added to prevent bacterial contamination during cell culture. PBS (Invitrogen) is a buffer solution, and is used to maintain a constant pH when reagents were diluted.

Table 2.1: Commonly used laboratory reagents

Reagent	Composition	Storage	Supplier
Fetal Bovine Serum (FBS; 100 ml)	-	-20 °C	Invitrogen
RPMI-1640 Medium (1X)	200 mM L-glutamine, 25 mM HEPES, pH indicator	4 °C	Invitrogen
Phosphate Buffered Saline (PBS; 1X)	0.138M NaCl, 0.0027M KCl (pH 7.2)	RT	Invitrogen
Penicillin/ Streptomycin (100 ml)	100 units/ml penicillin,100 µg streptomycin	-20 °C	Invitrogen
R1	1 % FBS in RPMI	4 °C	*
R10	10 % FBS in RPMI	4 °C	*
R20	20 % FBS in RPMI	4 °C	*

* Prepared in the laboratory

2.2 Ethical approval

This study included participants in the acute and chronic stage of HIV infection. Ethical approval for individuals with chronic HIV infection was obtained from the Research Ethics Committee of the Faculty of Health Sciences, University of Cape Town (reference number is 106/2002). Inclusion criteria for participants were as follows: HIV-infected, with CD4 counts >200 cells/mm^3, and an age of greater than 18 years old. Informed consent was obtained from each participant prior to participating in the study.

HIV-uninfected individuals were longitudinally followed whilst enrolled in a vaccine preparedness study and individuals that seroconverted were recruited into an acute infection cohort funded by the International AIDS Vaccine Initiative. Ethical approval for this cohort was obtained from Research Ethics Committee of the Faculty of Health Sciences, University of Cape Town (reference number is 134/2006). Inclusion criteria included age between 16-40 years, and all participants gave informed consent.

2.3 Peripheral Blood Mononuclear Cell (PBMC) processing

HIV-uninfected blood samples were obtained from Western Province Blood Transfusion Service (WPBTS) donors. PBMCs from chronically infected participants were obtained from the Nyanga Day Clinic and acute samples from the Uluntu Centre, both sites in Gugulethu, Cape Town. All samples were processed on the day of collection. Briefly, PBMCs were processed using Ficoll-density gradient centrifugation, freezing media added and cryopreserved in vapour phase liquid nitrogen for long-term storage. Table 2.2 lists the reagents required for PBMC processing.

2.3.1 PBMC isolation procedure

Ficoll-Hypaque (Sigma Aldrich) is used for density gradient centrifugation and for the separation of peripheral mononuclear cells (PBMCs) from blood plasma, erythrocytes and granulocytes (Figure 2.1). Ficoll-Hypaque consists of ficoll, a carbohydrate polymer, and a dense hydrophilic polysaccharide, metrizamide, which serves as a

Table 2.2: Reagents required for PBMC processing

Reagent	Composition	Storage	Supplier
Ficoll-Hypaque	Ficoll and metrizamide	4 °C	Sigma Aldrich
Dimethyl Sulfoxide (DMSO)	-	RT	Sigma Aldrich
Freezing media	20 % DMSO, 80 % FBS	Kept on ice	*
ViaCount Reagent	Nuclear and vibility dye, sodium azide	4 °C	Gauva Technologies
Washing media	1 % FBS PBS	RT	*

* Prepared in the laboratory

solute and provides a higher density without increasing viscosity. Ficoll has a density higher than that of PBMCs and after centrifugation, plasma and platelets are located at the top of the gradient since they have a lower density compared to red blood cells, ficoll and PBMCs. The next layer formed is the PBMC layer which consists of lymphocytes and monocytes, and below the ficoll layer are the red blood cells which have the greatest density.

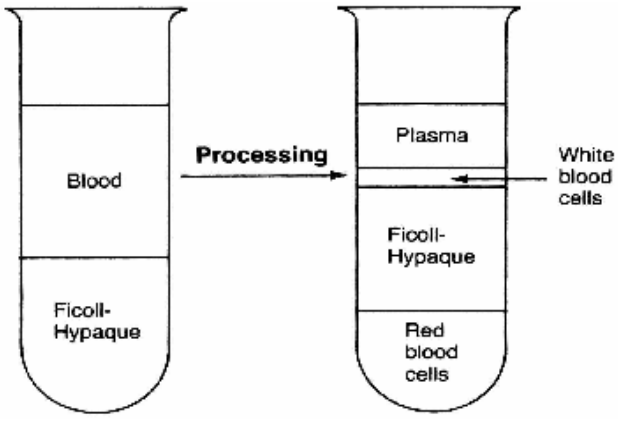

Figure 2.1: Diagrammatic representation of blood components as separated using ficoll-Hypaque density centrifugation (taken from McGukin *et al*., 2008).

Initially, 30 ml of Ficoll-Hypaque solution (that was allowed to reach room temperature before use) was added to a 50 ml Leucosep tube (Greiner Bio-one) containing a filter disc. This was then centrifuged for 1 min at 1257 X g, to allow the Ficoll-Hypaque solution to descend below the filter of the Leucosep tube. Donor

blood samples were poured into the 50 ml Leucosep tubes and centrifuged as before for 15 min. The PBMC layer was then removed and washed with 1 % FBS in PBS. The cell pellet was resuspended in 5-40 ml R1 (Table 2.1) for counting.

Samples were counted using an automated Gauva cell counter (Gauva Technologies) which counts the number of nucleated cells in a sample and tests the viability of the sample. For cell counting, a 1:20 dilution was made using the Gauva ViaCount reagent (10 µl of sample added to 190 µl of the ViaCount reagent; Gauva Technologies). This was incubated for 8 min at room temperature before counting. The Gauva ViaCount reagent consists of two dyes, a nuclear and viability dye. The nuclear dye stains nucleated cells and the viability dye stains dead cells. In this manner, viable and non-viable cells can be distinguished based on the differential permeabilities of the two dyes. This viability assay also allows for the exclusion of debris which is negatively stained by the nuclear dye.

2.3.2 Cyropreservation of PBMCs

Cryopreservation involves preserving cells at low temperatures in order to restore them with minimal damage for later analysis. Cyropreserved cells were stored in a vapour phase liquid nitrogen cryotank (Cryo 200, Forma Scientific). Cells were frozen in media containing the cryoprotectant reagent DMSO (Sigma Aldrich). It is a polar solvent that can penetrate membranes without damaging them during the freezing process and it protects cells from ice crystals that form. It also maintains the integrity of cell membranes during the freezing process, thereby limiting cell death.

Isolated PBMCs were first resuspended in a volume of FBS (Invitrogen) and then freezing media (Table 2.2) was added drop-wise to the suspension to obtain a final concentration of 10-15 $x10^6$ cells/ml. One ml aliquots were dispensed into pre-cooled 2 ml cryovials (Greiner Bio-one) and placed into pre-cooled “Mr Frosty” (Nalgene) containers. A “Mr Frosty” contains isopropanol and allows the temperature to gradually decrease at approximately -1 °C/minute, allowing samples to freeze slowly to limit cell death. “Mr Frosties” were stored in a -70 °C freezer overnight and cryovials were transferred the following day to liquid nitrogen.

2.4 Thawing of cryopreserved samples

Frozen PBMC samples were thawed by being placed in a 37 °C waterbath for 1-2 min until a tiny ice crystal remained. Ten ml of pre-warmed R1 (Table 1) was added drop-wise to the sample while gently swirling. To this suspension, a further 15 ml R1 was added and the sample was centrifuged at 289 X g for 10 min. The supernatant was decanted and the pellet was resuspended. DNAse I (0.002 %; Roche Diagnostics) was added to the sample. DNAse comes from bovine pancreas and it digests DNA by hydrolysing the phosphodiester linkages of double-stranded or single-stranded DNA to a mixture of oligo- and mononucleotides. Some inevitable cell death from cryopreservation leads to cell lysis and the release of viscous DNA in the sample, and DNAse treatment prevents cells from clumping. Samples were incubated at room temperature for 2 min. An additional 25 ml R1 was added to the sample and it was centrifuged as before. The pellet was resuspended in 3-5 ml (dependant on cell count, with 2-4 $x10^6$ cells/ml being optimal) of R20 (Table 2.1) and transferred into a 37 °C, 5 % CO_2 incubator overnight. Samples are rested overnight as this reduces background responses (Horton *et al.,* 2007).

2.5. Stimulation of thawed samples

2.5.1. Peptides used in this study

In order for an adaptive T cell immune response to develop or be detected, foreign bodies are phagocytised and proteins are processed to form peptides, small chains of amino acids. These peptides are presented to T cells by MHC molecules. To by-pass antigen-processing, peptides of 15 -18 amino acids in length were used to measure HIV-specific responses. All peptides were obtained lyophilised and resuspended in DMSO (Sigma Aldrich). Peptide pool stocks were then further dissolved in RPMI (Invitrogen) at a concentration of 40 μg/ml and stored at -80 °C. HIV peptides were assembled into Gag and Nef pools as listed in Table 2.3. These pools overlap by 10-11 amino acids to ensure that no epitopes are lost. These peptides were based on the Du422 strain of HIV. The Du HIV strains were used because they are close to a South African consensus sequence (Williamson *et al*., 2003).

Table 2.3: HIV-1 subtype C peptide pools used in this study

Peptide Pool *	Number of peptides
Gag	122
Nef	50

* Gag peptides were obtained from NIH Research and Reference Reagent Repository and Nef peptides were obtained from Clive Gray, National Institute of Communicable Diseases (NICD), Johannesburg.

2.5.2 Additional stimulation reagents

Whilst peptide stimuli were added to PBMC in order to stimulate cytokine production and therefore detect specific T cells, a number of additional reagents were included in the stimulation procedure. These are summarised Table 2.4 and their preparation and storage are listed in the Appendix. Co-stimulatory molecules anti-CD28 and anti-CD49d (BD Biosciences) were included in the stimulation process to promote TCR recognition (Waldrop *et al*., 1997) by synergistically providing a distinct signal to induce transcription factors and thus cytokine gene induction (Udagawa *et al*., 1996). CD28 is a receptor for CD80 (B7.1) and CD86 (B7.2), which are expressed on antigen presenting cells. CD49d is the alpha 4 integrin subunit of the $\alpha 4\beta 1$ lymphocyte homing receptor. It functions as an adhesion receptor and is expressed on lymphocytes, with an increased expression on activated cells.

A positive control was included to ensure that the assay had worked and the cells were functional. Both PMA/Ionomycin (Sigma Aldrich) and SEB (Sigma Aldrich) were used. Phorbol Myristate Acetate (PMA) is a strong mitogen that stimulates the T cell receptor (Touraine *et al*., 1977). Ionomycin is an ionophore, which is produced by the bacterium *Streptomyces conglobatus.* It is involved in transferring ions across membranes (Beeler *et al*., 1979) and when used in conjunction with PMA it increases intracellular Ca^{2+} molecules, thereby inducing activation of a cell and production of cytokines (Kawanishi and Joseph, 1992). Staphylococcal enterotoxin B (SEB) is produced by *Staphylococcus aureus* and it is a toxin commonly causing food poisoning in humans. Similarly to PMA, it is also a strong stimulator of T cells by inducing DNA synthesis and hence production of cytokines (Jenkinson *et al*., 1989).

The benefit of using SEB instead of PMA is that it does not downregulate CD4 expression (Pelchen-Matthews *et al*., 1993).

In order to measure intracellular markers by flow cytometry, the protein secretory inhibitors Brefeldin A (BFA) and monensin (Sigma Aldrich) were both used. These substances prevent extracellular secretion and hence cytokines are confined intracellularly. BFA is a lactone antibiotic produced by the fungus *Penicillium brefeldianum*, and causes the endoplasmic reticulum and the golgi apparatus to merge, thereby forming a blockade (as reviewed in Klausner *et al*., 1992). Monensin is an antibiotic from the bacterium *Streptomyces cinnamonensis* and inhibits protein transport by de-acidifying the golgi layers (Fliesler and Basinger, 1987).

Table 2.4: Reagents used for stimulation

Reagent	Storage	Supplier
anti-CD28 (1 μg/ml)	4 °C	BD Biosciences
anti-CD49d (1 μg/ml)	4 °C	BD Biosciences
Brefeldin A (BFA) (0.5 μg/ml)	-20 °C	Sigma Aldrich
Monensin (10 μg/ml)	-20 °C	Sigma Aldrich
Phorbol Myristate Acetate (PMA; 1 μg/ml)	-20 °C	Sigma Aldrich
Ionomycin (0.5 μg/ml)	-20 °C	Sigma Aldrich
SEB (2 μg/ml)	4 °C	Sigma Aldrich

2.5.3 Stimulation procedure

After cells were rested overnight they were counted and washed in 15 ml of pre-warmed R10 (Table 2.1) and centrifuged as before. The pellet was then resuspended in R10 stimulation mix. This mix consisted of 4.5 ml R10, 500 μl DNase I, anti-CD49d (1 μg/ml), anti-CD28 (1 μg/ml) and BFA (0.5 μg/ml). An additional 5 μl of monensin (10 μg/ml) (Sigma Aldrich) was added to the stimulation mix.

The cell suspension was then stimulated with either no peptide (unstimulated background control), the positive control PMA (1 μg/ml) and ionomycin (0.5 μg/ml) or SEB (50 μg/ml), or Gag and Nef peptide pools of HIV-1 subtype C, or specific peptides (used at 2 μg/ml). Included in each stimulation was 5 μl of anti-CD107a-FITC (see section 3.2.2). Samples were stimulated for 16 h in a 37 °C, 5 % CO_2 incubator (Thermo Electron Corporation), while PMA/ionomycin tubes were incubated for 4 h.

2.6 Surface and Intracellular Staining (ICS)

ICS involves staining for intracellular markers such as cytokines, and includes staining for surface markers that determine the phenotype of a cell. The term staining in this context refers to the use of antibodies that are conjugated to a fluorochrome that bind to markers of interest, which are then detected by a flow cytometer. Initially, cells were stained with antibodies that are expressed on the cell surface, thereafter were permeabilized allowing antibodies to enter and bind to intracellular markers of interest. The permeabilizing reagent consists of saponin, a plant derived antibiotic, which creates pores in the cell to allow antibodies to enter. This occurs by saponin binding to cholesterol molecules on cell membranes forming insoluble complexes, which results in rearrangement of lipids, hence producing a more permeable structure (Way, 1969). Samples were washed with a buffer containing saponin to maintain permeabilization, as this is a reversible process. Cells were subsequently fixed to preserve the staining. Reagents used in the ICS protocol are listed in Table 2.5.

Table 2.5: List of reagents for ICS

Reagent	Composition	Storage	Supplier
FACS Wash Buffer	1 % FBS PBS, 0.001 % sodium azide	4 °C	*
Cytofix/Cytoperm (1X)	Formaldehyde and saponin	4 °C	BD Biosciences
Perm/Wash (10X)	FBS and saponin	4 °C	BD Biosciences
CellFIX (10X)	Formaldehyde and sodium azide	RT	BD Biosciences

* Prepare in the laboratory

2.6.1 Antibody staining procedure

Table 2.6 contains a list of antibodies and the titrated volumes used for the polychromatic antibody assay. Once stimulations were complete, cells were washed with FACS wash buffer (Table 2.5) and centrifuged at 838 X g for 3 min at 4 ºC. All further centrifugation steps of 96 well microtiter plates were performed at this speed, time and temperature. The cell pellet was resuspended in 50 µl PBS containing a pre-titered volume of violet viability reactive dye ("Vivid"; Invitrogen), and incubated for 20 min at room temperature. Samples were washed with 150 µl FACS wash buffer and centrifuged, and this was repeated using 200 µl FACS wash buffer. A surface staining mix was prepared, which consisted of anti-CD19-Pacific Blue, anti-CD14-Pacific Blue, anti-CD8-Qdot 605, anti-CD4-PerCP-Cy5.5 and PBS. A final volume of 50 µl was added to each sample and the plate was incubated at room temperature for 20 min. Samples were then washed twice with FACS wash buffer as before. The pellets were then resuspended in 100 µl Cytofix/Cytoperm (BD Biosciences) and incubated at room temperature for 20 min. Samples were washed twice using 150 µl and then 200 µl 1X Perm/Wash buffer (BD Biosciences). An intracellular staining mix of anti-CD3-APC-H7, anti-IFN-γ-Alexa fluor 700, anti-TNF-α-PE-Cy7, and anti-APC-IL-2 was prepared in PBS and a final volume of 50 µl was added to each sample, followed by an incubation for 20 min at room temperature. Cells were washed as before with 1X Perm/Wash buffer and resuspended in 150 µl 1X CellFIX (BD Biosciences).

2.6.2 Compensation

2.6.2.1 What is compensation?

During acquisition of samples from a flow cytometer there may be spectral overlap between fluorochromes in which fluorescence from one fluorochrome is detected in more than one detector, as indicated in red in the Figure 2.2. To correct for this, the fluorescence spillover is mathematically subtracted, and this is termed compensation.

Table 2.6: Anti-human antibodies used in multicolour flow cytometry panel

Specificity*	Fluorochrome**	Clone	Catalogue Number	Titrated volume used in assay (µl)
CD3	APC-H7	*SP34.2*	BD 641397	2.5
CD14	Pacific Blue	*M5E2*	BD 558121	5
CD19	Pacific Blue	*SJ25-C1*	MHCD1928	1.25
CD8	Quantum dot 605	*3BS*	Q10009	0.5
CD4	Quantum dot 655	*S3.5*	Q10007	0.25
CD4	PerCP-Cy5.5	*L200*	BD552838	1
IFN- γ	Alexa fluor 700	*B27*	BD 557995	2
TNF-α	PE-Cy7	*MAB 11*	BD 557647	1.25
MIP1β	PE	*D21-1351*	BD 550078	0.625
CD107a	FITC	*H4A3*	BD 555800	5
IL-2	APC	*MQ1-17H12*	BD 551383	5
IL-2	PE	*MQ1-17H12*	BD 554566	5

All antibodies were supplied by BD Biosciences except for both the Quantum dots (Invitrogen) and CD19 Pacific Blue (Caltag)

* IFN-γ - Interferon gamma, TNF-α - Tumour necrosis factor alpha, MIP 1β -Macrophage inhibitory protein one beta, IL-2 – Interleukin two

** APC-H7 – allophycocyanin- H7, PerCP-Cy5.5 – Peridininchlorophyll protein-Cy5.5, PE-Cy7 – Phycoeryhrin-Cy7, PE-Phycoerythrin, FITC – Fluorescein isothyocyanate, APC – allophycocyanin

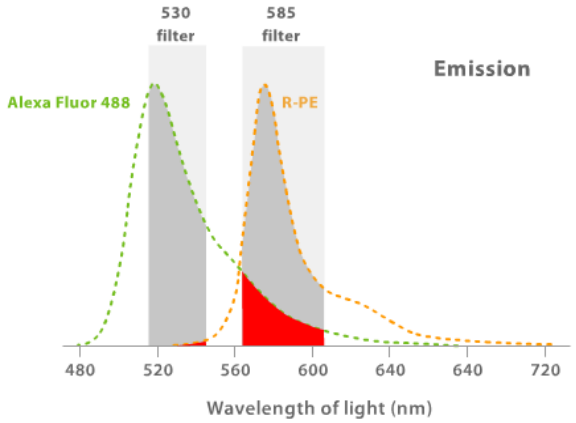

Figure 2.2: Emission spectra of two fluorochromes. Highlighted in red is the spectral overlap between the two (taken from www.probes.invitrogen.com).

This is accomplished by making a single stain of all antibodies used in the assay, i.e. one control for each fluorochrome. It is essential that each compensation control is as bright or brighter than staining on the cells of interest. This is to ensure that samples are compensated accurately so that the fluorescence spillover is corrected. Compensation controls which do not fluoresce at the same brightness as the sample will not be correctly compensated, as the fluorescent spillover of the compensation control will be less than the sample, therefore compensation will be inaccurate. All compensation was performed using FlowJo software version 8.1.3. (Treestar).

In most cases, CompBeads (BD Biosciences) were used to compensate samples. With certain fluorochromes, however, cells were required for accurate compensation, as will be discussed in Chapter 3.

2.6.2.2 Preparation of compensation beads

CompBeads Set (BD Biosciences) consists of two microparticle populations, the CompBeads anti-mouse Ig,κ (positive) and the CompBeads negative control. The positive microparticles bind to mouse κ chain immunoglobulins (antibody), resulting in a positive population, whereas the CompBeads negative control has no binding ability, therefore cannot bind to an antibody and forms the unstained population. CompBeads anti-rat set (BD Biosciences) was also used. It functions in the same manner but is specific for rat κ chain immunoglobulins. Table 2.7 lists the details of CompBeads Sets used in the assays.

To 5 ml Falcon tubes (BD Biosciences), 100 µl of Perm/Wash buffer (Table 2.5) was added. The CompBeads were vortexed thoroughly for 1 min to avoid aggregation. Subsequently, a drop of negative and positive beads was added to each tube. The corresponding volume of antibody that had been used during staining of samples was added to the tube. This was incubated at 4 °C for 15 min, and fixed by adding 100 µl of 1X CellFIX (BD Biosciences).

Table 2.7: Reagents used for compensation and staining

Compensation Kit	Components	Storage	Supplier
Anti-rat Ig,κ Compensation Beads	Beads in buffered solution containing BSA (Bovine serum albumin) and 0.09 % FBS	4 °C	BD Biosciences
Anti-mouse Ig,κ Compensation Beads	Beads in buffered solution containing BSA and 0.09 % FBS	4 °C	BD Biosciences
Live/Dead Fixable Violet Dead Cell Stain Kit	5 vials of 25 μg lyophilised dye and 1 vial of 500 μl DMSO	-20 °C	Invitrogen
Polymer Amine Beads (500 ml)	Beads in DI water and 0.1 % SDS (sodium dodecyl sulphate)	4 °C	Bangs Laboratories

2.6.2.3 Preparation of Vivid dye and pre-labelled amine beads for ViVid compensation

Live/Dead Fixable Violet Dead Cell Stain Kit (Invitrogen) (Table 2.8) was used to distinguish between viable and non-viable cells. It consists of a violet-fluorescent reactive dye that enters damaged cell membranes and reacts with the amine groups in the cytoplasm. The dye does react with surface amines but to a lesser extent, and cannot enter viable cells so does not enhance fluorescence significantly. In addition to violet, viability dyes are available in other colours, such as blue, aqua, green and red. These dyes are less problematic compared to other dead cell exclusion markers since they are not affected by permeabilization and fixing of cells (Perfetto *et al.*, 2006).

An aliquot of 40 μl of working stock (40 X 10^6 beads/ml) was added to 1660 μl PBS (Table 2.1) and centrifuged at 452 X g for 3 min. The bead pellet was resuspended in 80 μl of PBS and 20 μl of the 1:40 working stock of the violet-fluorescent reactive dye was added to this. This was incubated for 20 min in the dark at room temperature. Subsequently, 20 μl of un-labelled polymer amine beads (40 X 10^6 beads/ml) was added to the labelled beads and beads were fixed with 100 μl of 1 X CellFIX (BD Biosciences).

