B lymphocyte activation and exhaustion in chronic HIV: Novel surrogate markers of generalised immune activation and selective modulation of aberrant B cell responses using vasoactive intestinal peptide (VIP)

by
Timothy Dawson Reid

Thesis presented in fulfilment of the requirements for the degree of Master of Science in Medical Sciences (Medical Virology) in the Faculty of Medicine and Health Sciences at Stellenbosch University

Supervisor: Dr. Richard H Glashoff
Co-supervisor: Dr. Hayley Ipp

March 2015

The financial assistance of the National Research Foundation (NRF) towards this research is hereby acknowledged. Opinions expressed and conclusions arrived at, are those of the author and are not necessarily to be attributed to the NRF.

Declaration

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

March 2015

Abstract

Introduction

Chronic HIV-1 infection is characterized by immune activation and dysregulation of immune homeostasis, which impacts on multiple immune cell types. The B-cell compartment, which plays an important role in the producing neutralizing antibodies, is also dysregulated in HIV-1 infection. In this study we investigated peripheral blood B-cell subset distribution, and changes in expression of cellular activation, inhibition, and apoptosis signaling markers in both untreated chronic HIV-1 infected individuals and healthy uninfected controls. The neuropeptide immune modulator, vasoactive intestinal peptide (VIP) is known to selectively down-regulate activation of CD4+ T-cells in various disease settings including HIV-1, however to our knowledge, no studies have investigated the effect of VIP inhibition on B-cell activation.

Materials & Methods

A total of 21 HIV^{+ve} (CD4 count >250 cells/μl), and 19 HIV^{-ve} individuals were recruited from the Emavundleni voluntary testing and counseling clinic in Crossroads, Western Province, South Africa. Whole blood was stained to distinguish B-cell subsets (activated memory (AM: CD21-CD27+), resting memory (RM: CD21+CD27+), mature naïve (MN: CD21+CD27-), or tissue-like memory (TLM: $CD21^{lo}CD27^{lo}$). In addition expression of markers of B-cell activation (CD126, CD86, CD38, CD284, CD287), inhibition (CD72, CD85j, CD300a, CD305, CD307d), and apoptosis signaling (CD95), was assessed *ex vivo* by flow cytometry (BD FACSCanto II). For determination of functional responsiveness isolated B-cells (RosetteSep, Stemcell Technologies) were cultured for 18h (37°C, 5%CO_2) without stimulation or stimulated with TLR ligands (LPS or R848). Stimulation experiments were also performed in the presence or absence of VIP.

Results

Chronic HIV-1 infection affected B-cell subset distribution. The percentage (%) of TLM was increased by 59.24%, and %RM was decreased by 22.73% (both $p<0.01$). Total expression of the VIP receptor VPAC2 was decreased by 47.35% ($p=0.0296$). Subsets had a mixed phenotype *ex vivo*; HIV infection upregulated CD38 (by 59.56%, $p=0.0004$), CD72 (by 60.70%, $p=0.0396$), CD307d (by 68.63%, $p=0.0015$) on AM, while RM B-cells had increased expression of TLR4 (by 107.04%, $p=0.0057$) and TLR7 (by 208.14%, $p=0.0199$). TLM B-

cells (i.e. exhausted phenotype) displayed upregulated TLR7 (by 550%, p=0.0128) and CD307d (by 72.40% p=0.045) expression. MN B-cells had increased CD72 expression (by 70.98%, p=0.0026). R848 upregulated CD86 expression by 42.20% on AM (p<0.01), and by 56.06% on RM B-cells (p<0.01), which was significantly downregulated with VIP inhibition (both p<0.05). Similarly, CD95 expression on RM, TLM, and MN B-cells increased by 31.10% (p<0.001), 21.46% (p<0.01), and 39.92% (p<0.01) with R848 stimulation respectively, which was also significantly downregulated with VIP inhibition.

Conclusion

These data indicate that B-cells in untreated HIV infection display increased levels of activation, and also the potential for increased susceptibility to apoptosis as evidenced by increased FAS (CD95) expression. VIP significantly down-regulated markers of activation, inhibition, and apoptosis signaling. Dysregulation of B-cells is thus apparent in asymptomatic stable chronic HIV-1 infection, which may impact on both inefficient neutralizing antibody production and hypergammaglobulinemia. The ability of VIP to prevent stimulation-associated marker upregulation may indicate that VIP is a potential therapeutic agent. Its immuno-modulatory properties were demonstrated to limit B-cell hyperactivation, and selectively down-regulate apoptosis and mark it out for further investigation.

Opsomming

Inleiding

Immunaktivering en ongekoppelde immuun-homeostase is kenmerke van chroniese MIV-infeksie. Ons het perifere bloed B-sel subgroep-verspreiding, en veranderinge in die uitdrukking van merkers van aktivering, inhibisie, en apoptose in 'n onbehandelde MIV-1 besmettende groep ondersoek (in vergelyk met 'n gesonde onbesmettende kontrole). Die immuun-moduleerder, vasoaktiewe intestinale peptied (VIP) is bekend om aktivasie van geaktiveerde $CD4^+$ T-selle te verminder, maar tot ons kennis, is daar geen studies wat die effek van VIP-inhibisie op B-sel aktivering ondersoek het, in die konteks van MIV-1 infeksie.