2.7 Flow Cytometry

Flow cytometry is a powerful technique that can be used to investigate immune responses. Characteristics of an individual cell can be identified within a cell suspension, by detecting cell surface and intracellular markers. It requires a specialised instrument, a flow cytometer, in order to measure the fluorescence of each cell.

A flow cytometer works by a pressurised fluidic system allowing cells in a suspension to be taken up through the flow cell (indicated by the arrow in Figure 2.3). During this process the cells pass the laser individually and the antibody-conjugated fluorochromes that are bound to a cell become excited by the laser and emit light at particular wavelengths, which are then directed by the mirrors and filters (optics) of the flow cytometer to detectors. These wavelengths are measured by photomultiplier tubes (PMT) or detectors that convert the optical signals into digital signals, thus allowing visualization on a computer screen.

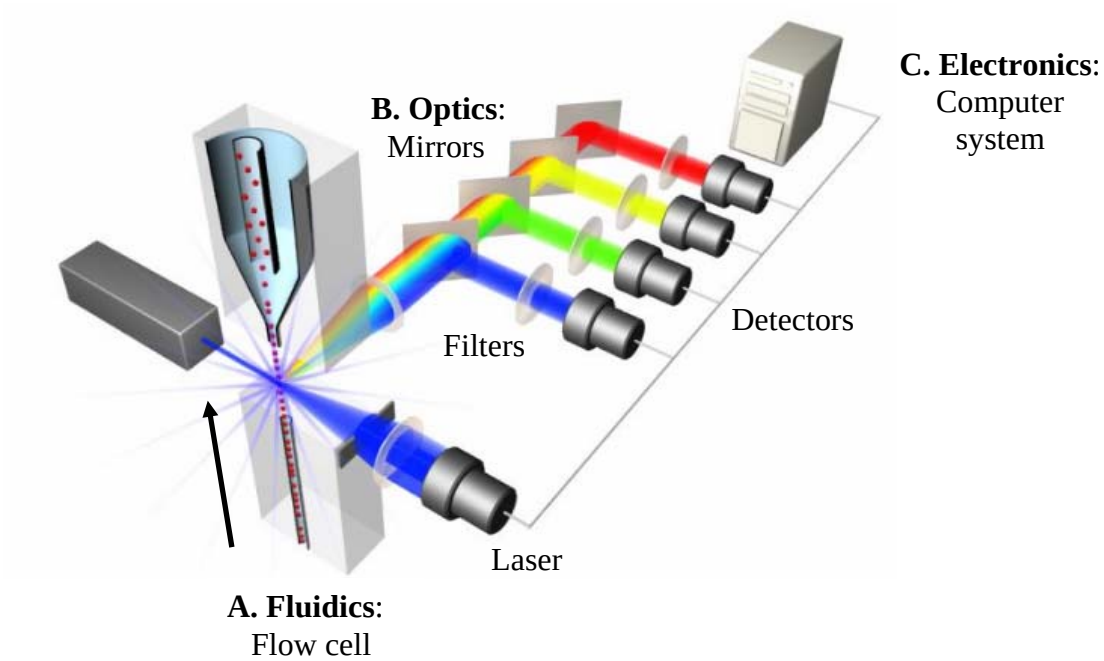

Figure 2.3: Diagrammatic representation of a flow cytometer, showing the three main characteristics of a flow cytometer, namely (**A**) fluidics, (**B**) optics and (**C**) electronics (taken from www.probes.invitrogen.com).

2.7.1 Data acquisition and gating strategy

After staining, samples were stored at 4 ºC in the dark and acquired within 24 h. Samples were acquired on the LSRII (Becton Dickinson) with FACSDiva software version 6.0 (Becton Dickinson) was used. The number of events collected was between 200000 and 1000000.

2.7.2 Analysis, including statistics

All analyses were performed using FlowJo version 8.5.2 (Treestar). GraphPad Prism (version 5.0) and Pestle (version 1.5.4) and SPICE version 4.1.6 (both from Mario Roederer, Vaccine Research center, NIAID, NIH) were used to analyse data. Statistical analysis was performed on GraphPad, version 5.0. The non-parametric Mann-Whitney U test was used to determine if the medians between two groups were significantly different. Correlations were investigated by the non-parametric Spearman rank correlation.

CHAPTER 3

OPTIMIZATION OF FLOW CYTOMETRY PANELS AND PROCEDURES

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CHAPTER 3
OPTIMIZATION OF FLOW CYTOMETRY PANELS AND PROCEDURES

This chapter describes the multiple steps required for development of a polychromatic flow cytometry panel, as well as optimization of the staining and stimulation procedures.

3.1 Optimization of a polychromatic flow cytometry panel

3.1.1 Initial considerations for development of a polychromatic flow cytometry panel

The starting point for developing a panel is knowledge of the configuration of the flow cytometer to be used. It is important to know how many lasers the instrument has and how many channels are available in order to know how many markers can be included in the panel. The LSRII (BD Biosciences) that was used in this study has three lasers (blue, violet and red) and in total has 12 channels available, as shown in the Figure 3.1.

3.1.1.1 Selection of markers

The aim of this study was to establish a five functional panel to detect HIV-specific responses in cryopreserved PBMC in HIV infection. The panel required a viability marker, since dead cells in cryopreserved PBMC can bind antibodies non-specifically and increase background responses (Horton *et al*., 2007). Antibodies against CD14 and CD19 were also included to exclude monocytes and B cells, in order to further reduce background responses, thereby increasing the sensitivity of the assay. Since this project was investigating T cell responses, a CD3 marker was required and both T cell subset markers, CD4 and CD8, were included. LSRII channels were limited, and thus no T cell differentiation markers could be included in the panel, which would have been informative.

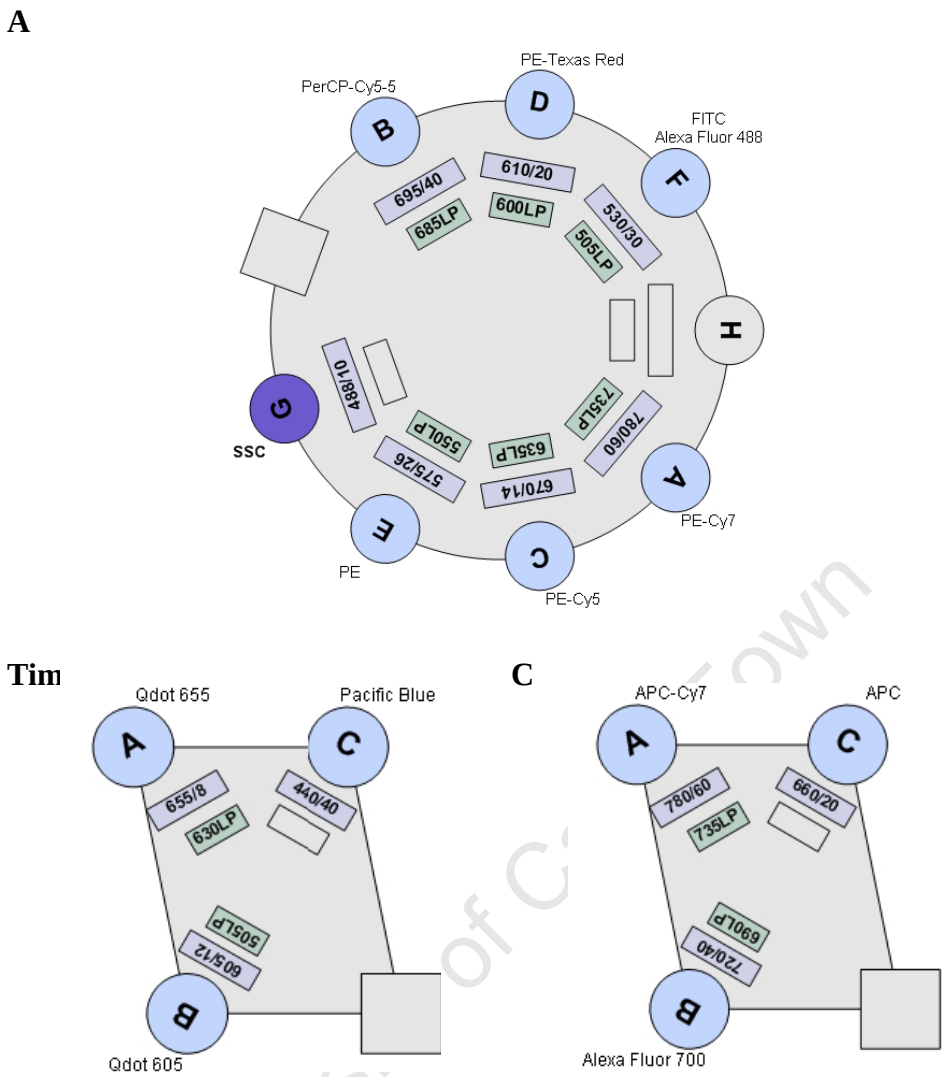

Figure 3.1: Configuration of the South African Tuberculosis Vaccine Initiative (SATVI) LSRII (BD Biosciences) showing the number of channels for each laser. (**A**) Blue laser, an octagon, and the 2 trigons (**B**) Violet, and (**C**) Red.

Five functional markers were included, namely cytokines and a degranulation marker. The five functions that were to be investigated were IFN-γ, IL-2, TNF-α, MIP-1β and CD107a. As explained in Chapter 1 (section 1.2.1.), IFN-γ is an antiviral cytokine and has been implicated with control of diseases, IL-2 is required for T cell proliferation upon antigen stimulation, TNF-α production is required for the recruitment of neutrophils and monocytes, while MIP-1β is a chemokine, and is responsible for the recruitment of lymphocytes and monocytes (as reviewed in Clore and Gronenborn,

1995). MIP-1β binds the HIV co-receptor CCR5, thus may compete with HIV for binding to $CD4^+$ T cells expressing this molecule. CD107a is a surrogate marker for cytotoxicity. During the degranulation process, CD107a is transiently expressed on the inner membrane of the cytotoxic granule (Suni *et al*., 2005), which then becomes exposed during degranulation and it can be detected on the surface of the cell (Betts *et al.,* 2003).

3.1.1.2 Selection of fluorochromes

Antibody selection is the next step in building a panel, as antibodies chosen should ideally be those that are bright, give the best separation, but also limit fluorescent spillover. One limiting factor in developing a panel is that not all markers are available commercially on all fluorochromes.

In polychromatic flow cytometry, there is a greater choice of fluorochromes compared to those available for the FACS Calibur, since there are more lasers and hence more fluorescent channels available. However, even with a greater number of choices, LSRII configurations are different and panels are not readily transferable from one instrument to another. Taking this into account, one can try to include new fluorochromes, such as Quantam dots (Qdots) and tandem dyes that can't be detected on a FACS Calibur. It is useful to use Qdots as the narrower emission spectra leads to less fluorescence being read in other detectors (see section 3.1.1.3) and hence reduced spillover. Tandem dyes have two fluorochromes bound together, and are excited by the wavelength of the first fluorochrome but emit at the wavelength of the second fluorochrome. For this reason, APC and APC-H7 can be used in the same panel. Tandem dyes can be unstable and degrade, thereby increasing fluorescent spillover, if exposed to light, high temperatures and paraformaldehyde-based fixatives (www.bdbiosciences.com). The H7 molecule is an analogue of Cy7 and APC-H7 has been engineered to be more stable than APC-Cy7.

Once markers of interest have been chosen it is easier to then work from an existing, optimized panel and a list of proposed markers can then be made. Table 3.1 lists an existing panel in use in the South African Tuberculosis Vaccine Initiative (SATVI)

laboratory that was used as a base to build a functional panel. Since the viability marker fluoresces in the Pacific Blue channel (and markers CD14 and CD19 were also placed on Pacific Blue), CD3 was moved to the APC-H7 channel. An attempt was made to include CD4 and CD8 on Qdots to maximise the use of the violet laser. CD107a was placed on the FITC fluorochrome as anti-CD107a-PE has been shown to have a higher background response in unstimulated $CD8^+$ T cells (Betts *et al*., 2003), IL-2 was placed on APC, permitting MIP-1β to be in the PE channel, TNF-α in the PE-Cy7 channel, leaving IFN-γ on Alexa 700.

Table 3.1: Antibodies of interest

Detector	Optimized Panel	Proposed Panel
SSC		
PE-Cy7	CD45RA-PE-Cy7	TNF-α-PE-Cy7
PerCP-Cy5.5	CD8-PerCP-Cy5.5	CD8-PerCP-Cy5.5/CD4-PerCP-Cy5.5
PE-Cy5, PerCP-Cy5	-	-
Alexa 610PE, ECD, TxR	-	-
PE	CD27-PE	MIP-1β-PE
FITC, Alexa 488, CFSE	IL-2-FITC	CD107a-FITC
Qdots 705-525	-	-
AmCyan	-	
Pacific Blue	CD3-Pacific Blue	Vivid/CD14/CD19-Pacific Blue
Cascade Blue	-	CD4-Qdot 655
or Qdots	-	CD8-Qdot 605
APC-Cy7, Alexa 750	-	CD3-APC-H7
Alexa 700	IFN-γ-Alexa 700	IFN-γ-Alexa 700
APC, Alexa 647	CCR7-APC	IL-2-APC
or Qdots 705-640	-	-

3.1.1.3 Spectral overlap between fluorochromes

A critical factor to take into consideration when developing a polychromatic flow cytometry panel is the spectral overlap between fluorochromes. Since there are many antibodies that are used in a single sample, it is useful to choose, as far as possible, fluorochromes with minimal fluorescence spillover and hence compensation requirements. A useful website that shows the excitation and emission spectra of fluorochromes is the Invitrogen Spectraviewer (www.invitrogen.com). At this resource, five different fluorochromes can be plotted together to give an idea of spectral overlap, as shown in Figure 3.2. This can be used to broadly guide antibody

selection. From this it can be seen that the use of APC and Qdot 655 may be highly problematic as their emission spectra completely overlap (Figure 3.2A).

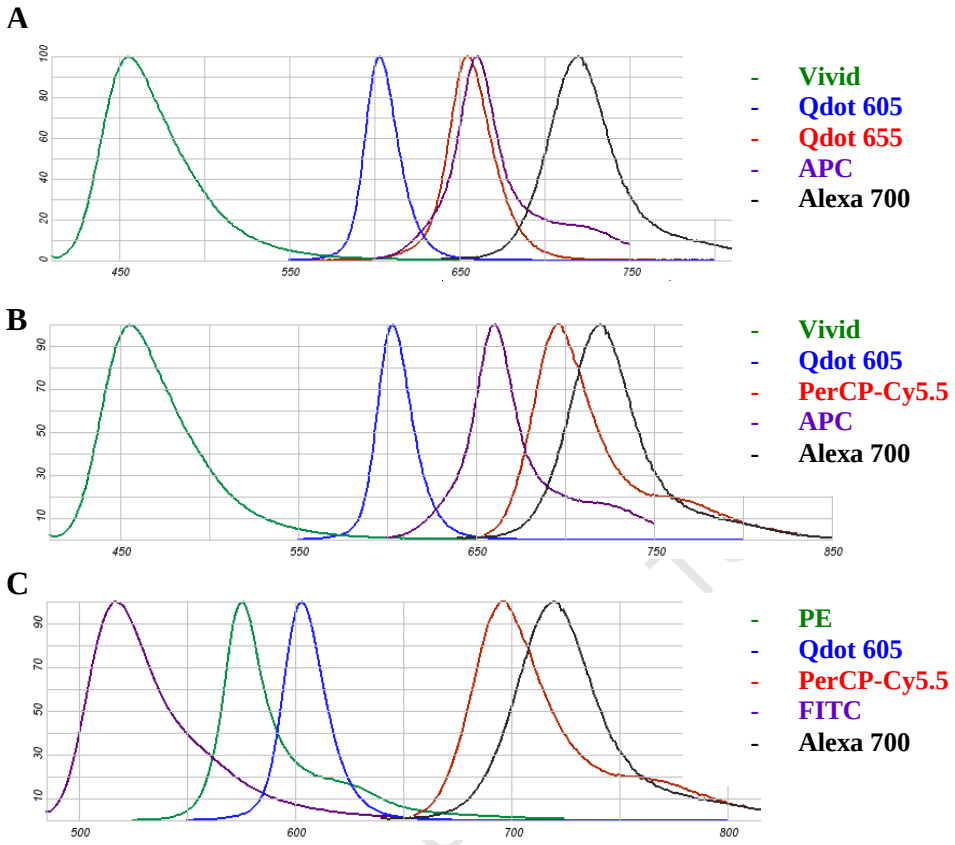

Figure 3.2: Emission spectra of fluorochromes using Spectraviewer (Invitrogen). (**A**) shows that Qdot 655 overlaps with APC and in (**B**) PerCP-Cy5.5 may be a better candidate to reduce this overlap. (**C**) shows the degree of Qdot 605 overlap between fluorochromes of interest.

3.1.2 Titration of antibodies

Titrations were performed in order to determine the optimum volume of antibody to use in the assays. It is crucial for titrations to be performed on all antibodies to ensure that they are used at a volume that provides the best separation and lowest background, the latter particularly important for antibodies binding to cytokines (Lamoreaux *et al*., 2006). Often the volume recommended by suppliers is not optimal.

Every antibody is different in the sense that they each have different amounts of fluorochrome conjugated to the antibody and each clone has different binding

affinities. Even high volumes may not provide the best separation between the negative and positive populations. The optimum volume to use in an assay is also dependent on the staining procedure, i.e. surface or intracellular staining. The optimal amount of antibody was determined by measuring the median fluorescent intensity (MFI) of the positive and negative population in a titration series. A titration curve was then drawn, plotting the MFI versus the antibody volume. The positive population curve was the one of interest, in order to observe when saturation occurs, shown by the curve levelling out. The optimum volume was taken as the volume just prior to saturation. Examples are described below.

3.1.2.1 Titration of amine reactive dye (viability marker)

The violet reactive fluorescent viability marker ('Vivid'; Invitrogen) was chosen as the dead cell marker. Similar to all antibodies used in an assay, Vivid also required a titration. Figure 3.3 illustrates the titration of Vivid, where the positive population represents dead cells, and the negative population is the live cells. The 1:64 dilutions was chosen since there is a clear separation between positive and negative populations and the negative population is not too bright, as is apparent in lower dilutions. Vivid staining was performed, before surface staining, due to Vivid possibly binding to the amine groups of the other antibodies in the surface staining mix.

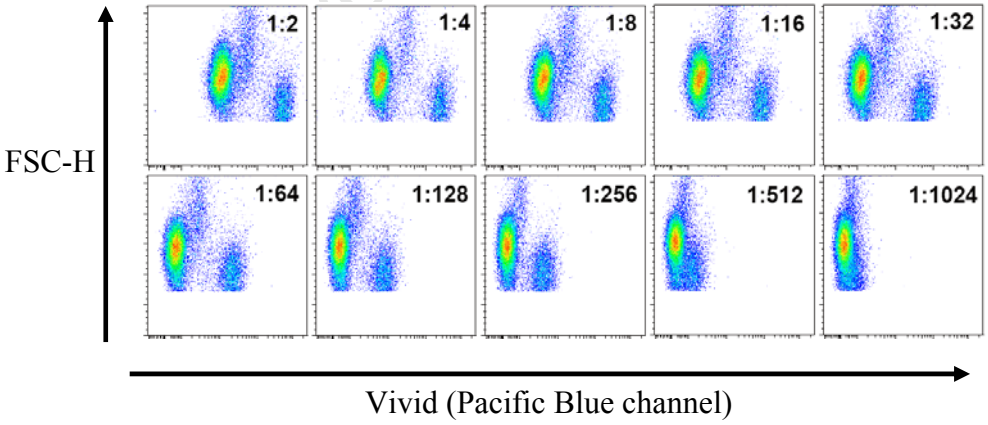

Figure 3.3: Flow plots representing Vivid titration. A two-fold dilution series was prepared and PBMC were stained, permeabilized and fixed before acquisition took place. The value on the top right hand corner of each flow plot represents the dilution of Vivid and populations shown were gated on singlets.

3.1.2.2 Titration of cell surface markers

Subsequent to viability staining, staining for cell surface markers is the next step in an ICS assay. Cells were stained with surface antibodies as described in chapter 2. As an example, titration of the CD8-Qdot 605 antibody is shown in Figure 3.4. At lower concentrations of the antibody no positive population could be identified. The MFI graph revealed that 0.5 µl of this antibody was the optimum to use in assays.

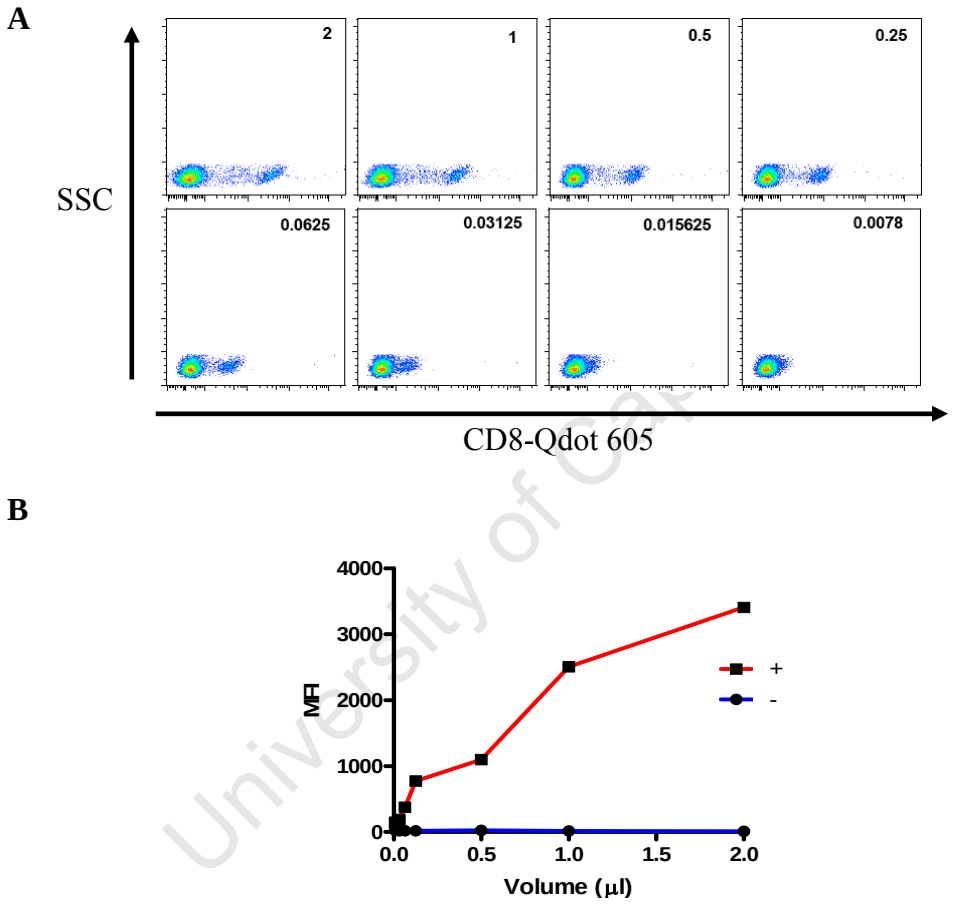

Figure 3.4: CD8-Qdot 605 titration. (**A**) represents the flow plots, where the volume of antibody added is shown on the top right hand corner of each plot. A two-fold dilution series was performed and PBMC were surface-stained, permeabilized and fixed. Cells were gated on singlets and lymphocytes. (**B**) shows the titration curve in which the MFI was calculated for the negative (-) and positive (+) population for all volumes tested.

3.1.2.3 Titration of intracellular markers

Staining for intracellular markers requires the cells to first be permeabilized in order for the antibody to enter and bind to its corresponding marker, as described in chapter 2. CD3 is a surface marker, however it was stained intracellularly due to internalization that may occur during stimulation (Luton *et al*., 1994). The titration results are shown in Figure 3.5. The optimum volume to use for staining of CD3 using anti-CD3-APC-H7 was chosen to be 2.5 µl. As can be seen from Figure 3.5, CD3 separation was better at lower (1.25-5 µl) than higher amounts (10-20 µl) of antibody. This illustrates just how critical it is to perform antibody titrations.

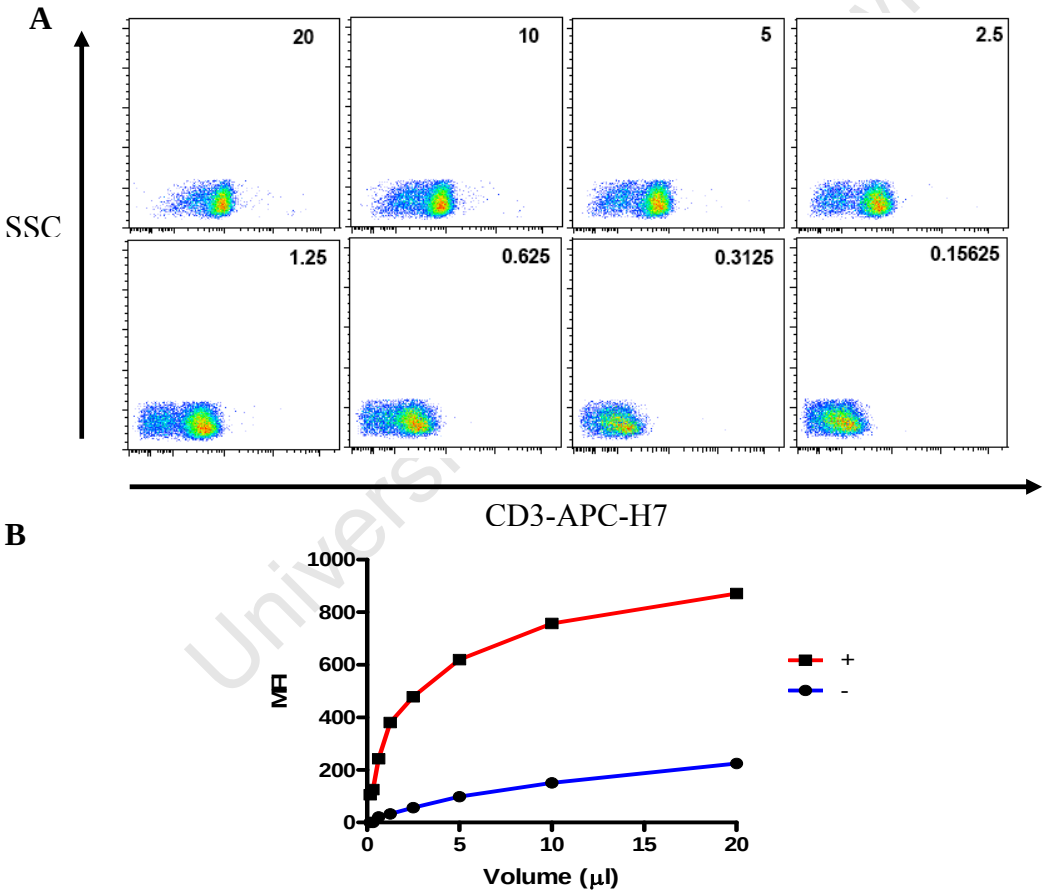

Figure 3.5: CD3-APC-H7 titration. (**A**) shows the flow plots with the volume of antibody added shown on the top right hand corner of each plot. A two-fold dilution series was performed and PBMC were permeabilized, intracellularly stained and fixed. Cells were gated on singlets and lymphocytes. (**B**) shows the titration curve in which the MFI was calculated for each negative (-) and positive (+) population.

All cytokines were stained intracellularly, and the TNF-α-PE-Cy7 titration is shown as an example in Figure 3.6 (over the page). The MFI plot reveals that the optimum volume to use in a 50 µl staining mix is 2.5 µl. When titrating anti-cytokine antibodies, it is necessary also to take into account background responses (responses in unstimulated cells; Figure 3.6D), to ensure that the volume selected gives a low background response.

3.1.2.4 Signal/noise ratio to determine the optimum antibody volume

Another way in which the optimal antibody volume can be determined is by calculating the MFI ratio (Lamoreaux *et al*., 2006). In this case, the positive population is the signal and the negative population is the noise. The ratio is the signal (MFI positive population) divided by the noise (MFI negative population). The highest ratio value obtained is the optimum volume to use, as seen in the Figure 3.7, which was 2.5 µl, as obtained with the previous method.

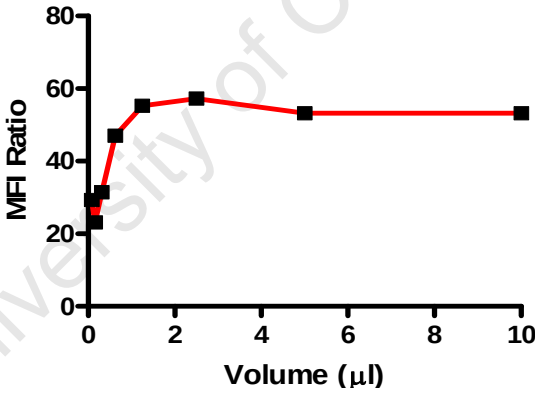

Figure 3.7: Signal/noise ratio titration curve of TNF-α-PE-Cy7. The ratio was determined by the signal divided by the noise.

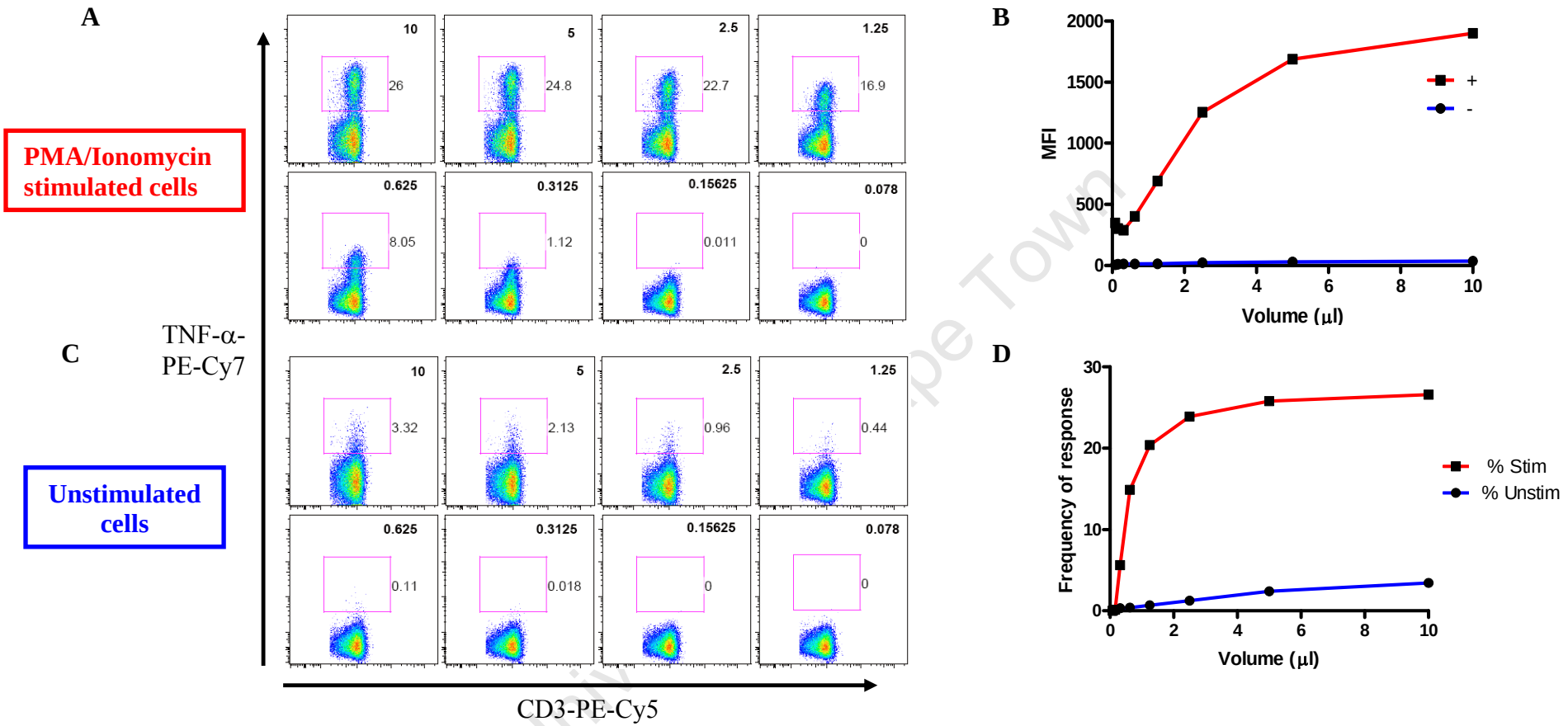

Figure 3.6: TNF-α PE-Cy7 titration. Shown in (**A**) and (**C**) are the flow plots with the volume of antibody in the top right hand corner of each plot. A two-fold dilution series was performed and PMA/ionomycin and unstimulated PBMC respectively were permeabilized, intracellularly stained with anti-CD3-PE-Cy5 and TNF-α-PE-Cy7, and fixed. Cells were gated on singlets, lymphocytes and $CD3^+$ T cells. (**B**) shows the titration curve in which the MFI was calculated for the negative (-) and positive (+) population for all volumes tested. (**D**) shows the percentage of TNF-α in PMA/ionomycin stimulated and unstimulated PBMCs to show background cytokine production at each volume tested.

3.1.3. Building a polychromatic flow cytometry panel

Building a panel involves adding antibodies consecutively to determine whether populations stain optimally and whether particular fluorochromes can be used together on selected markers. This section discusses different antibodies and certain dyes that increase the accuracy of the results obtained, such as a viability marker. It also describes important controls (Fluorescence Minus One) for developing a polychromatic panel. Initially, a nine colour panel was to be optimised, but due to limitations of the LSRII that was used as well as time constraints, an eight colour panel incorporating the measurement of four functions was developed.

3.1.3.1. Inclusion of a viability marker

The violet fluorescent reactive dye (Vivid) was chosen to discriminate between dead and live cells. Since cryopreserved PBMC samples were used in this study, it was prudent to include such a dye to exclude dead cells and improve quality of data by reducing background. Figure 3.8 shows that the background responses were decreased for both $CD4^+$ and $CD8^+$ T cells when Vivid was included in the staining procedure. Typically the viability of cryopreserved PBMC used in this study ranged from 57 %-94 % (median 74 %).

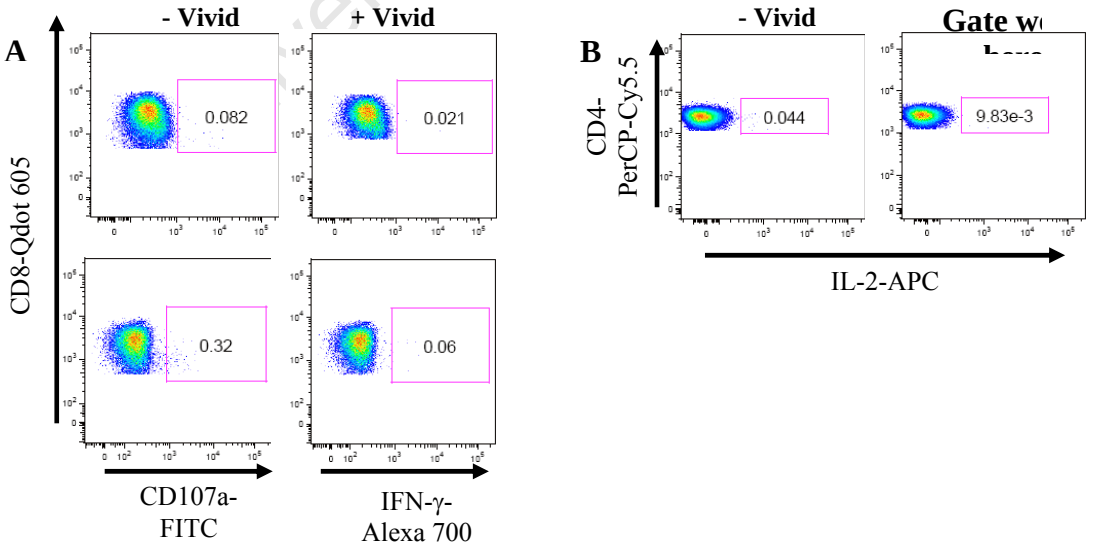

Figure 3.8: A viability marker reduces non-specific background. The percentages of cytokines produced by unstimulated **(A)** $CD8^+$ and **(B)** $CD4^+$ T cells when Vivid is included (right panel) and excluded (left panel).

3.1.3.2 Inclusion of a "dump" channel

The Pacific Blue channel, which also includes the viability marker, was used as a dump channel, not only to exclude dead cells from the analysis, but also to exclude monocytes ($CD14^+$ cells) and B cells ($CD19^+$ cells; Figure 3.9). This also aided in improving the quality of data obtained in terms of obtaining true T cell cytokine responses.

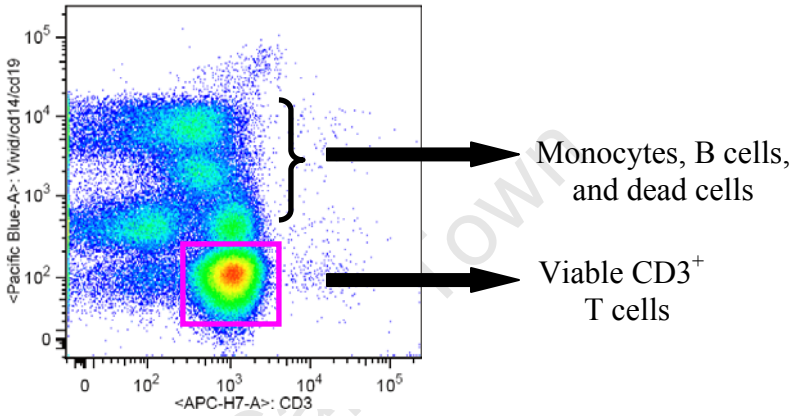

Figure 3.9: Use of a dump channel. The different populations in the Pacific Blue channel were used to exclude B cells, monocytes and dead cells in the analysis. Cells were gated on singlets.

3.1.3.3 Inclusion of Quantum dot fluorophores

Quantum dots, commonly known as Qdots, are tiny inorganic crystals (nanoparticles) that consist of a semiconductor core coated with a zinc layer to stabilize the core (Chattopadhyay *et al*., 2006). The name of each Qdot refers to the centre emission wavelength. These fluorophores have a narrow emission wavelength and are more photostable compared to organic fluorophores and are therefore excellent candidates for polychromatic analysis to reduce spectral spillover (Figure 3.10). Qdots are best excited by the violet or UV laser, however they are excited by other lasers and their emission spectra remain the same. It is extremely important to titrate Qdot antibodies accurately, due to their brightness. Another important point when preparing a staining master mix including Qdots is to centrifuge the mixture to remove aggregates, which may appear very bright on the flow cytometry plot.

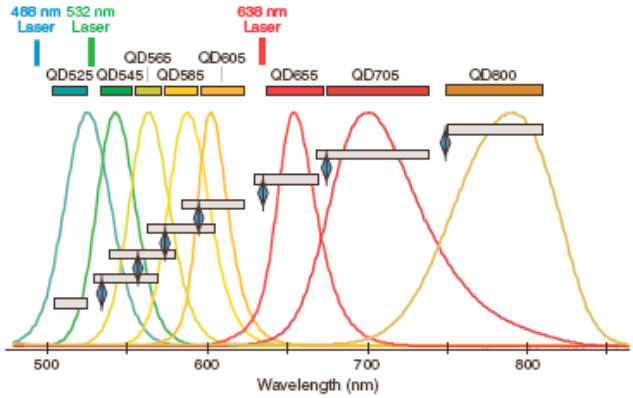

Figure 3.10: Emission wavelength of different Qdots (taken from Chattopadhyay *et al*., 2006).

The LSRII that was used in this study had a trigon violet laser, and since Pacific Blue was used as the "dump channel", two channels remained for Qdot use, namely Qdot 605 and Qdot 655. These were included on the CD4 and CD8 markers, and staining is shown in Figure 3.11. Whilst the use of the two Qdots showed good separation between CD4 and CD8, and they were compatible for use with CD3-APC-H7 and the Pacific Blue channel, subsequent experience in the SATVI laboratory showed that Qdot 655 was incompatible with the APC channel due to a large degree of spillover with this LSRII configuration, and it had to be dropped from further development.

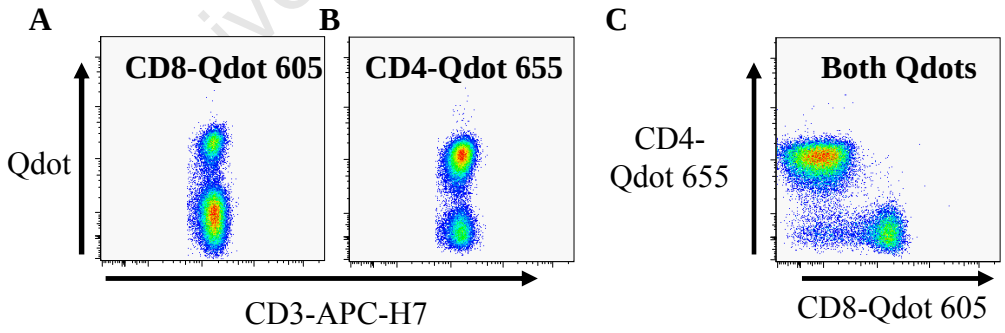

Figure 3.11: Flow plots of Quantum dot staining. (**A**) CD8-Qdot 605 and (**B**) CD4-Qdot 655 and (**C**) cells stained with both anti-CD4-Qdot 655 and anti-CD8-Qdot 605. A PBMC sample was surface stained (Vivid, CD4, and CD8, permeabilized, and intracellularly stained for CD3 and fixed. Flow plots represent cells gated on singlets, lymphocytes and then $CD3^+$ viable cells.

3.1.3.4 Fluorescent Minus One (FMO) controls

FMOs are used to determine whether the panel of fluorochromes chosen can be used in combination with one another. When developing a flow cytometry panel that includes many antibodies being used in conjunction, it is vital to make sure that there is no major spillage of one fluorochrome into another channel, thereby compromising its detection. This is achieved by performing FMOs (Figure 3.12A). Plotting the FMO channel versus all the other fluorochromes reveals any fluorescence spillover. To determine this experimentally, cells were stained with all antibodies in the panel excluding the one of interest. This is performed for all antibodies in the panel. FMOs are particularly important for cytokine markers, due to the fact that they are rare events, and phenotypic markers are brighter as they are highly expressed. Thus, it is vital to make sure that the fluorescence of bright phenotypic markers are not spilling into a cytokine channel so that there is optimal and accurate detection of cytokine markers. An example is shown here in Figure 3.12B, where there is no spillover of CD8-Qdot 605 into IFN-γ-Alexa 700 and IL-2-APC channels.

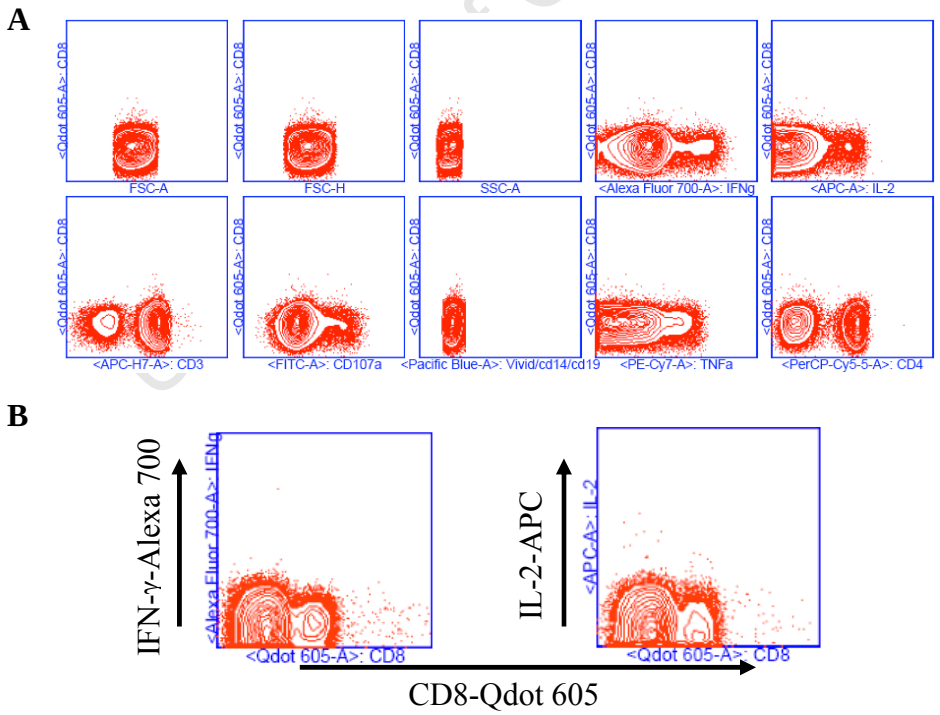

Figure 3.12: Flow plots of FMOs. (**A**) CD8-Qdot 605 FMO, showing each channel plotted against FMO channel (**B**) IFN-γ-Alexa 700 and IL-2-APC FMOs, showing no fluorescent spillover of CD8-Qdot 605 into either of the two cytokine channels.

Another important use for an FMO is to distinguish between negative and positive populations that are not well separated due to continuous expression of markers, such as memory markers. In this way gates are set using the FMO, and then applied to the sample.