Materiaal & Metodes

$MIV+^{we}$ individue (CD4-telling >250 selle/µl) , en $MIV-^{we}$ kontroles is gewerf uit die vrywillige toetsing en berading Emavundleni kliniek, Crossroads, Westelike Provinsie, Suid-Afrika. B-sel subgroepe is gedefinieer as geaktiveerde geheue (AM: $CD21^-CD27^+$), rusende geheue

(RM: $CD21^{+}CD27^{+}$), volwasse naïef (MN: $CD21^{+}CD27^{-}$), of weefsel-agtige geheue (TLM: $CD21^{lo}CD27^{lo}$). Merkers van aktivering (CD126, CD86, CD38, CD284, CD287), inhibisie (CD72, CD85j, CD300a, CD305, CD307d), en apoptose signalering (CD95) is via vloeisitometrie (BD FACSCanto II) op B-selle *ex vivo* en ook op geïsoleerde B-selle (RosetteSep, Cell Technologies) ondersoek. Vir die bepaling van funksionele responsiwiteit, geïsoleerde B-selle (RosetteSep, StemCell Technologies) was vir 18h (37°C, 5%CO2) gekweek, sonder stimulasie of gestimuleer met TLR ligande (LPS of R848). Stimulasie eksperimente het ook in die teenwoordigheid of afwesigheid van VIP plaasgevind.

Resultate

Chroniese MIV-1 infeksie het B-sel subset verspreiding geraak. Die persentasie (%) van TLM is verhoog deur 59,24%, en% RM het met 22.73% afgeneem (beide p <0,01). Totale uitdrukking van die VIP reseptor VPAC2 het met 47,35% afgeneem (p = 0,0296). Subgroepe het 'n gemengde *ex vivo* fenotipe; MIV-infeksie het CD38 (deur 59,56%, p=0,0004), CD72 (deur 60,70%, p=0,0396), CD307d (deur 68,63%, p=0,0015) op AM verhoog, terwyl RM B-selle het verhoogde uitdrukking van TLR4 (deur 107,04%, p=0,0057) en TLR7 (deur 208,14%, p=0,0199). TLM B-selle (die uitgeputtende fenotiep) het verhoogde TLR7 (deur 550%, p=0,0128) en CD307d (deur 72,40% p=0.045) uitdrukking gewys. MN B-selle het verhoogde uitdrukking van CD72 (deur 70,98%, p = 0,0026). R848 het CD86 uitdrukking op AM deur 42,20%, en op RM deur 56,06% toegeneem (beide p <0,01). Dit het met VIP inhibisie beduidend afgeneem (beide p <0.05). CD95 uitdrukking was soortgelyk verhoog op RM, TLM, en MN B-selle met 31.10% (p <0.001), 21,46% (p <0,01), en 39,92% (p <0,01) met R848 stimulasie. Al drie het beduidend afgeneem met VIP inhibisie.

Gevolgtrekking

Hierdie data dui daarop dat B-selle in onbehandelde MIV-infeksie vertoon verhoogde aktiveringsvlakke, en ook die potensiaal vir verhoogde vatbaarheid vir apoptose soos bewys deur verhoogde uitdrukking van FAS (CD95). VIP het beduidend merkers van aktivering, inhibisie, en apoptose af-gereguleer. Wanfunksie van B-selle is dus in asimptomatiese stabiele chroniese MIV-1 infeksie duidelik, wat impak kan hê op beide oneffektiewe neutraliserende teenliggaampie produksie, en hiepergammaglobulinimie. Die vermoë van VIP stimulasie-verwante merker opregulasie te voorkom kan aandui dat VIP 'n potensiële terapeutiese agent is. VIP se immuno-moduleerende eiendomme is gedemonstreer om B-sel hieperaktiveering te beperk, en selektief apoptose afreguleer, en merk dit vir verdere ondersoek.

Acknowledgements

I would like to express my most heartfelt gratitude and thanks to my supervisor, Dr. Richard Glashoff, for his encouragement and support throughout the duration of my MSc. His insight and encouragement throughout the revision process was greatly appreciated. Thank you for helping me become a better scientist.

I would also like to thank my co-supervisor, Dr Hayley Ipp for her positive input and support, especially with the more clinical aspects of the study. Thank you Hayley for always finding a different approach to the task at hand, even after the millionth revision.

Special thanks to Jan de Wit for his technical assistance, and always being available to have a chat .

Thanks to Danni Ramduth from BD Biosciences for the massive amount of input during the multicolour flow cytometry training and subsequent data acquisition.

Thanks to the rest of the staff and students at the Division of Medical Virology for their contribution to the day-to-day laughs and seriousness of academia.