3.1.3.5 Problems encountered during optimization

Originally a nine colour panel, with five functions, was to be optimised. Cells expressing cytokines are rare events, therefore staining for these cells should be at optimum levels to allow for accurate and sensitive detection. To evaluate this experimentally, the frequency of cells expressing cytokines is compared with and without the addition of a new antibody, to determine whether it influences the response obtained.

During this study, anti-MIP-1β-PE had to be removed from the panel as IL-2 responses drastically decreased when stimulated with SEB, as illustrated in Figure 3.13. This reduction in APC signal was not a result of compensation, as all compensation percentages were below 30 % as determined from analysis in FlowJo (Treestar). The amount of IL-2 detected did not differ substantially when IL-2 alone or three additional cytokine-specific antibodies were added (Figure 3.13A and B). However, addition of the MIP-1β PE-conjugated fluorochrome resulted in a near complete abrogation of the IL-2 response (Figure 3.13C). It is therefore vital to perform such analyses during development of a panel to detect incompatibility between markers and fluorochromes. Due to time constraints, the nine colour functional panel could not be further optimized to include a MIP-1β antibody, and MIP-1β was dropped from the panel.

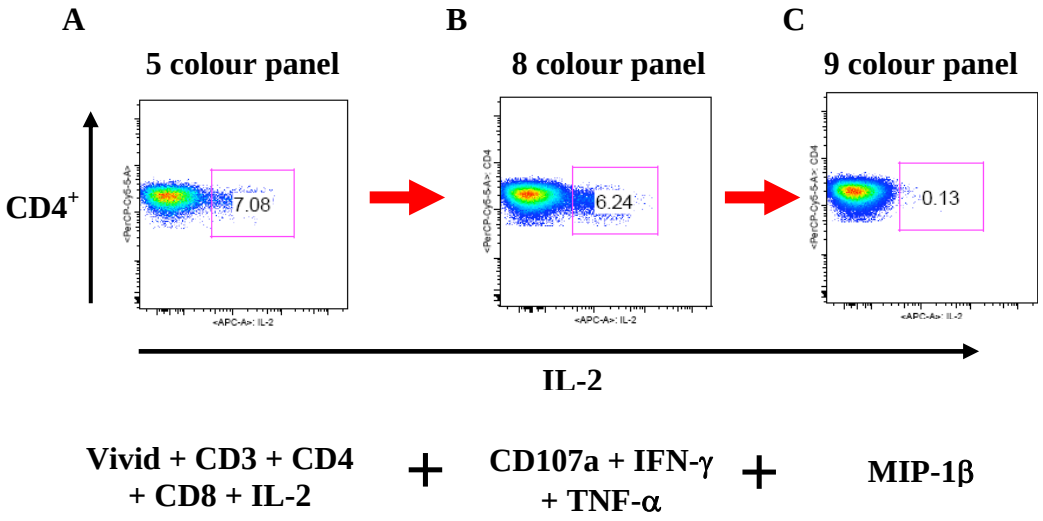

Figure 3.13: Incompatibility of particular fluorochromes leads to a decrease in IL-2 detection. (**A**) PBMC stained with CD3-APC-H7, CD4-PerCP-Cy5.5, CD8-Qdot 605 and IL-2-APC antibodies. (**B**) Addition of IFN-γ-Alexa 700, CD107a-FITC and TNF-α-PE-Cy7 antibodies. (**C**) Addition of MIP-1β-PE antibody.

3.1.3.6 Validating the polychromatic flow cytometry panel (eight colours)

MIP-1β-PE was dropped from the panel, leaving a four functional flow cytometry panel. It was then necessary to confirm that this eight colour panel was optimized in terms of cytokine detection. To determine whether percentages were influenced upon addition of fluorochromes, a sample was stimulated with SEB and stained with the full eight colour panel, or with all the surface markers and just one of the functional markers (Figure 3.14). Results obtained from this analysis showed that percentages were similar in all panels compared to the full eight colour panel, and that no fluorochrome was influencing another channel in which responses were decreased significantly, thus validating the eight marker polychromatic panel.

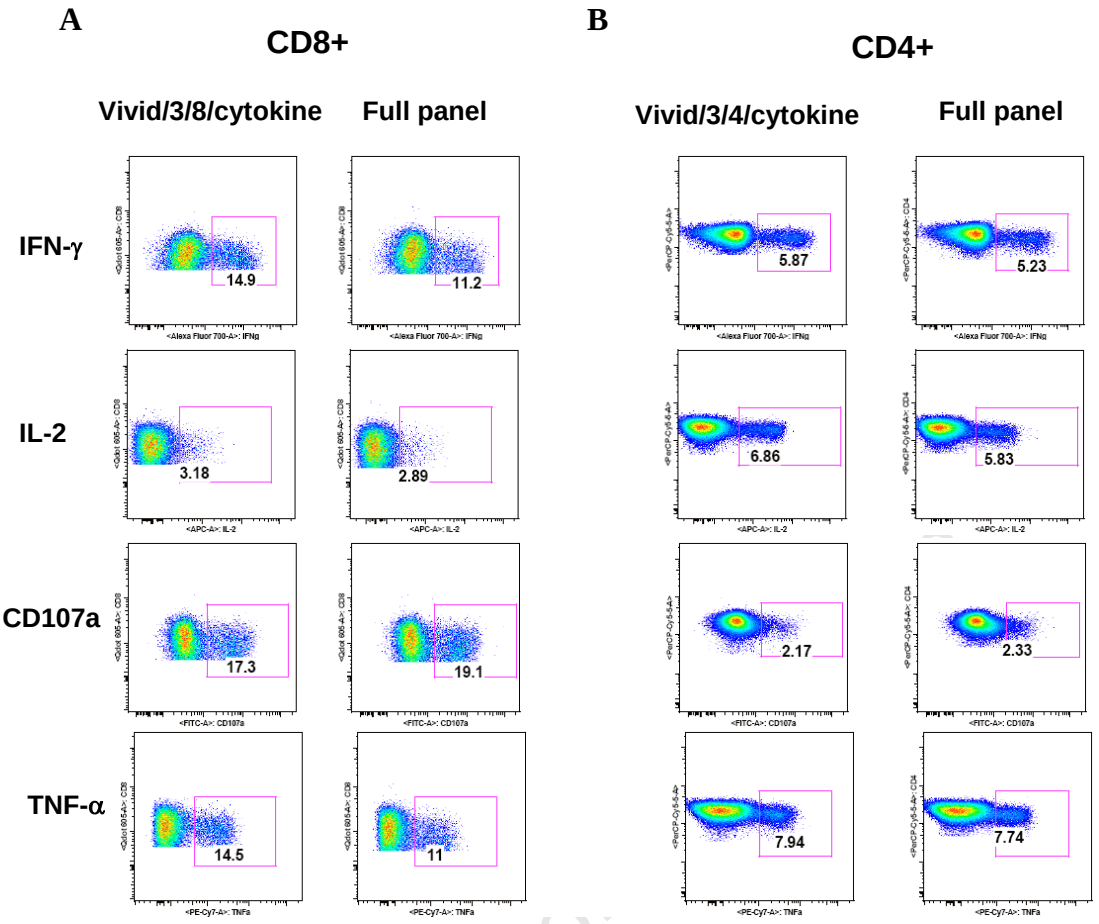

Figure 3.14: Optimisation of a four functional panel. Showed in (**A**) are $CD8^+$ cytokine responses and in (**B**) $CD4^+$ cytokine responses. PBMC were stained with Vivid, CD8, CD4 or both, and intracellularly stained with CD3 and cytokine antibodies, either individually or in combination.

3.1.3.7 Gating strategy

Figure 3.15 shows the gating strategy for detecting cytokine responses using the optimized polychromatic panel. There are a number of ways in which gating can be performed, and it is important to establish a gating strategy for optimal analysis and visualisation of the data. For instance, T cells that produce cytokine may down-regulate CD3 and CD8 expression, thereby becoming dim cells. It is important to bear this in mind since gating $CD4^+$ cells versus $CD8^+$ cells will exclude these dim cells from the CD8 gate and underestimate responses. To overcome this, surface markers were gated versus a cytokine to correct for these dim cells (as shown by the black arrow in Figure 3.15).

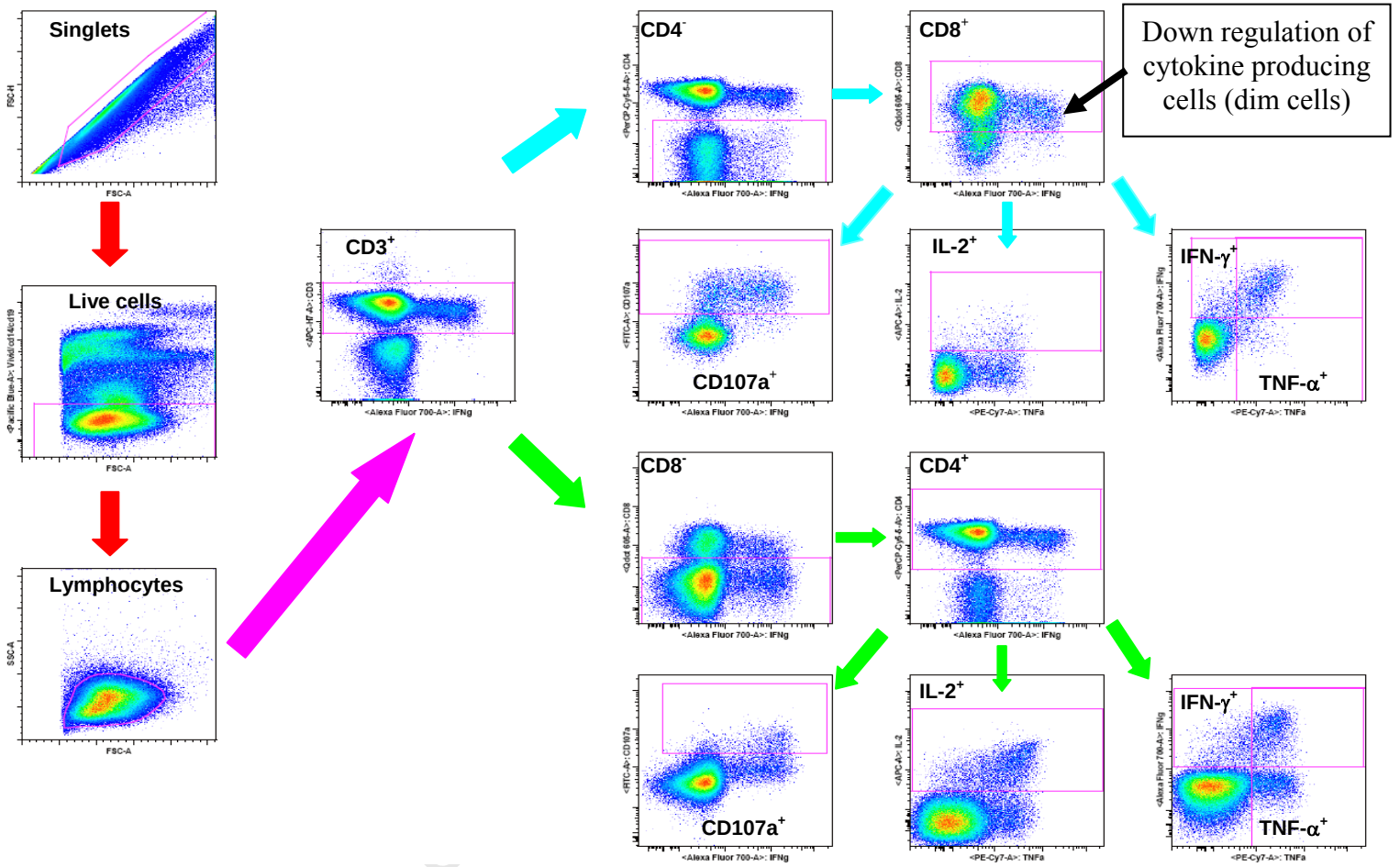

Figure 3.15: Gating scheme used for the polychromatic flow cytometry panel. Cells were first gated on singlets, live cells and then lymphocytes. From this gate, $CD3^+$ cytokine-producing T cells were gated, and then further gated on $CD4^+$ and $CD8^+$ T cells. Gates for each of the four functions were set based on the negative control and kept constant for all samples analysed.

Gating in this manner does not distinguish between $CD4^+$ and $CD8^+$ double positive T cells, as with a $CD4^+$ versus $CD8^+$ plot. Thus, gating on $CD4^-$ and $CD8^-$ (after the $CD3^+$ gate) and then $CD8^+$ and $CD4^+$ respectively ensured that these two subsets were differentiated and double positive cells are excluded.

Sometimes there may be interference in terms of bubbles or a small blockage in the flow cell during acquisition, which may not be noticed during this process. It is worthwhile to examine a time plot when analysing data to exclude any blocks which may have caused shifts in fluorescence. As illustrated in Figure 3.16, during the beginning of acquisition this occurred, which caused inconsistent acquisition. A time gate was therefore included to exclude these events.

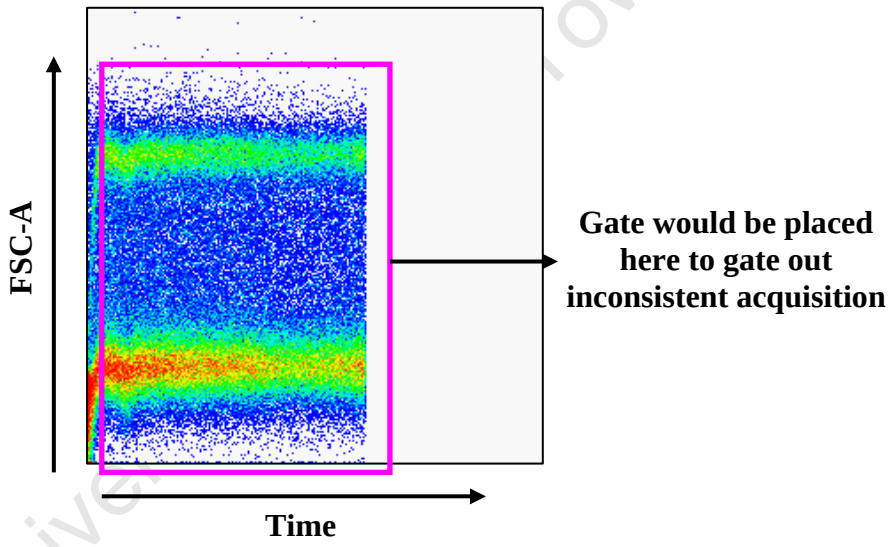

Figure 3.16: A time plot to examine consistency during acquisition of sample. The pink block indicates cells that were gated.

3.1.4 Determination of a cut-off value for positivity

There are a number of ways of determining what constitutes a positive response. For this study, a threshold value was used. This was performed by analysing the background cytokine responses of 20 HIV-infected individuals (Figure 3.17). All medians were below 0.05 % for both $CD4^+$ and $CD8^+$ T cell subsets. This threshold was also obtained for the acute infected cohort. Therefore, a threshold of 0.05 % was chosen and a positive response was taken as net percentage (subtraction of the background unstimulated sample value) and > 0.05 %. All net responses that were ≤ 0.05 % were set to zero. In polyfunctional analysis where cells producing more than one cytokine were measured, this threshold was reduced to 0.0 1%, since median background responses all fell below this value.

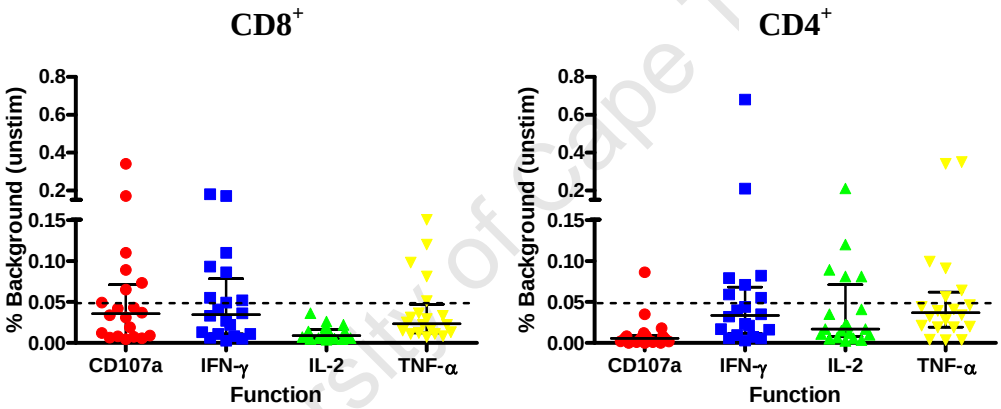

Figure 3.17: Establishment of a cut-off value for the determination of positive HIV-specific responses. These graphs represent 20 HIV-infected unstimulated responses of the four functions examined for (**A**) $CD8^+$and (**B**) $CD4^+$ T cells. The solid black lines represent the median of responses for each cytokine and the dotted black line represents the cut-off chosen (0.05 %).

3.1.5 Compensation requirements

As mentioned in chapter 2, a compensation control consists of a sample (usually cells) stained with only one antibody conjugated to a fluorochrome (a single stain). However, compensation beads are available and can be used instead of cells, and are extremely useful when cells are limited. When using these beads, it is essential to

make note of the species of antibody in use. There are different compensation beads for each species of antibody, i.e. if your antibody is from mouse then, anti-mouse compensation beads are required.

Compensation controls need to be brighter than stained samples, as described in chapter 2. To determine whether beads stain brighter than, or as bright, as cells, PBMC were stained singly with each antibody used in the panel, and single-stained beads were also prepared. The fluorescence of the cell and bead stain with the same antibody was plotted together to visualize fluorescence (Figure 3.18). The three antibodies that required cells for compensation were PE-Cy7, APC and Alexa 700, since the positive population of single stained cells (second peak) had a brighter fluorescence compared to beads. Thus, singly-stained cells were used for these fluorochromes for accurate compensation.

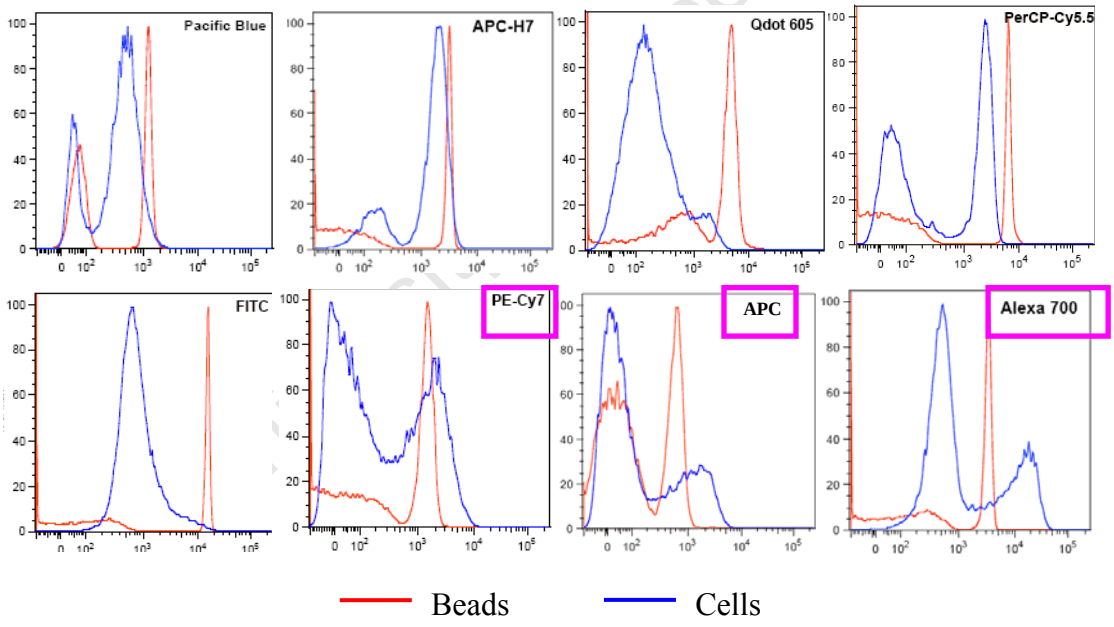

Figure 3.18: Comparison of the fluorescence of single stained cells and single stained compensation beads for all 8 colours in polychromatic flow panel. Beads staining peaks are show in red and cells in blue. PE-Cy7, APC and Alexa 700 required cells for compensation.

3.2 Optimization of the stimulation protocol

Cytokines are produced in response to a stimulus, and therefore it is important to ensure that the stimulation protocol is optimal. During the stimulation procedure, a number of reagents are added and the sample is incubated for a particular time period. Since there were five functions to be detected, and each with potentially different kinetics of production and intracellular transport, the stimulation process was optimized.

3.2.1 Transport inhibitor selection: BFA versus Monensin

The majority of studies use Brefeldin A (BFA) as the protein transport inhibitor, which blocks transport from the endoplasmic reticulum to the golgi apparatus (as reviewed in Klausner *et al*., 1992), hence trapping cytokines intracellularly. Since CD107a (a marker for degranulation) was included as one of the functions investigated, the addition of monensin was required (Betts *et al*., 2003). However, TNF-α detection is greater with BFA compared to monensin (O'Neil-Andersen and Lawrence, 2003). Therefore, a comparison between these two protein inhibitors was performed to investigate the effect on all five functions.

The results revealed that the combination of both inhibitors increased MIP-1β and TNF-α detection, the latter substantially (Figure 3.19). However, IL-2 levels were decreased slightly whilst CD107a and IFN-γ remained fairly constant when one or both inhibitors were used. Thus, both inhibitors were included in the stimulation assay.

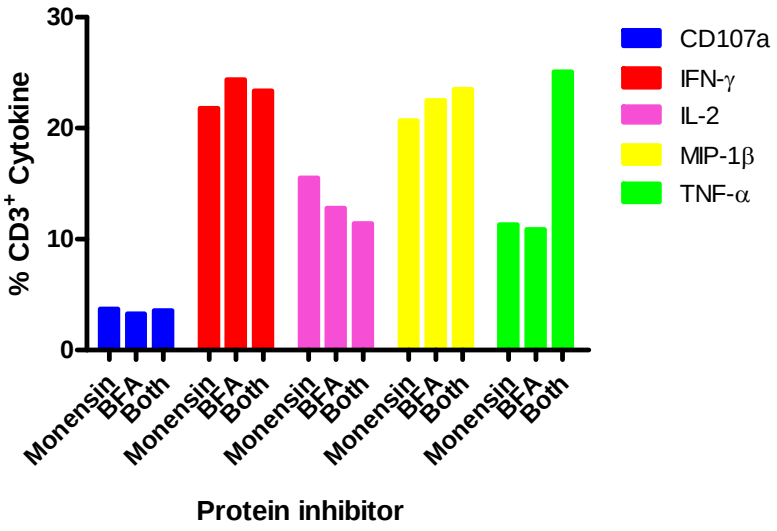

Figure 3.19: The effect of BFA and monensin on cytokine detection. A PBMC sample was stained and gated on lymphocytes. Shown here are the $CD3^+$ T cell frequencies of five functions investigated, when either one or both of the transport inhibitors were included in the stimulation assay.

3.2.2 Determination of the incubation period for stimulations

The duration of the incubation period is an important factor to be investigated as cytokines may be produced at different times in response to an antigen. For example, some may be produced early during stimulation period and remain at peak levels for a number of hours, whereas others may require more time before they can be detected. Since five functions were initially being investigated in this study, a 16 hour stimulation time course was performed and all five markers examined. Two HIV-uninfected PBMC samples were incubated at 37 °C and every 2 h samples were stimulated and removed from the incubator and placed at 4 °C for staining and acquisition the following day. Samples were stimulated with immobilised anti-CD3 monoclonal antibody (BD Biosciences) as described in Veenstra *et al.* (2007). BFA and monensin were added at the start of incubation period as well as anti-CD107a-FITC antibody. It was necessary to include anti-CD107a-FITC antibody during stimulation as this marker is transiently expressed during stimulation (as reviewed in Fukuda, 1991) and a previous study showed that inclusion of a CD107a antibody during stimulation greatly increased detection compared to surface staining during ICS (Betts *et al.*, 2003).

The kinetics of five functions for both $CD8^+$ and $CD4^+$ T cells are shown in Figure 3.20. IL-2 production peaked at 12 h and 10 h for $CD8^+$ and $CD4^+$ T cells, however the other functions peaked at 6 h for $CD8^+$ T cells, and 16 h for $CD4^+$ T cells. Thus, a 16 h stimulation period was required, since the other cytokine levels did not decrease significantly at 16 h. It is important to note that CD107a levels were not significantly reduced during the 16 h stimulation process, thus it appears that an incubation of longer than 6 h does not affect the ability of the CD107a antibody to stain optimally.

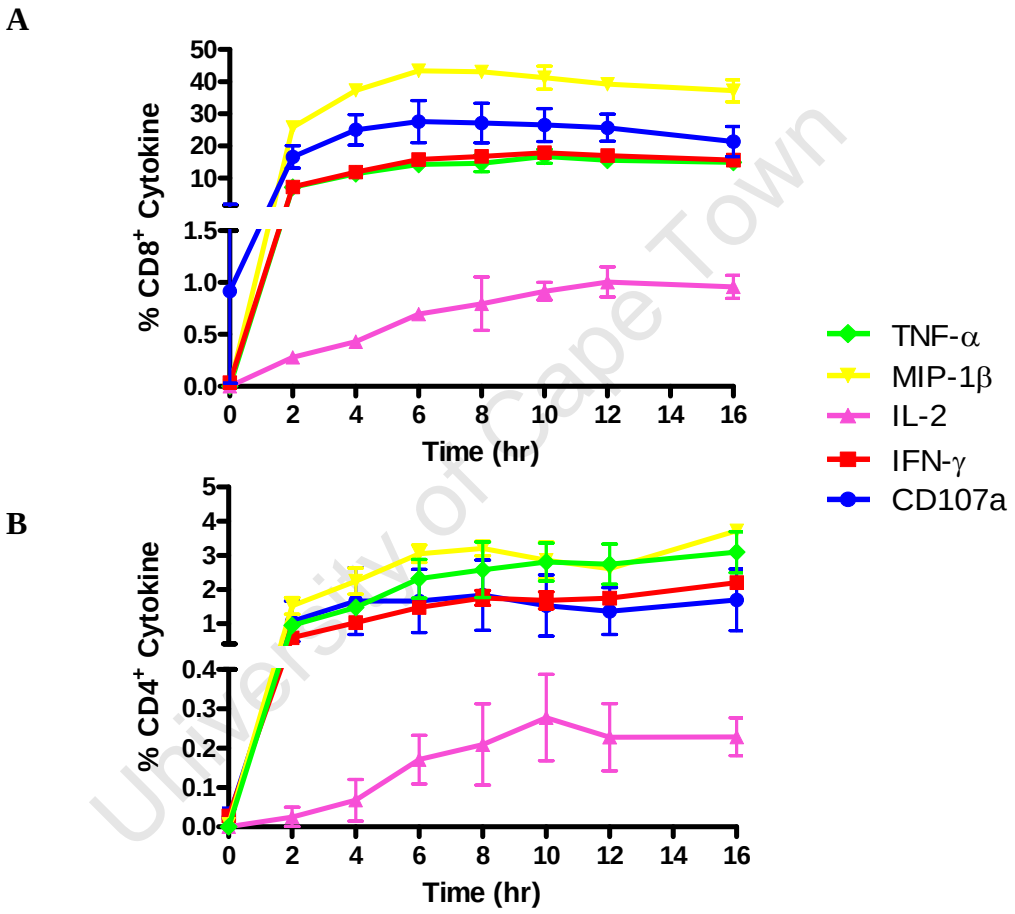

Figure 3.20: Time course of cytokine production. Expression levels of cytokines produced by (**A**) $CD8^+$ and (**B**) $CD4^+$ T cells stimulated by anti-CD3 over various times of stimulation.

3.3 Conclusions

The aim of this work was to develop a multicolour flow cytometry panel to investigate five different T cell functions. Several steps were followed to develop the panel. Initially, the markers of interest and then fluorochromes were chosen according to the LSRII that was to be used. The antibodies were titrated, and each antibody was added sequentially to the panel to ensure their compatibility and optimal staining. FMO controls were performed for all antibodies to confirm antibody compatibility and that there was no significant fluorescent spillover into other channels. During the development of the panel, the MIP-1β antibody had to be dropped due to reduction in IL-2 detection. The optimal incubation period for maximal induction of cytokine responses was determined to be 16 h. The use of Brefeldin-A and monensin together was found to best inhibit cytokine transport. A gating strategy for optimal analysis was determined, and a threshold value for a positive response was set at >0.05 %, calculated from the median background cytokine production in unstimulated cells. Thus, an optimised stimulation protocol and an eight colour polychromatic flow cytometry panel were developed to apply to the investigation of HIV-specific T cell responses in chronic and acute HIV infection, as described in Chapter 4.

CHAPTER FOUR

INVESTIGATION OF T CELL RESPONSES IN CHRONIC AND ACUTE HIV INFECTION

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CHAPTER FOUR

INVESTIGATION OF T CELL RESPONSES IN CHRONIC AND ACUTE HIV INFECTION

HIV-specific T cell responses were investigated in acute and chronic infection using an eight-colour flow cytometry panel. The acute cohort was a longitudinal study, in which the individuals were followed one year post infection, and the chronic cohort was a cross-sectional study. This chapter describes the magnitude and breadth of HIV-specific T cell responses in both these cohorts as well as a comparison between the two cohorts. Responses were measured by intracellular cytokine staining (ICS) using overlapping Gag and Nef peptides based on the HIV-1 subtype C genome.

4.1 HIV-specific T cell responses in a chronic cohort

HIV-specific T cell responses were investigated in 20 chronically infected individuals. The characteristics of the participants are listed in Table 4.1. Participants were recruited from the Nyanga Day Clinic, Cape Town, South Africa, and all participants were female. The median age of the cohort was 35. Four of the 20 individuals had been on antiretroviral therapy (ART) for at least 1 year at the time of sample collection. Of these four individuals, three had $CD4^+$ counts lower than the median $CD4^+$ counts. Although the time of infection was not known, all participants had been HIV-infected for more than 1 year. The $CD4^+$ cell counts of these individuals ranged from 188-1306 cells/ul (median = 619 cells/ul). Viral loads were not available for these individuals.

Table 4.1: Characteristics of 20 study participants

Patient ID	Age	ART*	$CD4^+$ Count
NY 017	36	Y	377
NY 021	33	N	581
NY 032	32	N	763
NY 044	39	Y	1306
NY 045	31	Y	530
NY 062	28	N	624
NY 066	26	N	908
NY 074	36	N	390
NY 076	36	N	640
NY 167	31	N	302
NY 168	35	N	567
NY 217	32	N	783
NY 219	37	N	642
NY 224	47	N	672
NY 234	35	N	943
NY 240	26	N	188
NY 263	35	Y	614
NY 267	40	N	545
NY 273	35	N	768
NY 292	41	N	574

* Antiretroviral therapy

4.1.1 HIV-specific cytokine responses

Single cytokine responses specific for HIV Gag and Nef peptide pools were investigated. Representative flow plots for two different study participants are shown in Figure 4.1. For each participant the negative control (unstimulated cells), Gag and Nef peptide pool stimulations are shown. The $CD8^+$ T cell responses are shown for study participant NY 32, and the $CD4^+$ T cell responses are for NY 292. Positive responses were observed for Gag-specific $CD8^+$ T cells (CD107a, TNF-α and IFN-γ), Nef-specific $CD8^+$ T cells (CD107a and IFN-γ), Gag-specific $CD4^+$ T cells (CD107a, IL-2, TNF-α and IFN-γ) and Nef-specific $CD4^+$ T cells (IL-2 and IFN-γ), after background subtraction and using a threshold of 0.05 %.

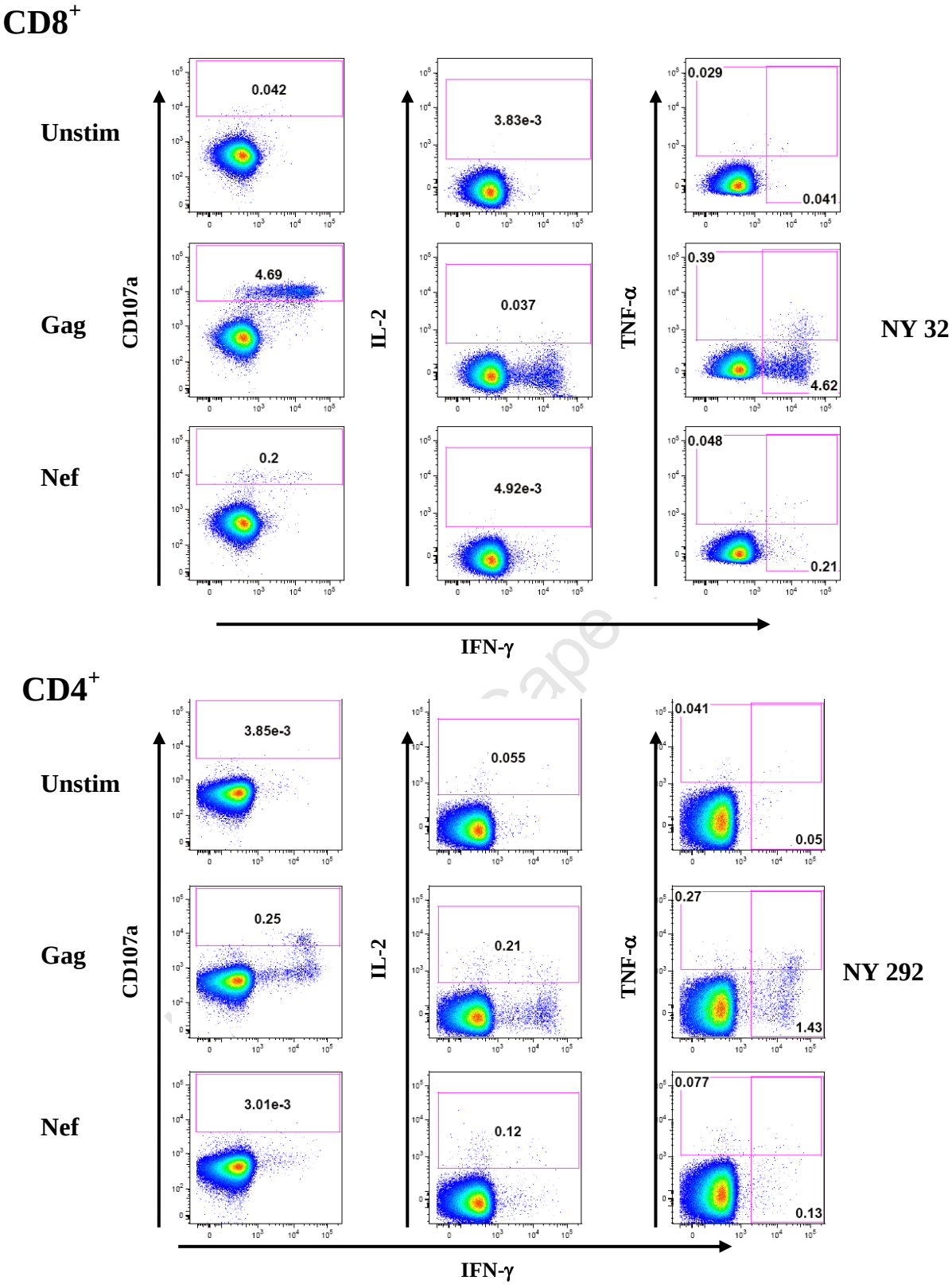

Figure 4.1: Representative flow plots of Gag and Nef-specific responses as measured by polychromatic flow cytometry for two different study participants. Shown are $CD8^+$ and $CD4^+$ T cell responses for the four functions investigated.

The HIV-specific T cells responses from 20 chronically infected individuals are shown in Figure 4.2. Where positive responses were obtained, the proportion of cells that were producing each function was determined (Figure 4.2A). Approximately fifty percent of HIV-specific $CD8^+$ T cell responses were due to CD107a expression, with the remaining 50 % being made of IFN-γ production. Whilst not significant, there was a greater tendency towards degranulation than IFN-γ production. A striking observation was that Gag and Nef-specific $CD8^+$ T cells produced no IL-2, and in only two individuals did a small proportion (<10 %) of the response consist of TNF-α production. In addition to the proportion of responses, the magnitude of HIV-specific $CD8^+$ T cells were dominated by CD107a and IFN-γ (Figure 4.2B, left panel). HIV-specific $CD8^+$ CD107a and IFN-γ responses were significantly higher in magnitude compared to $CD8^+$ TNF-α responses ($P<0.0001$ for both Gag and Nef). Gag and Nef were targeted in a similar manner, with CD107a dominating the response, followed by IFN-γ, as seen in Figure 4.4C. In contrast to $CD8^+$ T cells, Gag-specific $CD4^+$ T cells showed a broader range of proportion of cytokines secreted by individuals, with IFN-γ highest (approximately 35 %), followed by TNF-α (30 %) and only about 15 % of the response being IL-2 production (Figure 4.2A, right panel). This pattern was similar for Nef-specific $CD4^+$ T cells. Analogous to $CD8^+$ T cells, the magnitude of Gag and Nef-specific $CD4^+$ T cells mirrored the proportion of responding $CD4^+$ T cells, where HIV-specific $CD4^+$ T cells showed a broader range of cytokine levels. However, CD107a expression was rare in $CD4^+$ cells and IFN-γ was of a lower magnitude than for $CD8^+$ T cells (Figure 4.2B). In contrast, TNF-α and IL-2 were of a greater magnitude compared to $CD8^+$ T cells. Gag- and Nef-specific $CD4^+$ IFN-γ and TNF-α responses were significantly higher in magnitude than CD107a responses ($p=0.001$, $p=0.0152$ for Gag; $p<0.0001$ $p=0.0002$ for Nef respectively). Figure 4.4C summarises the magnitude of HIV-specific T cell cytokine responses. For $CD4^+$ T cells, IFN-γ dominates the Gag-specific response, followed by TNF-α, IL-2 and then CD107a, whereas for Nef, TNF-α dominates, followed by IFN-γ, IL-2 and then CD107a. Thus, in chronic HIV infection, HIV-specific $CD8^+$ T cell responses are characterised by CD107a and IFN-γ expression, while HIV-specific $CD4^+$ T cells produce IFN-γ, TNF-α, and IL-2. These patterns did not differ significantly for T cells targeting Gag or Nef.

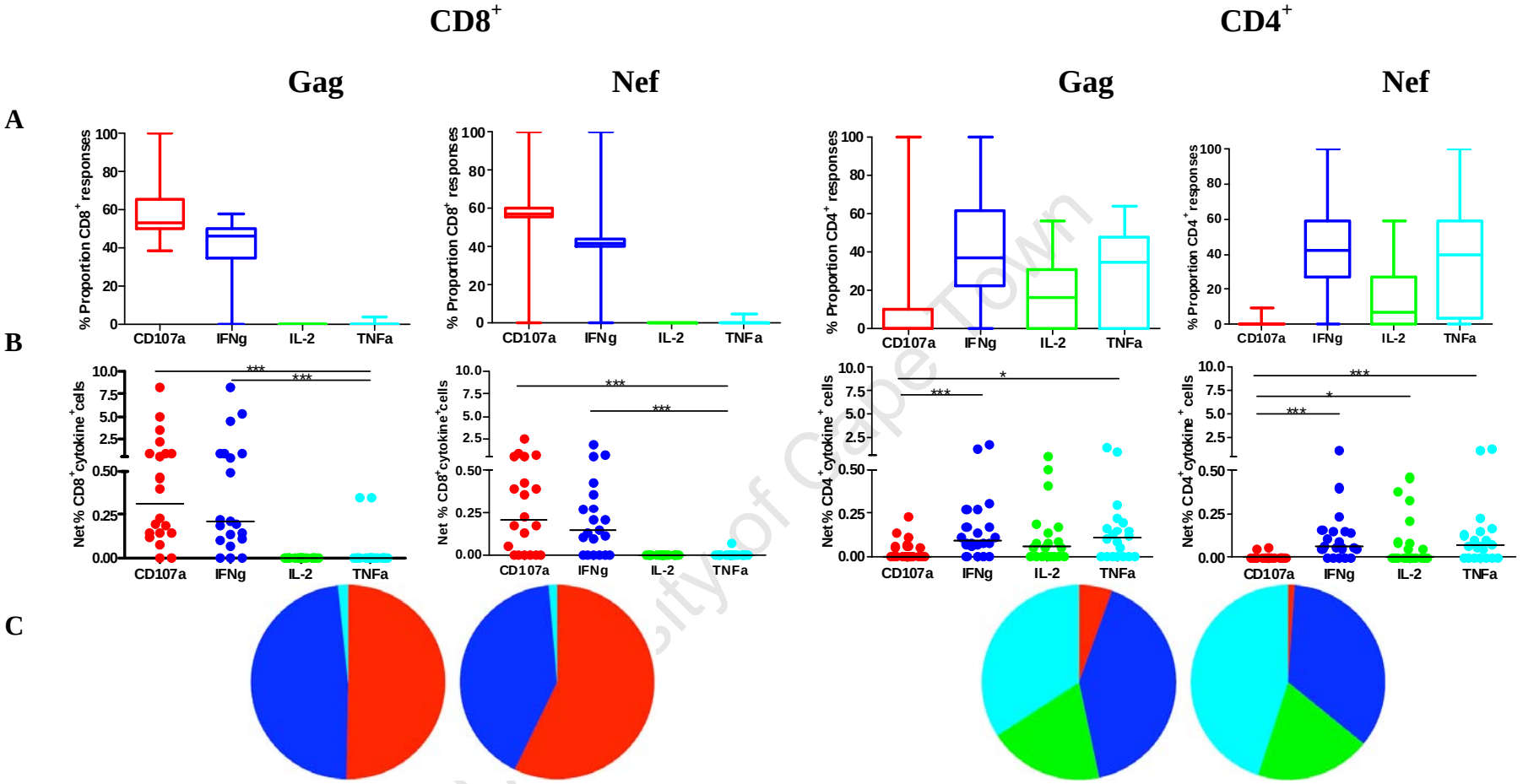

Figure 4.2: Cross-sectional analysis of HIV-specific T cell responses in 20 chronically infected individuals. Shown in (**A**) is the proportion of responding cells, in (**B**) the magnitude of responses, and in (**C**), proportion of magnitude of responses represented by pie charts. Samples were stimulated with Gag or Nef peptide pools for 16 h, followed by staining and acquisition. Horizontal lines represent medians. Significant differences are shown by an asterisk.

4.1.2 The total magnitude of HIV-specific T cell responses

The magnitude of total cytokine responses i.e. all HIV-specific cells secreting one or any combination of cytokines or degranulating, were determined. The total magnitude of responses was calculated by adding all the boolean combinations, to give a total magnitude of response. Here, a less-stringent cut-off of 0.01 % was used for cells producing more than two functions, since median background responses were well below 0.01 %. This is because non-specific binding of two cytokine antibodies to the same cell is rarer than non-specific binding of a single cytokine antibody. A cut-off of 0.01 % also ensured that all positive responses were greater than 10 events. For the single cytokine-producing cells, (cells producing IFN-γ *only*, TNF-α *only*, etc.), the cut-off value of 0.05 % remained, since median background responses for these populations were 0.05 %. Total HIV-specific $CD8^+$ T cell responses were substantially higher in magnitude than $CD4^+$ T cell responses (median 0.045 % versus 0 % for Gag, and 0.289 % versus 0.229 % for Nef), as shown in Figure 4.3A. There was no significant difference between Gag and Nef response magnitudes within either T cell compartment. When considering the frequency of responses, it was observed that all individuals mounted a CD8+ response, whilst 80 % (16/20) individuals mounted a CD4+ response. The frequency of Gag-specific $CD8^+$ T cell responders was greater compared to Nef-specific $CD8^+$ responders (20/20 and 15/20, respectively), and seventy five percent (15/20) of individuals had both a Gag and a Nef response (Figure 4.3B). Gag was also more highly targeted than Nef in the $CD4^+$ compartment (15/20 and 9/20 responders, respectively). Many of these individuals (8/15) had both a Gag and Nef response. No correlations were observed with either the total HIV-specific $CD8^+$ or $CD4^+$ T cell responses and $CD4^+$ counts, or between total HIV-specific $CD8^+$ and $CD4^+$ T cell responses (data not shown). Thus, the magnitude and frequency of total cytokine responses of HIV-specific $CD8^+$ T cells were greater than HIV-specific $CD4^+$ T cells.

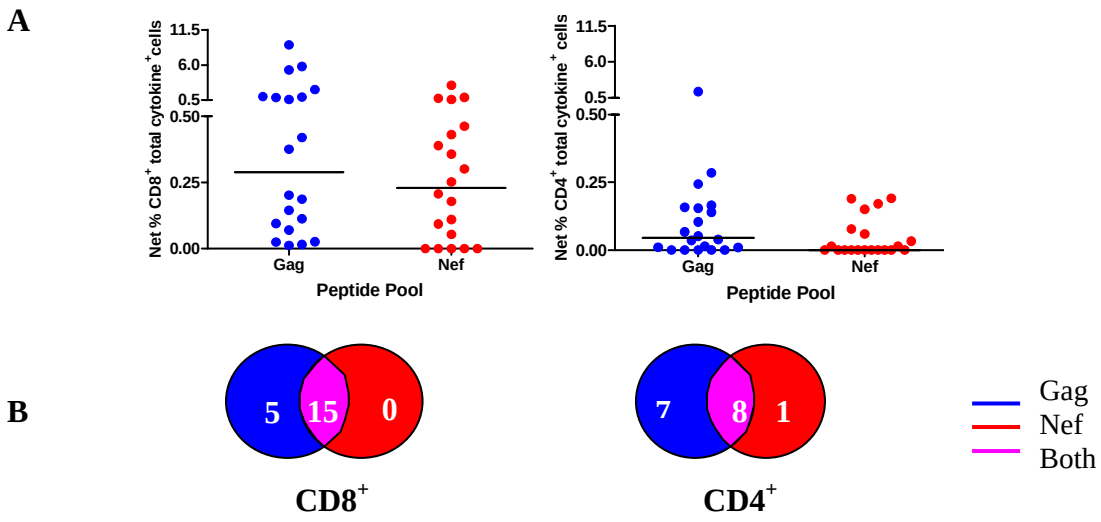

Figure 4.3: The total magnitude of HIV-specific $CD8^+$ T and $CD4^+$ T cell responses, Shown in (**A**) are $CD8^+$ (left panel) and $CD4^+$ T cells (right panel). (**B**) shows the frequency of positive responders to either Gag, Nef or both peptides. Samples were stimulated with Gag and Nef peptide pools for 16 h, stained and acquired. Horizontal lines represent medians.

4.1.3 HIV-specific polyfunctional T cell responses

The combinations of the four functions produced simultaneously were examined. Figure 4.4 shows HIV-specific $CD8^+$ and $CD4^+$ T cell responses. Both T cell compartments showed poor polyfunctionality towards Gag and Nef in chronic infection, with no cells detected that produced the three cytokines and degranulated. HIV-specific $CD8^+$ T cells showed similar functionality compared to HIV-specific $CD4^+$ T cells, with tri-functional cells present in both compartments but at a low frequency (8/20 and 3/20 respectively). A common phenotype for both Gag and Nef were $CD8^+$ T cells producing CD107a and IFN-γ simultaneously, whilst for CD4+ T cells two-functional cells were more diverse, reflecting the broader array of cytokines produced by CD4+ T cells. The proportion of response magnitudes are shown in pie charts (Figure 4.4A and 4.4B). Nef-specific $CD8^+$ T cell responses were dominated by monofunctional cells, producing either CD107a or IFN-γ, whereas Gag-specific $CD8^+$ T cell responses had a greater magnitude of dual-producing CD107a and IFN-γ cells. Tri- functional cells were present for Gag and Nef-specific $CD8^+$ T cells, although at a very low magnitude and proportion of the response (Figure 4.4C). The proportion of Gag-specific dual-producing $CD8^+$ T cells was significantly higher than Nef dual-

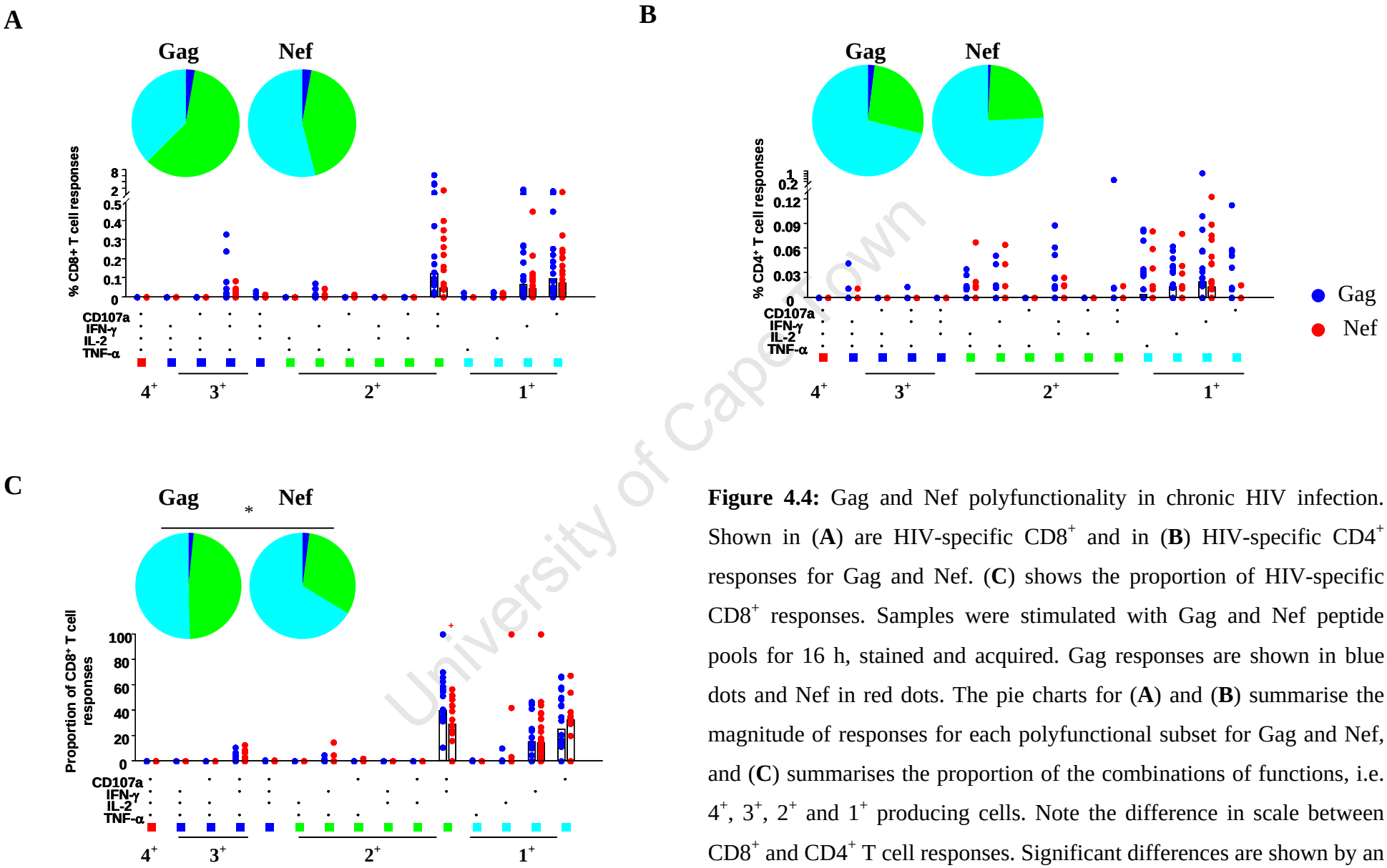

Figure 4.4: Gag and Nef polyfunctionality in chronic HIV infection. Shown in (**A**) are HIV-specific $CD8^+$ and in (**B**) HIV-specific $CD4^+$ responses for Gag and Nef. (**C**) shows the proportion of HIV-specific $CD8^+$ responses. Samples were stimulated with Gag and Nef peptide pools for 16 h, stained and acquired. Gag responses are shown in blue dots and Nef in red dots. The pie charts for (**A**) and (**B**) summarise the magnitude of responses for each polyfunctional subset for Gag and Nef, and (**C**) summarises the proportion of the combinations of functions, i.e. 4^+, 3^+, 2^+ and 1^+ producing cells. Note the difference in scale between $CD8^+$ and $CD4^+$ T cell responses. Significant differences are shown by an asterisk.

producers (p= 0.039; Figure 4.4C). Gag and Nef-specific $CD4^+$ T cell responses were both dominated by monofunctional T cells (Figure 4.4.B). There was also no difference between Gag and Nef in the proportion of functions for $CD4^+$ T cell responses (data not shown). The four individuals on ARVs did not shower a greater capacity for polyfunctionality compared to untreated individuals. Thus, HIV-specific T cells showed poor polyfunctionality in chronic HIV infection.

4.1.4 HIV-specific polyfunctional T cells produce more cytokine per cell

The median fluorescent intensity (MFI) was calculated to determine whether polyfunctional T cells (in this case 3^+ cytokine producers) were producing more cytokine per cell compared to mono- or dual functional cells. Shown in Figure 4.5 is the IFN-γ MFI of Gag or Nef-specific $CD8^+$ and $CD4^+$T cells. Polyfunctional cells have a greater MFI compared to less functional cells, and this is true for $CD8^+$ and $CD4^+$ T cells. The difference is most striking between the most polyfunctional cells (3^+) compared to monofunctional cells (p=0.0035) for IFN-γ MFI of $CD8^+$ T cells (Figure 4.5A). Even though there were fewer $CD4^+$ T cell polyfunctional responses, a similar trend for IFN-γ MFI was found. This trend was only examined for IFN-γ, since there were not sufficient responses to determine whether this trend was true for the other three functions investigated. Thus, the amount of IFN-γ decreases with the polyfunctionality of cells.

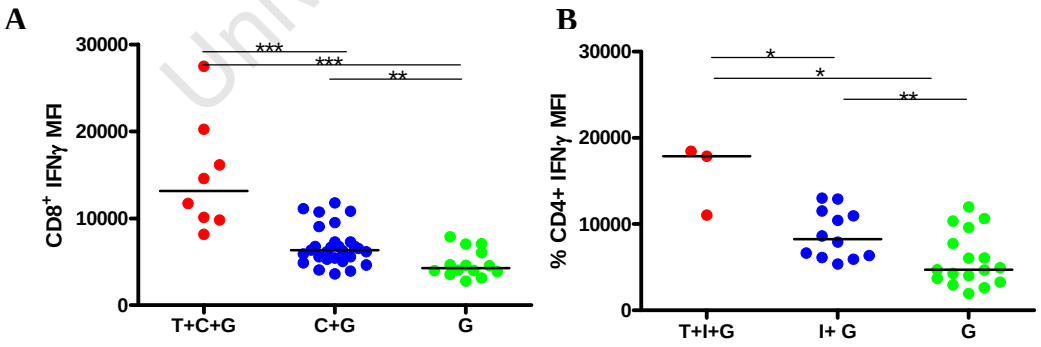

Figure 4.5: Amount of IFN-γ produced decreases as polyfunctionality decreases. Shown in (**A**) is the $CD8^+$ IFN-γ MFI and in (**B**) the $CD4^+$ IFN-γ MFI. Samples were stimulated with Gag and Nef peptide pools for 16 h, stained and acquired. The horizontal line represents the median. The Mann Whitney non-parametric test, was used to determine statistical differences. G = IFN-γ; C = CD107a; T = TNF-α; I = IL-2. Significant differences are shown by an asterisk.

4.2 HIV-specific responses in an acute longitudinal study

Seven acutely infected individuals were recruited from a vaccine retention study and individuals were followed monthly for up to a year post infection. The characteristics of the study participants are listed in Table 4.2. Longitudinal analysis was only performed on five of these individuals, since two individuals left the study for unknown reasons, and for these two individuals only incident samples were analysed. The study participants were all female. Two individuals spontaneously controlled viral loads to < 5 000 copies/ml (001 and 008). $CD4^+$ counts were > 500 cells/μl for all participants, except for participant 007, who also had the highest viral load. Participants were evaluated for T cell responses to Gag and Nef peptide pools, as well as specific peptide responses that were determined by the IFN-γ ELISPOT assay. The ELISPOT data was provided by Hoyam Gamieldien of the Division of Medical Virology, University of Cape Town.

4.2.1 HIV-specific T cell total cytokine responses to peptide pools over time

Due to the limited number of individuals in the acute cohort (n = 7), responses obtained were grouped according to incidence visit (containing a single time point), month 3-6 (M 3-6) and month 9-12 (M9-12), to determine any possible trends. Figure 4.5 shows the HIV-specific T cell total cytokine responses over time. At a gross level, $CD8^+$ T cell responses were initially high at incidence and decreased at M3-6 and increased again at 9-12 months post infection (Figure 4.6A). However, the difference between response magnitudes at the three different time points for $CD8^+$ T cell responses was not significant. In contrast, a highly striking loss of $CD4^+$ T cell responses was observed over time, where responses were highest at incidence and were then nearly completely lost over the period investigated. This was reflected in a significant difference between $CD4^+$ T cell responses at incidence compared to M3-6 ($p=0.02$). When only those individuals with longitudinal data were included in the analysis, the dynamics of responses are illustrated more clearly (Figure 4.4C and D). There was one individual (001) who showed an opposite trend compared to the other participants, in which Gag-specific $CD4^+$ T cells were present at month 9-12. This data suggests that $CD4^+$ responses are present initially, and are then lost as duration of HIV infection time increases.

Table 4.2: Characteristics of participants recruited during acute infection

Patient ID	Age	Incidence		Month 3		Month 4		Month 6		Month 9		Month 12	
		CD4 Count	Viral load	CD4 Count	Viral load	CD4 Count	Viral load	CD4 Count	Viral load	CD4 Count	Viral load	CD4 Count	Viral load
001	19	548	NA	759	2020	555	6980	543	4890	-	-	615	2420
002	29	548	7480	592	4510	587	7410	-	-	-	-	-	-
004	38	867	11000	829	NA	-	-	865	14400	612	18200	-	-
007	28	177	228000	274	599000	-	-	-	-	-	-	-	-
008	23	787	16900	776	6550	-	-	703	<400	-	-	-	-
349[a]	34	-	-	-	-	-	-	-	-	-	-	-	-
420[a]	34	-	-	-	-	-	-	-	-	-	-	-	-

[a] Only incidence samples available for these participants
NA- Not available

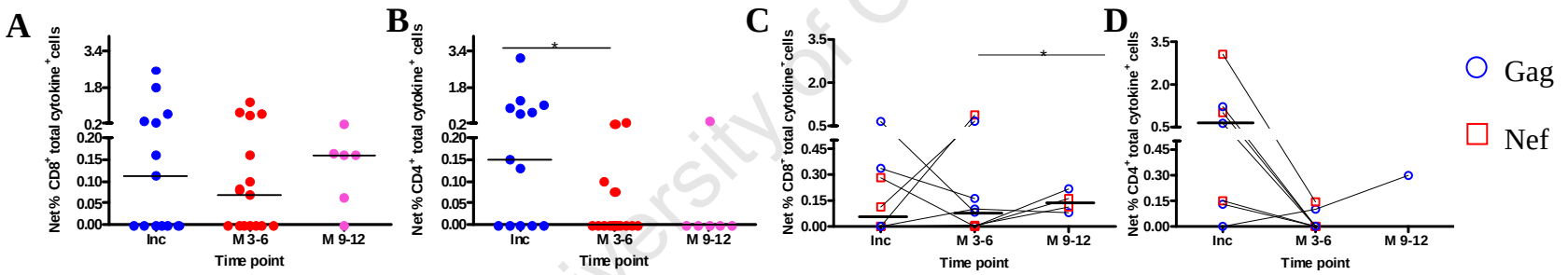

Figure 4.6: HIV-specific $CD8^+$ (**A**) and $CD4^+$ T (**B**) cell responses over the first year of infection. Each dot represents either a Gag or Nef response. Samples were stimulated for 16 h with Gag or Nef peptide pools followed by ICS. Horizontal lines represent the median values for each data set. Significant difference was observed between time points as denoted by an asterisk, determined using the non-parametric Mann Whitney U Test. Inc = incidence; M3-6 = month 3-6; M9-12 = month 9-12.

4.2.2 HIV-specific polyfunctional T cell responses over time

The polyfunctional nature of the HIV-specific responses was investigated next. Figure 4.7 illustrates HIV-specific polyfunctional T cell responses in the seven participants studied over the first year of infection. Gag and Nef-specific $CD8^+$ T cell responses had similar profiles for both $CD8^+$ and $CD4^+$ T cells over the time period investigated, as shown in the pie charts. At incidence, $CD8^+$ T cell responses consisted of one, two, three and four-functional cells (Figure 4.7A). $CD8^+$ T cell responses showed a loss in polyfunctionality over the time investigated, where four-functional cells were present at incidence (Figure 4.7A) but were lost at month 3-6 and three-functional cells decreased substantially by month 3-6 in both magnitude and frequency (Figure 4.7B). By month 9-12, all $CD8^+$ Nef-specific three-functional cells had disappeared (Figure 4.7C). The three-functional cells present at incidence were simultaneously producing IFN-γ, TNF-α and degranulating, indicating the development of a strong effector-type HIV-specific $CD8^+$ phenotype early on in infection, targeting both Gag and Nef. Gag and Nef-specific $CD4^+$ T cells showed a similar trend, where one, two, three and four-functional cells were present at incidence, but functionality decreased over the first year of infection (Figure 4.7D-F). $CD4^+$ three-functional T cells also comprised IFN-γ and TNF-α-producing cells, but differed from $CD8^+$ T cells in that they produced IL-2 and did not degranulate. There was a significant difference ($p=0.008$) between Gag and Nef-specific $CD4^+$ T cell responses at month 3-6 due to the presence of three-functional cells (IFN-γ, TNF-α, IL-2). Nef-specific $CD4^+$ T cell polyfunctionality was lost earlier compared to Nef-specific $CD8^+$ T cells, and a complete absence of $CD4^+$ Nef-specific responses were observed (Figure 4.7F). The pie charts show the proportion of four, three, two or one cytokine producing cells, and it can be clearly seen that over time from infection, both $CD8^+$ and $CD4^+$ Gag and Nef responses lose functionality. Thus, in both T cell compartments, four-functional cells were only detectable at incidence and the results clearly show the loss in polyfunctionality over time.

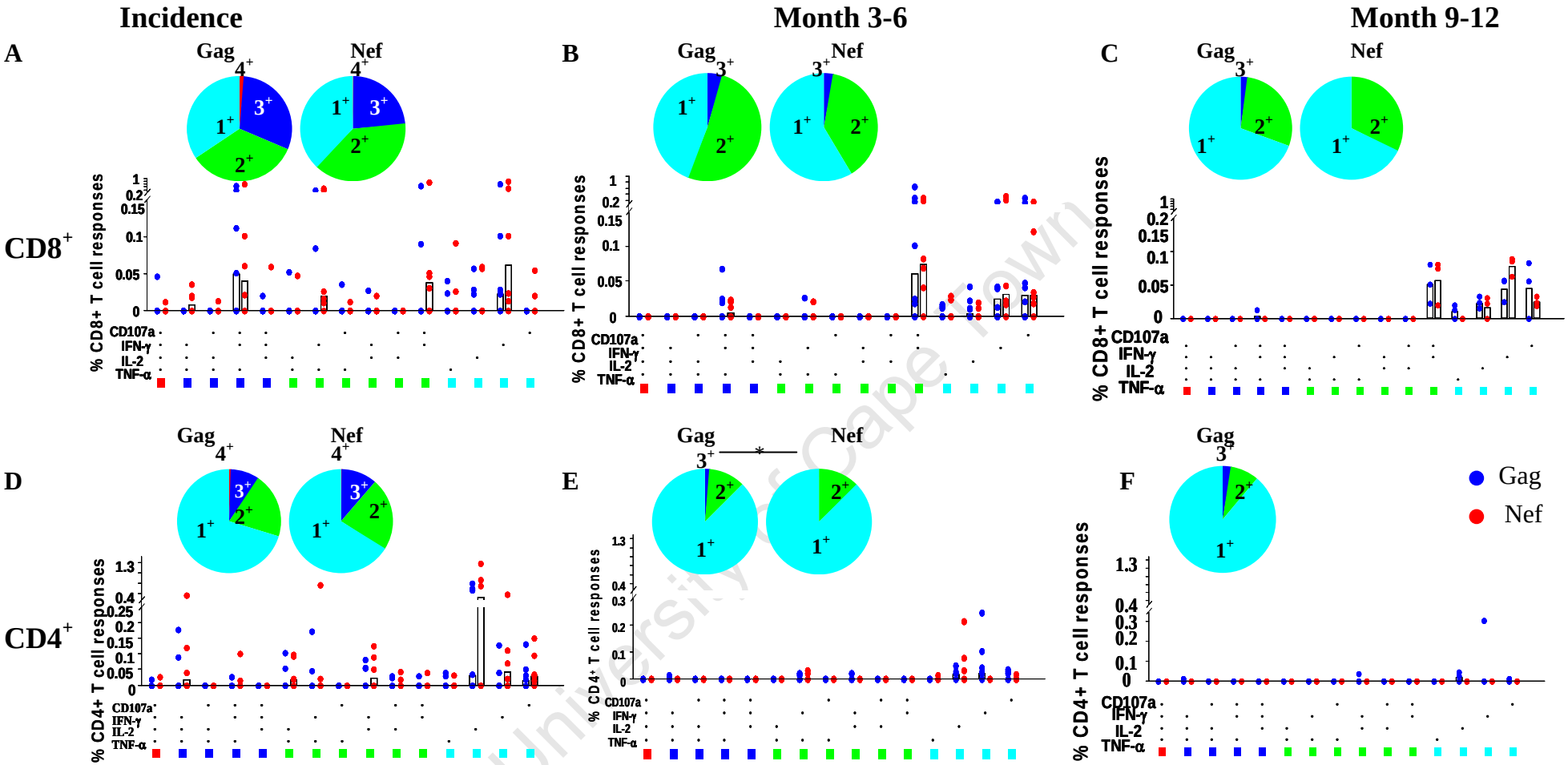

Figure 4.7: Loss of polyfunctional T cell responses over time. Shown in (**A**)-(**C**) are $CD8^+$ T cells and in (**D**)-(**F**) $CD4^+$ T cells. Samples were stimulated for 16 h with Gag (blue dots) or Nef (red dots) peptide pools, followed by ICS and acquisition. Box charts represent median responses. Pie charts summarise the magnitude of responses for each combination of functions, 4^+ (red), 3^+ (dark blue), 2^+ (green), and 1^+ (light blue). Significant differences are shown by an asterisk.

4.2.3 Participant HIV-specific T cell cytokine responses over time

The kinetics of HIV-specific T cell responses were investigated, to determine whether particular cytokine functions were preferentially lost over the course of HIV infection (Figure 4.8). Gag and Nef-specific $CD8^+$ T cell responses were present initially, except for participant 001, and 004, which developed Gag and Nef-specific IFN-γ and CD107a responses only later. Frequently, Gag or Nef responses developed early (by 3 months post infection). Interestingly, these cells appeared to maintain IFN-γ production and the ability to degranulate, but largely lost the ability, if it was detectable, to produce TNF-α, e.g. the Nef-specific responses in participants 004 and 008 (Figure 4.8B). An exception was the Gag-specific $CD8^+$ response in participant 008, that maintained TNF-α production. All other responses consisted of only IFN-γ and CD107a, in line with results shown in Figure 4.7, where later time points were dominated by these two cytokines. There were few $CD4^+$ responses detectable, but two participants maintained or developed $CD4^+$ HIV-specific IL-2 responses (participants 001 and 008; Figure 4.8C and 4.8D), whilst participant 007 lost its IL-2 responses.

In summary, participants with a Gag or Nef-specific $CD8^+$ T cell IFN-γ response also had a comparable CD107a response in terms of magnitude, and both of these were usually maintained or increased over time. IL-2 and TNF-α $CD8^+$ T cell responses were rare and sub-dominant compared to IFN-γ and CD107a responses, and were not frequently maintained over time. HIV-specific $CD4^+$ T cell responses consisted mainly of IL-2 responses that were maintained over time in some participants.

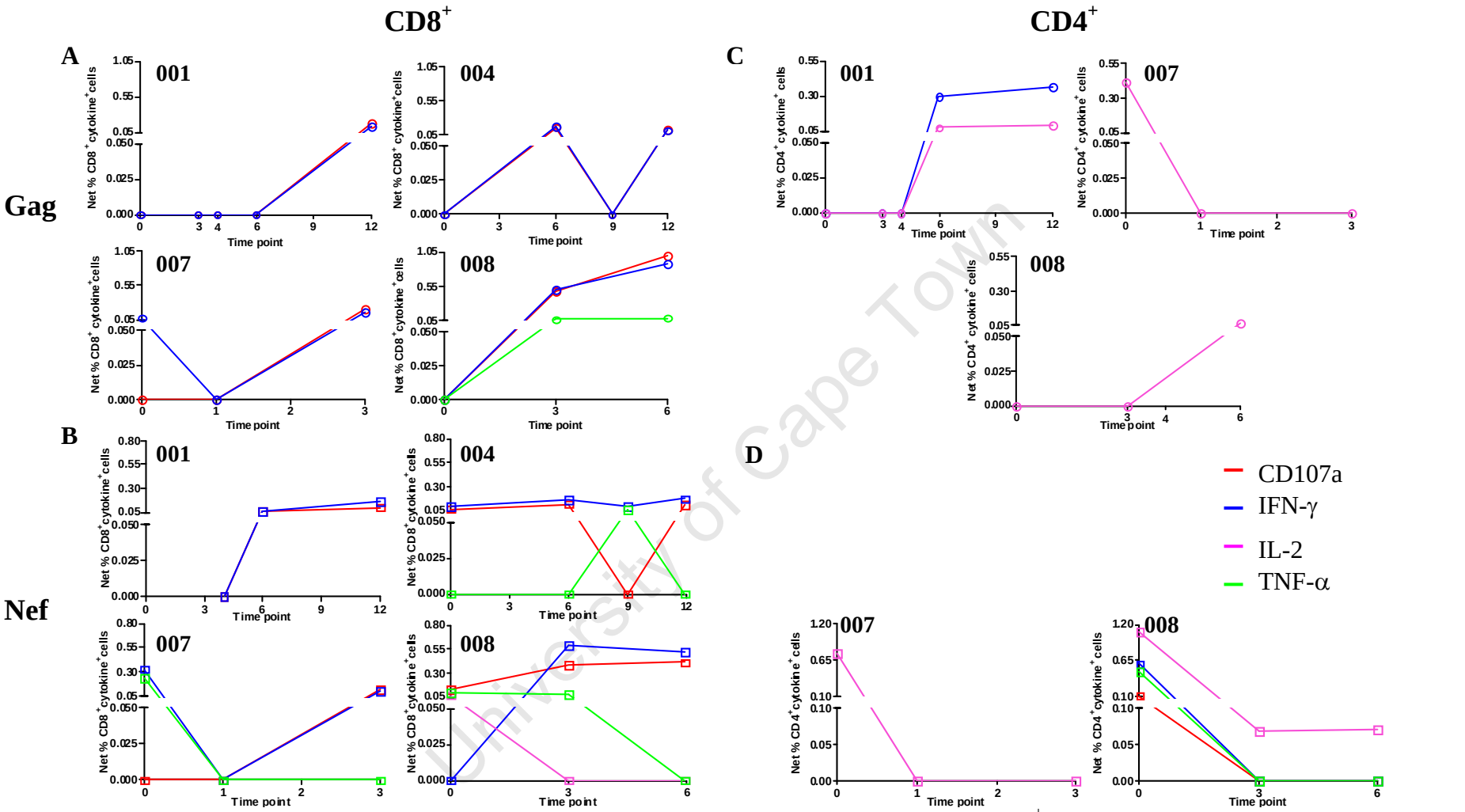

Figure 4.8: The magnitude of HIV-specific T cell responses over time. Shown in (**A**) and (**B**) are $CD8^+$ T cell Gag and Nef responses and in (**C**) and (**D**) are $CD4^+$ T cell Gag and Nef responses. Samples were stimulated with Gag and Nef peptide pools for 16 h stained and acquired. The number at the top left corner of each graph represents the participant ID. Time points represent months post infection whilst "0" represent the incidence visit.

4.2.4. Changes in magnitude of $CD8^+$ specific peptide responses over time

Participants in this cohort were initially analysed for responses by the IFN-γ ELISPOT assay, which identified specific reactive peptides, thus allowing the use of those peptides in the ICS assay. Determining changes to specific peptide responses over time may give more accurate insights than tracking differences to peptide pools, since responses to pools will include multiple responses that may arise and decline over time. Figure 4.9 shows two participants that had detectable $CD8^+$ responses to specific peptides (as outlined in Table 4.3). Both participants show a different pattern of responses over time even with respect to the same specific peptide (Nef 19). Participant 004 had Gag 38-specific $CD8^+$ T cell responses producing IFN-γ and IL-2 at a similar magnitude, which were only present at month 12. The IL-2 response was not detected in the Nef peptide pool (Figure 4.8A), but this may be because the response is borderline, being just above the cut-off threshold of 0.05 %. Nef 19-specific $CD8^+$ T cells consisted of IFN-γ and CD107a responses, corresponding with the data observed in Figure 4.8B in terms of these two functions. However, there was also a TNF-α $CD8^+$ T cell response detected to the Nef pool which was probably due to another peptide and not Nef 19 (Figure 4.8B). The ELISPOT responses for Gag 38 and Nef 19 were 260 and 100 s.f.u per 10^6 cells respectively, which are low but were still detectable with ICS (Table 4.3). It is also important to note that for both Gag 38 and Nef 19 additional functions (IL-2 and CD107a, respectively) were observed, which were missed in the IFN-γ ELISPOT assay, which is limited to measuring IFN-γ.

Interestingly, there was a stepwise decrease in functionality observed for participant 008 for both peptides tested. There were $CD8^+$ T cells producing all four functions at incidence, but at month 3, IL-2 responses were lost, and then at month 6 TNF-α was lost, but CD107a and IFN-γ responses were maintained (Figure 4.9B). This data largely confirms what was observed for the peptide pool responses in Figure 4.8. The TNF-α response was absent at month 6 (Figure 4.9B), however was still present in the peptide pool response, but this may be because it was secreted by T cells specific for another Gag peptide. CD107a and IFN-γ responses specific for Gag 19 and Nef 19 were of a lower magnitude compared to the peptide pools, and again, this was

probably due to other peptides that were also contributing to the overall response to Gag and Nef.

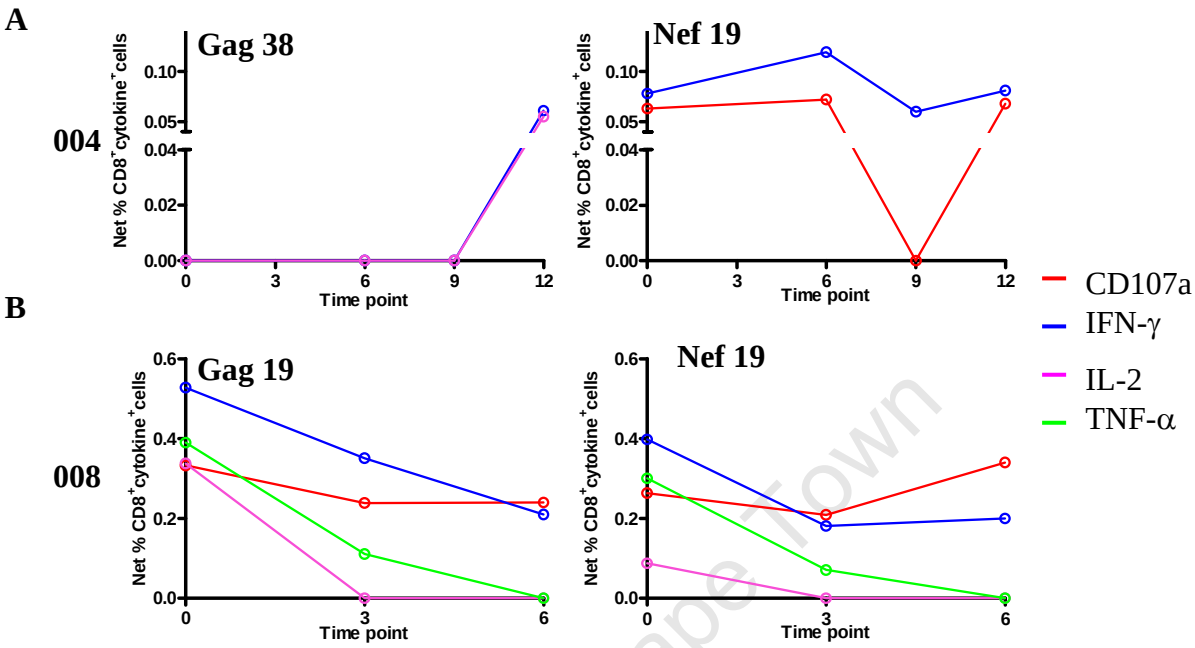

Figure 4.9: The change in $CD8^+$ T cell magnitudes of specific HIV peptide responses over time. Shown here are two participants, (**A**) 004, and (**B**) 008. Specific peptides for each participant are shown on the top left corner of each graph. Samples were stimulated with the respective peptide for 16 h, stained and acquired. The colours represent the different cytokine responses.

The ELISPOT data reveals that there was an increase in IFN-γ responses for both Gag 19 and Nef 19, from month 3 to month 6, however this increase in response was only observed in the Gag 19 IFN-γ ICS response, whereas the Nef 19 IFN-γ response decreased in magnitude (Table 4.3).

Thus, specific detectable responses were rare, as determined by ICS, and did not always compare well with IFN-γ ELISPOT data. This may be accounted for by the high threshold of 0.05 % that was used after background responses had been subtracted. This data also shows that it important to measure more than one function, as seen in these examples, since T cells are not only single cytokine producers. Thus, longitudinal analysis allows for the investigation of T cell cytokine kinetics, and

Table 4.3: Individual peptides used in the study and the responses obtained with ELISPOT and flow cytometry.

Patient ID	Time point[a]	Protein	Peptide Sequence[b]	Phenotype[c]	IFN-γ Net s.f.u per 10^6 cells[d]	CD107a (Net %)	IFN-γ (Net %)	IL-2 (Net %)	TNF-α (Net %)
004	Inc	Gag 38 (p24)	^{152}LNAWVKVIEEKAFSP^{166}	-	ND[e]	0	0	0	0
		Nef 19	^{73}QVPLRPMTYKAAFDL^{87}	CD8	ND	0.063	0.088	0	0
	M 6	Gag 38 (p24)	^{152}LNAWVKVIEEKAFSP^{166}	-	130	0	0	0	0
		Nef 19	^{73}QVPLRPMTYKAAFDL^{87}	CD8	0	0.072	0.119	0	0
	M 9	Gag 38 (p24)	^{152}LNAWVKVIEEKAFSP^{166}	CD8	ND	0	0	0	0.064
		Nef 19	^{73}QVPLRPMTYKAAFDL^{87}	-	ND	0	0	0	0
	M 12	Gag 38 (p24)	^{152}LNAWVKVIEEKAFSP^{166}	CD8	260	0	0.061	0.055	0
		Nef 19	^{73}QVPLRPMTYKAAFDL^{87}	CD8	100	0.068	0.081	0	0
008	Inc	Gag 19 (p17)	^{73}EELKSLYNTVATLYC^{87}	CD8	ND	0.263	0.398	0.088	0.3
		Nef 19	^{73}QVPLRPMTYKAAFDL^{87}	CD8	ND	0.3	0.5	0.3	0.39
	M 3	Gag 19 (p17)	^{73}EELKSLYNTVATLYC^{87}	CD8	ND	0.209	0.181	0	0.07
		Nef 19	^{73}QVPLRPMTYKAAFDL^{87}	CD8	90	0.239	0.51	0	0.11
	M 6	Gag 19 (p17)	^{73}EELKSLYNTVATLYC^{87}	CD8	850	0.34	0.2	0.2	0
		Nef 19	^{73}QVPLRPMTYKAAFDL^{87}	CD8	1170	0.24	0.21	0	0

[a] M= month; Inc= incidence
[b] Obtained from the Los Alamos database
[c] Phenotype identified by flow cytometry
[d] Provided by Hoyam Gamieldien, Division of Medical Virology, University of Cape Town
[e]ND- Not done

examining specific single peptide responses can provide more accurate information on kinetics compared to stimulating with peptide pools.

4.3 Comparison of HIV responses in acute and chronic infection

4.3.1 Magnitudes of single cytokine responses

Cytokine response magnitudes and frequencies were compared between individuals in the acute phase of HIV infection (n = 7), and those chronically infected (n = 20) (Figure 4.10). The responses shown here for acute infection refer to any responses in the first three months of infection. Although low numbers of acutely-infected participants limits the conclusions that can be drawn from this analysis, several descriptive observations can be made. In the $CD8^+$ T cell compartment, CD107a and IFN-γ tend to be greater in magnitude in chronic infection compared to acutely-infected individuals. IL-2 $CD8^+$ responses were completely absent in chronic infection but are found in acute infection (1/7 and 2/7 responders, for Gag and Nef, respectively). While there were few $CD8^+$ TNF-α responses in both phases of infection, the frequency of responders was greater in acute infection compared to chronically infected individuals (e.g. 3/7 versus 1/20 for Nef). Although there were few $CD4^+$ T cell responses in acute infection compared to chronic $CD4^+$ T cell responses, when present they were in a similar range to each other. There was, however, a greater magnitude of $CD4^+$ IFN-γ responses in chronic compared to acute HIV infection ($p=0.0224$). Even though Figure 4.7 illustrates a striking loss in $CD4^+$ T cell responses over time, in a larger cohort of 20 chronically infected individuals, some do have $CD4^+$ T cell responses, for example 16/20 individuals had IFN-γ responses.

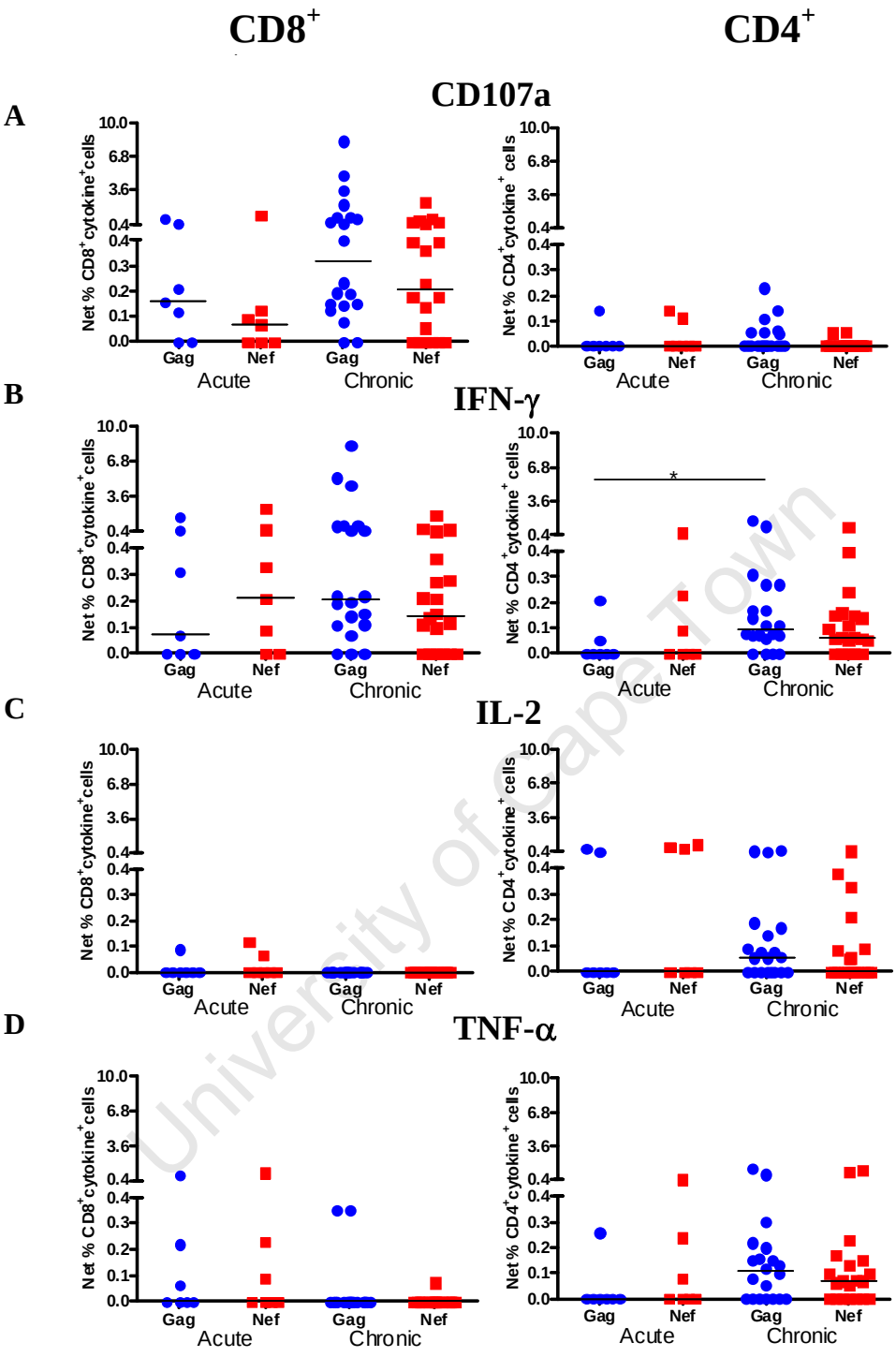

Figure 4.10: The magnitude of HIV-specific cytokine responses in chronic and acute HIV infection. Shown in (**A**) CD107a (**B**) IFN-γ (**C**) IL-2 and in (**D**) TNF-α responses for $CD8^+$ and $CD4^+$ T cells respectively. Samples were stimulated with Gag and Nef peptide pools for 16 h, stained and acquired. Significant differences are denoted by an asterisk as determined by Mann-Whitney U test. Horizontal lines represent medians.

4.3.2 Total cytokine production

The magnitude of the total HIV-specific cytokine responses was compared in chronic and acutely-infected individuals (Figure 4.11). Acute infection here refers to any responses in the first three months of infection in the seven participants evaluated. In chronic infection, Gag and Nef-specific $CD8^+$ T cell responses were higher in magnitude compared to acute infection, however this difference was not significant. However, the opposite was true for $CD4^+$ T cells, where the magnitude of Gag and Nef-specific T cell responses were higher in acute infection. Similarly, there are a greater frequency of $CD4^+$ responders in the acute phase of infection compared to chronically-infected individuals (4/7 versus 9/20 responders for Gag respectively).

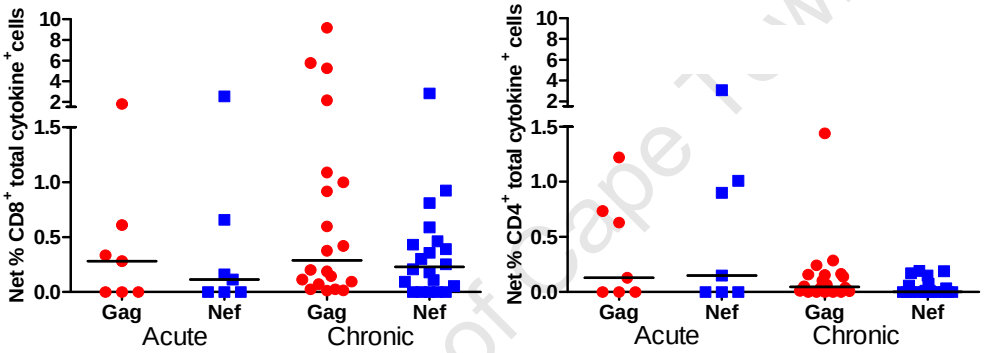

Figure 4.11: The magnitude of total cytokine HIV-specific T cell responses in chronic and acute infection. Samples were stimulated with Gag and Nef peptide pools for 16 h, stained and acquired.

4.3.3 Polyfunctional T cell responses

The polyfunctional responses in acute and chronic infection were compared next. Refer to Figure 4.4A and B and Figure 4.7. It can be clearly seen that early on in infection, both T cell compartments are polyfunctional (Figure 4.7A and D), with 4^+ and 3^+ cytokine-producing cells present. However, there is a loss in polyfunctionality over time (Figure 4.7). A few, low magnitude 3^+ cytokine-producing cells were present in chronically infected individuals for both Gag and Nef in both T cell compartments, which was true only for Gag in the acute cohort, but this may be due to a smaller cohort evaluated. Thus, it appears that as duration of infection increases, polyfunctional T cell responses are largely lost, which was confirmed in the larger chronically infected cohort.

4.4 Conclusions

HIV-specific T cell responses were investigated in 20 chronically-infected individuals and 7 individuals with acute infection that were followed longitudinally over the first year of infection. HIV-specific $CD8^+$ T cell responses were greater in magnitude than $CD4^+$ responses and predominantly produced IFN-γ and degranulated ($CD107a^+$), with a complete absence of IL-2 production in chronic infection. HIV-specific $CD4^+$ T cells were of a lower magnitude and had a broader cytokine repertoire, producing IFN-γ, TNF-α and IL-2, but rarely degranulated. There were poor polyfunctional $CD8^+$ and $CD4^+$ T cell responses in chronic infection. $CD8^+$ T cells showed a similar capacity for polyfunctionality compared to $CD4^+$ T cells, in which a small proportion of three functional were present and no four-functional cells were detected, but most responses were two and one-functional cells. Gag and Nef stimulated a similar magnitude and frequency of HIV-specific cells. In acutely-infected individuals, there was a decline in polyfunctionality over time, as well as a striking loss of $CD4^+$ T cell responses by 6 months post infection. In comparison to chronic infection, there were greater $CD4^+$ T cell responses in acute infection, and a greater $CD8^+$ T cell response in chronic infection compared to acute infection. In summary, it appears that HIV-specific T cell polyfunctional responses are lost over time, and this is most striking in the $CD4^+$ T cell compartment.

CHAPTER FIVE
DISCUSSION

Several correlates of viral control have been identified in HIV infection, but it is still unclear whether they actually result in control of HIV replication, or are a consequence of lower viral loads that may be caused by as yet unidentified immune factors. The advent of new technologies such as polychromatic flow cytometry have greatly enhanced our ability to try to determine correlates of control, including being able to investigate additional markers that traditionally were not evaluated.

The major aim of this study was the development of a nine-colour polychromatic flow cytometry panel. The panel was to consist of a viability marker, CD3, CD8 and CD4 and five functional markers, namely IFN-γ, IL-2, TNF-α, MIP-1β and CD107a. These five markers were chosen as they are commonly used to determine HIV-specific T cell responses (Almeida *et al*., 2007; Betts *et al*., 2006; Duvall *et al*., 2008; Streeck *et al*., 2008).

During optimization of this panel, it was observed that upon addition of MIP-1β PE antibody, the signal in the IL-2-APC channel was drastically reduced. This loss in IL-2 detection was not expected as there is minimal fluorescent spillover of PE into the APC channel, as observed in the Invitrogen Spectraviewer (www.invitrogen.com). The compensation matrix in FlowJo also showed that all percentages were < 30 % and there was no apparent spillover of PE in the APC FMO. Thus, it was unclear as to what was the cause of this reduction in signal. It may be due to the LSRII configuration, since it consists of an octagon blue laser, and no green laser. The green laser provides better resolution compared to the blue laser since it increases immunofluorescence sensitivity (Perfetto and Roederer, 2007). All panels developed in the SATVI laboratory consist of a maximum of 8 markers, and no 9-colour panels have been developed to date, although attempts have been made. Thus, due to time constraints, an eight-colour panel was optimized and MIP-1β was removed from the panel.

Despite this result, a nine-colour panel may still be developed since there are further optimization steps that can be performed. When developing a polychromatic flow cytometry panel, it is essential to try markers on all available fluorochromes to observe which antibody combinations work well together, however, due to time and resource constraints, this was not possible. Thus, alternative optimization steps such as placing IL-2 on different fluorochromes may have resulted in obtaining a nine-colour panel. One option may be to use IL-2-FITC, since this fluorochrome is bright and usually used for dim markers such as cytokines, and then transfer CD107a to APC, which is commercially available. This then leaves MIP-1β on PE. Since CD107a is a surface marker, and thus a brighter marker than intracellularly stained markers, the effect of the PE fluorochrome may not be so evident. An alternative would be to try transferring CD107a to APC, IL-2 could be placed on PE and MIP-1β on FITC. Another option is to try IL-2 on one of the fluorochromes that the other surface markers were on, such as PerCP-Cy5.5.

Apart from optimizing a flow cytometry panel, the stimulation protocol was also optimized in terms of incubation period and transport inhibitor selection. In this study, two HIV-uninfected PBMC samples were stimulated with immobilised anti-CD3 monoclonal antibody as previously shown in Veenstra *et al.* (2007), which non-specifically stimulates the T cell receptor (TCR). It was found that the optimum incubation period for all five functions investigated was 16 hours, but a disadvantage of using a longer incubation period may be an increase in cell death (Waldrop *et al*., 1997; Maino *et al*., 2008). The majority of studies use a 6 hour incubation to preserve cell viability, and some studies have shown that cytokine levels peak at 6 hours (Picker *et al*., 1995), but remain at peak levels until 18 hours (Horton *et al*., 2007). It has been documented that longer incubation periods do result in increased cell death, since incubations of 12 hours or longer were significantly different to shorter incubation times in terms of cell viability (Waldrop *et al*., 1997). Since cryopreserved samples were used, cell viability is of great concern, thus emphasizing the importance of including a viability marker in the staining procedure to ensure good quality data was obtained.

It would be of interest to observe whether the kinetic profile of cytokine secretion is similar with other stimuli, such as SEB, PMA/ionomycin or even viral peptides. Published studies show that the kinetics of SEB stimulated PBMC are similar to the results obtained in this study, where cytokines (IL-2, TNF-α, and IFN-γ) peaked around 6 hours and remained at that level till 15 hours (Sandberg *et al*., 2001). PMA/ionomycin stimulated cells, however, resulted in a more rapid expression of cytokines (IL-2, IL-4 and IFN-γ), which peaked at 4 hours, and drastically decreased at ten hours (Picker *et al*., 1995). However, in macaque PBMC stimulated with SIV peptides, IFN-γ and TNF-α were optimally detected at 6 hours, however the next time point investigated was 20 hours, so this does not clearly indicate whether responses increased for additional time and before responses started to wane at 20 hours (Gaudin *et al*., 2004).

Protein transport inhibitors were evaluated for their effect on the cytokine and degranulation markers of interest. The majority of studies use Brefeldin A (BFA) as the protein transport inhibitor, which blocks transport from the endoplasmic reticulum to the golgi apparatus (as reviewed in Klausner *et al*., 1992). Since CD107a was included as one of the functions investigated, monensin was required, as it has been shown to enhance CD107a detection (Betts *et al*., 2003). Our analysis, however, revealed no difference in CD107a detection with either of the inhibitors. For TNF-α, it was also found that detection was the same with BFA compared to monensin in the $CD8^+$ T cell compartment. This lack of difference in TNF-α detection between monensin and BFA has been previously observed (O'Neil-Andersen and Lawrence, 2002). Another finding with respect to TNF-α detection was that when both inhibitors were used together, this greatly increased TNF-α detection. Thus, both protein transport inhibitors were included in the optimized protocol. These findings, however, were based on a single HIV-uninfected PBMC sample, and this experiment would need to be repeated on at least two more samples to confirm the result.

Besides the optimization to the stimulation protocols mentioned in this study, there are other variables during stimulation and ICS that could have been evaluated. Another contributing factor that may influence cytokine responses is the time of addition of protein transport inhibitors during the stimulation procedure. Here, BFA

and monensin were added from the beginning of the incubation period, however there have been studies that added BFA 1-2 hours after stimulation (Suni *et al*., 1998; Waldrop *et al*., 1997). This variable was not tested in this study, due to time constraints. Co-stimulatory molecules anti-CD28 and anti-CD49d were added in our stimulation procedure. The use of co-stimulatory molecules has been investigated and it was found that they were beneficial in one study, with an increase in cytokine responses observed (Waldrop *et al*., 1997), whereas in another study results were inconclusive (Horton *et al*., 2007). The staining procedure in our protocol was performed in 96 well microtiter plates with incubations periods of 20 min at room temperature, as described in Lamoreaux *et al.* (2006). However, other staining protocols use tubes and longer incubation periods at lower temperatures (Ramduth *et al*., 2005). The type of vessel used for ICS was shown not to affect cytokine responses (Horton *et al*., 2007). The benefit of using a plate versus tubes is that it is high throughput; if there are a large number of samples to stimulate and stain, it makes the procedure far easier working with one plate rather than a large number of tubes.

It is also worthwhile to examine different gating strategies. When plotting different markers against each other, some combinations offer better separation compared to others, thus responses are not lost by inaccurate gating due to poor separation. This is especially important for cytokine responses. It is also vital to evaluate surface down-regulation of phenotypic markers, as observed in some samples in this study. $CD3^+$ and/or $CD8^+$ T cell $cytokine^+$ populations may become dim due to down regulation of these markers. To account for this, $CD3^+$ or $CD8^+$ T cell populations were plotted versus a cytokine channel for initial gating, as described in Betts *et al.* (2006).

Determination of what constitutes a positive response is an important issue. This can be calculated in a number of ways, such as using a two to three-fold above background cutoff (Trigona *et al*., 2003), using a 90^{th} percentile cut-off based on background responses (Duvall *et al*., 2008), using two standard deviations above background responses as a cut-off, as well as having a ten event cut-off for polyfunctional T cells (Betts *et al*., 2006), or using a threshold value (Shacklett *et al*., 2003). Cut-off values may also be determined by evaluating cytokine responses in HIV-uninfected PBMC samples, and using the mean plus 4 times the standard deviation as the cut-off, as previously performed in our laboratory. There are also

rigorous statistical methods that offer stringent determination of positive responses, as shown in Trigona *et al*. (2003). Here, a threshold value of 0.05 % was used for single cytokine responses, determined by calculating the background responses of the four functions in the 20 chronically infected individuals. All net responses below 0.05 % were set to zero. For cells secreting more than one cytokine, this threshold was lowered to 0.01 % in the Spice analysis, which then included positive responses that were > 10 events, since these cells are unlikely to represent non-specific staining. This threshold was also used for calculating the total cytokine response for cells secreting >1 cytokine, since unstimulated background responses in the 2^+, 3^+ and 4^+ cells were in the range 9.56×10^{-3} and 3.26×10^{-4}.

The second part of this study was to apply the polychromatic flow panel and optimized stimulation protocol to investigate T cell responses in acute and chronic HIV infection. Gag and Nef peptide pools only were used due to limited sample availability. Many studies have shown that high frequencies of Gag and Nef $CD8^+$ and $CD4^+$ responses are detectable in HIV-infected individuals (Addo *et al*., 2003; Betts *et al*., 2001; Edwards *et al*., 2002; Kaufmann *et a*l., 2004; Ramduth *et al*., 2005), thus these proteins were chosen as antigens. Using intracellular staining, it was found that in chronically infected individuals, cytokines responses are detectable for both $CD4^+$ and $CD8^+$ T cell responses. The cytokine profiles, however, differ between the two compartments, where $CD8^+$ T cells predominantly produce IFN-γ and degranulate with little TNF-α production. This result agreed with previous literature, with the only difference observed was that MIP-1β (not tested here) was most frequently produced, followed by IFN-γ and CD107a expression (Betts *et al*., 2006). Similarly, our results confirm previous literature in which no IL-2 production from $CD8^+$ T cells was detected (Betts *et al*., 2006), although others have shown that these cells can be present during chronic infection (Duvall *et al*., 2008). HIV-specific $CD4^+$ T cells show a broader cytokine expression range, which was dominated by IFN-γ and TNF-α, followed by IL-2, with little capacity to degranulate. There is evidence, though, that Gag-specific $CD4^+$ T cells are capable of degranulating (Duvall *et al*., 2008). A likely reason for these discrepancies may be due to the fact that a small study cohort was used and individuals in the cohort were not chosen on their basis of having a high magnitude of HIV-specific responses, as performed in Duvall *et al*. (2008). The

differences between cytokine expression by $CD8^+$ and $CD4^+$ cells likely reflects their different functional roles in HIV infection, were $CD8^+$ cells are the effectors and are directly cytotoxic, while $CD4^+$ cells are limited primarily to a helper response. Our findings also show that the specificity of $CD4^+$ and $CD8^+$ T cell responses did not differ in their cytokine profiles between Gag and Nef proteins in terms of polyfunctionality, as previously shown (Betts *et al*., 2006).

In terms of total magnitude of responses, Gag-specific $CD8^+$ T cell responses were of a lower magnitude of responses compared to previous literature (medians were <0.5 % and <2 % respectively) (Duvall *et al*., 2008). Similarly, Gag-specific $CD4^+$ T cells were lower in total magnitude compared to previous literature (medians were 0 and 0.25 % respectively) (Duvall *et al*., 2008). This may be due to the fact that an additional function, the chemokine MIP-1β, was included in this published study, and almost 100 % of HIV-specific $CD8^+$ T cells are capable of producing MIP-1β, thus influencing the magnitude of the total response considerably. HIV-specific $CD8^+$ T cells have shown a vast breadth in response, capable of recognizing all HIV proteins (Betts *et al*., 2006). In this study, it was found that the magnitudes between Gag and Nef were similar in the $CD8^+$ T cell compartment, however, the frequency of total cytokine responses differed between Gag and Nef. All 20 chronically infected individuals had a Gag-specific $CD8^+$ response, as observed in Duvall *et al.* (2008). However, Gag-specific $CD4^+$ T cells, showed a lower frequency of responders (15/20 compared to 16/16; Duvall *et al*., 2008).

A comparison between the ICS data obtained in this study and IFN-γ ELISPOT results showed that they did not always agree with one another. For instance, if an IFN-γ response was obtained using the ELISPOT assay, an ICS IFN-γ response may not have been detected. This was most likely due to low magnitude ELISPOT IFN-γ responses, which may have been undetectable in ICS due to the cut-off threshold, which may be more stringent than the ELISPOT cut-off, or background responses being higher in ICS (possibly from the addition of co-stimulatory molecules). It may also reflect a difference in sensitivity between the two assays. Previous studies have shown significant correlations between flow cytometry and ELISPOT (Asemissen *et al*., 2001; Karlsson *et al*., 2003), suggesting that these two methods are comparable.

Results may also have differed based on the differentiation of the $CD8^+$ and $CD4^+$ T cell subsets in ICS, and their IFN-γ responses, compared to a bulk amount of T cell IFN-γ response obtained in the ELISPOT. The advantage of ICS is that cytokines in addition to IFN-γ can also be detected, and are indeed present. In addition to measuring more parameters, the phenotype of the responding cell is identified, thus distinguishing between $CD4^+$ and $CD8^+$ T cell responses.

T cells are capable of exerting a number of functions, including the secretion of multiple cytokines, and expression of many cytokines simultaneously. Recently, it has been established that T cells that are polyfunctional have been associated with viral control or non-progression in HIV infection (Betts *et al*., 2006; Duvall *et al*., 2008; Kannanganat *et al*., 2007b). Thus, polyfunctional T cells were investigated in chronic and acute HIV infection. It was found that in chronic infection, both HIV-specific $CD4^+$ and $CD8^+$ T cell polyfunctionality were lower in frequency and in some cases, lower in magnitude, and 4^+ producing cells were absent in chronic infection, compared to acute HIV infection. Previously, polyfunctional (5^+ and 4^+) HIV-specific T cells have been described in untreated chronic infection (Almeida *et al*., 2007; Betts *et al*, 2006; Duvall *et al*., 2008)..

A major finding of this study, when comparing chronic and early HIV infection, was that there was a remarkable loss in polyfunctionality, which cannot be observed in a cross-sectional study. This illustrates the value of longitudinal studies. The loss in polyfunctionality reveals that specific cytokine responses over time decrease in terms of magnitude, and then become completely absent. For instance, this was observed for IL-2, which was the first cytokine response to diminish followed by TNF-α, but cells maintained the ability to degranulate and produce IFN-γ, suggesting an effector phenotype. This maintenance of an effector phenotype was confirmed in the chronic cohort. Our data clearly indicated that there was indeed a loss in polyfunctionality, where cells present early in infection (<3 months) were diminished in terms of polyfunctionality as duration of infection increased, which has been observed previously (Streeck *et al*., 2008). This results in poor functionality in chronic infection.

This loss in polyfunctionality in the $CD4^+$ T cell compartment, as observed over time, was confirmed by showing that if polyfunctional (3^+) HIV-specific $CD4^+$ T cell responses were present in chronic infection, they were of a low frequency (3/20), whereas there was as dramatic loss in polyfunctionality observed in the smaller acute cohort. The decrease in functionality may be due to high viral loads (Streeck *et al*., 2008). This seems unlikely in our study, since all the individuals who were followed for more than one time point in the acute cohort had decreasing viral loads from initial time point to last visit. Thus, it appears that high viral loads were not responsible for T cell functionality, although sustained viremia cannot be excluded as a reason for loss of polyfunctionality. However, all the individuals displayed decreasing $CD4^+$ cell counts from the initial time point to the last visit. Thus, this loss in polyfunctionality may be accounted for by the loss of HIV-specific $CD4^+$ T cells and hence loss in $CD4^+$ T cell help for $CD8^+$ T cells, which is important for the development of memory cells (Janssen *et al*., 2003; Shedlock *et al*., 2003; Sun *et al*., 2004). Why was there a loss in HIV-specific $CD4^+$ T cells? A reason that may account for this is the fact that HIV-specific $CD4^+$ T cells are preferentially targeted, and thus depleted in HIV infection (Douek *et al*., 2002). The connection between the depletion of $CD4^+$ T cells and the loss in polyfunctional HIV-specific T cells has been observed in the bronchoalveolar lavage (BAL) of HIV infected individuals (Brenchley *et al*., 2007). It was shown that BAL had preserved $CD4^+$ T cells, which were highly polyfunctional compared to the blood, where there were significantly fewer HIV-specific $CD4^+$ T cells. It was also shown that polyfunctional HIV-specific $CD8^+$ T cells were present more frequently in the BAL compared to blood. Hence, the loss of polyfunctionality may be due to the depletion of HIV-specific $CD4^+$ T cells.

During antigenic stimulation, T cells proliferate, mature and differentiate into specialised subsets of T cells. These subsets can be identified due to different surface expression markers as well as by the functions they play, thus phenotype is linked to function. This is of particular interest, since new functions may be acquired upon differentiation, and other functions may be lost. It was observed in this study that specific epitopes showed a loss in function over time. This loss in functionality could be a result of T cells differentiating, thus losing the ability to produce certain cytokines. An example in this study revealed that IL-2 was the first cytokine to be lost, and this may be a result of cells differentiating into effector and effector memory

T cells, which primarily produce IFN-γ and have the capacity to be cytotoxic (Champagne *et al*., 2001). Furthermore, it has been shown with increased number of cell divisions, IL-2 production decreased, and IFN-γ production increased (Gett and Hodgkin, 1998). Thus, sustained viremia may cause differentiation and increased cell division, resulting in certain functions being lost over time.

Despite several studies showing polyfunctional cells associate with control of HIV and other infections, their function is still controversial. HIV studies have shown the importance of polyfunctional T cells in individuals who naturally control virus, have low viral loads and do not progress to disease (Betts *et al*., 2006; Duvall *et al*., 2008; Kannanganat *et al*., 2007b). Thus, due to a limited amount of literature surrounding this topic, it is still unclear whether polyfunctionality controls viral replication or is the result of low viral loads. Recently, a study by Streeck *et al.* (2008) attempted to address the question of polyfunctionality as the cause or consequence of HIV control, by following acutely-infected individuals longitudinally. It was observed that persistent antigen recognition influenced functional profiles of $CD8^+$ T cells, concluding that polyfunctionality (or the lack there of) was a consequence of viremia. Interestingly, it was observed that if a specific epitope escaped recognition, this resulted in a decrease in monofunctionality (or an increase in polyfunctionality), i.e. since there was no more persistent antigenic stimuli for these responses since the epitope had escaped, the response could possibly mature. This finding (an increase in polyfunctionality) was not observed in the acutely-infected individuals in this study, but only a limited number of specific reactive peptides were examined over time, and these could be ones that did not induce escape.

In spite of observing only a limiting number of polyfunctional responses in chronic infection, our data confirms previous findings that polyfunctional T cells secrete more cytokine per cell compared to a monofunctional cell, as determined by $CD8^+$ T cell IFN-γ median fluorescent intensity (MFI) (Precopio *et al*., 2007; Darrah *et al*., 2007; Kannanganat *et al*., 2007a; Duvall *et al*., 2008). Due to few responses, this could not be determined for the other functions, although this has been shown to be true previously for IL-2 and TNF-α in vaccinated individuals, as well as for $CD4^+$ T cells (Darrah *et al*., 2007; Kannanganat *et al*., 2007a). Thus, polyfunctional cells appear to

have enhanced effector functions due the secretion of more cytokine per cell, which may account for the protection that polyfunctional cells play in *Leishmania major* infection (Darrah *et al*., 2007), and their correlation with viral control (Betts *et al*., 2006; Kannanganat *et al*., 2007b; Duvall *et al*., 2008)

Although there have been great advances in flow cytometry allowing multiple functional markers to be investigated, thus enhancing the potential to discover correlates of control, the question still remains: what should be measured, and which immunological method should be used to provide the most relevant information? HIV-specific T cells are capable of producing a wide array of cytokines, including several that are rarely measured in the majority of HIV studies, such as IL-5, IL-8, IL-13, and granulocyte-macrophage colony-stimulating factor (GM-CSF; Goonetilleke *et al*., 2006). Flow cytometry, is a powerful technique, allowing the measurement of a number of markers, however, the number of functional markers that can be included is still limited, and we do not necessarily know which are the most important cytokines or functions to measure. A further limitation is that many of the markers that are used are surrogates may not necessarily or accurately reflect direct T cell functioning. The panel developed in this study included IL-2 and CD107a markers to measure proliferative and cytotoxic activity indirectly. Are these markers true indicators of function? Markers such as granzyme A or B, perforin or even granulysin may be more reliable markers of cytotoxic activity. CD107a is a surface-expressed marker so it does not prove that degranulating cells can actually kill target cells, although CD107a expression has been shown to directly correlate with cytotoxic activity (Betts *et al*., 2003). Furthermore, T cell IL-2 responses have been shown to correlate with proliferative ability (Harari *et al*., 2004). Thus, verifying the use of surrogate markers is extremely important, and proliferation and viral suppression assays may be more biologically relevant to T cell functioning.

In conclusion, the development of a polychromatic flow cytometry panel has led to an enhanced knowledge of T cell responses, and this is being applied to develop other panels in our laboratory, such as the investigation of memory differentiation and immune activation in HIV infection. The major findings in this study, although limited by a small cohort, was the striking decrease of $CD4^+$ T cell responses over the course of early infection, and a significant loss in polyfunctionality over time infected,

which was confirmed in the chronic cohort. It is still unknown whether polyfunctionality is the cause or consequence of viral control, emphasizing the importance of longitudinal studies to help resolve this issue. Thus, it is important to continue such investigations to further understand HIV-specific immunity and correlates of control in order to aid development of an effective HIV vaccine.

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APPENDIX
Preparation and storage of reagents

DNAse (Roche Diagnostics)
Working stocks of 0.2 μg/ml (500 μl) in R1 were stored at -20 °C. A 1:10 dilution was made with 4.5 ml R1 and 500 μl of this dilution was added to each sample.

Brefeldin A (Sigma Aldrich)
Stock solutions of 5 μg/ml were prepared with 100 % ethanol and stored at -20 °C. These were further diluted 1:10 with PBS for use at final concentration of 0.5 μg/ml.

Monensin (Sigma Aldrich)
10 mg/ml stocks were prepared with 100 % ethanol and stored at -20 °C. It was used at a final concentration of 10 μg/ml.

Phorbol Myristate Acetate (PMA) (Sigma Aldrich)
0.1 mg/ml stocks of PMA were prepared with DMSO and stored at -20 °C. A 1:100 dilution was made with PBS for a final concentration of 1 μg/ml.

Ionomycin (Sigma Aldrich)
5 μg/ml stocks of ionomycin were prepared with 100 % ethanol and stored at -20 °C. A 1:10 dilution with PBS was made for a final concentration of 0.5 μg/ml.

Staphylococcal enterotoxin B (SEB)
0.5 mg/ml aliquots in PBS were stored at -80 °C. An aliquot was further diluted 1:10 using PBS to make 50 μg/ml working stocks and stored at 4°C.

Violet viability reactive dye (Invitrogen)
50 μl of DMSO was added to a vial of Violet viability reactive dye (25 μg/ml), which was stored at -20 °C. This was further diluted in a 1:40 dilution with dH_2O, and 2.5 μl of this dilution was used to stain samples, as determined by a titration.

Polymer Amine Beads (Bangs Laboratories)
Beads were diluted to a final concentration of 40 X 10^6 beads/ml with PBS and stored at 4 °C. 40 μl of this stock was used for compensation.

Perm/Wash Buffer (BD Biosciences)
A 1:10 dilution was prepared with dH_2O to make a 1 X stock solution and stored at 4 °C, with a shelf life of 3 weeks.

CellFIX (BD Biosciences)
A 1:10 dilution with dH_20 (1X stock) was prepared and stored at 4 ºC, with a shelf life of 1 month.