Thank you to the staff of NHLS Tygerberg for performing the routine blood analyses used in this study

Most importantly, thank you to my family; my wife Liezl, and my son Finley for their endless support throughout the entire process, from the late nights at the lab, to writing up at the crack of dawn. Thank you for all your patience, love and encouragement

Thanks to my parents, and parents-in-law for continued support and funding.

The financial assistance of the National Research Foundation (NRF), Poliomyelitis Research Foundation (PRF), and Stellenbosch University (SU), towards this research is hereby acknowledged. Opinions expressed and conclusions arrived at, are those of the author and are not necessarily to be attributed to the NRF, PRF, or SU.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

$+^{Ve}$	Positive
$-^{Ve}$	Negative
AAA	ATPases Asscociated With Diverse Cellular Activities
Ab	Antibody
ADM	AIDS-Defining Malignancy
ADP	Adenosine Diphosphate
Ag	Antigen
AID	Activation-Induced Cytidine Deaminase
Amem	Activated Memory B-Cells (Also AM)
ANOVA	Analysis of Variance
AP-1	Activator Protein 1
APC	Antigen Presenting Cell
APC	Allophycocyanin (Fluorochrome)
ART	Antiretroviral Therapy
ARV	Antiretroviral [Medication]
BAFF	B-cell Activating Factor
BCMA	TNFRS17 or Tumor Necrosis Factor Receptor Superfamily Member 17
BCR	B-cell Receptor
BD	Beckton Dickenson
BLIMP-1	PRDM1, or PR-Domain Zinc Finger Protein 1
Blys	B Lymphocyte Stimulator
Bnabs	Broadly-Neutralising Antibodies
BTLA	B- and T Lymphocyte Attenuator
C	Constant (Gene Segment)
CCR	C-C Motif Chemokine Receptor
CD	Cluster of Differentiation
CR2	Complement Receptor 2

CS&T	Cytometer Setup and Tracking
CVID	Common Variable Immunodeficiency
CXCL	C-X-C Motif Ligand
CXCR	C-X-C Motif Chemokine Receptor
Cy	Cyanine
D	Diverse (Gene Segment)
DAMP	Damage-Associated Molecular Pattern
DC	Dendritic Cell
DC-SIGN	Dendritic Cell-Specific Intercellular Adhesion Molecule-3-Grabbing Non-Integrin
DNA	Dioxyribonucleaic Acid
ds	Double-Stranded
ELISA	Enzyme-Linked Immunosorbent Assay
FACS	Fluorescence-Activated Cell Sorting
FCS	Foetal Calf Serum
FcRL4	Fc Receptor-Like 4
fDC	Follicular Dendritic Cell
FITC	Fluorescein Isothiocyante
FMO	Fluorescence Minus One
FSC-A	Forward Scatter Area
FSC-H	Forward Scatter Height
GALT	Gastrointestinal-Associated Lymphoid Tissue
GIT	Gastrointestinal Tract
HAART	Highly Active Antiretroviral Therapy
HIV	Human Immunodeficiency Virus
HSC	Haematopoetic Stem Cell
HPT	HIV Prevention and Testing (Clinic)
ICL	Idiopathic CD4+ Lymphocytopenia
IDO	Indoleamine 2,3-Dyoxigenase

Ig	Immunoglobulin
Ig_h	Immunoglobulin Heavy Chain
Ig_l	Immunoglobullin Light Chain
IL	Interleukin
IFN	Interferon
ITIM	Immunoreceptor Tyrosine-Based Inhibitory Motifs
IQR	Interquartile Range
J	Joining (Gene Segment)
-L	Ligand
L	Linking (Gene Segment)
LAIR1	Leukocyte-Associated Ig-Like Receptor 1
LILR	Leukocyte Immunoglobulin-Like Receptor
LPS	Lipopolysaccharide
Mϕ	Macrophage
MALT	Mucosa-Associated Lymphoid Tissue
Mdc	Myeloid Dendritic Cell
MFI	Mean Fluorescent Intensity
MHC	Major Histocompatibility Complex
MN	Mature Naïve B-Cells
mRNA	Messenger RNA
NASBA	Nucleic Acid Sequence-Based Amplification
NF-Kb	Nuclear Factor Kappa Beta
NHL	Non-Hodgkin Lymphomas
PACAP	Pituitary Adenylate Cyclase-Activating Polypeptide
PAMP	Pathogen-Associated Molecular Pattern
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate-Buffered Saline
PD-1	Programmed Death 1

pDC	Plasmacytoid Dendritic Cell
PE	Phycoerythrin
PerCP	Peridinin Chlorophyll
PLB	Plasmablasts
PMTCT	Prevention of Mother-to-Child Transmission
-R	Receptor
R848	TLR7/8 Agonist - Imidazoquinoline Compound
RMem	Resting Memory B-Cells (Also RM)
RNA	Ribonucleaic Acid
RPMI	Roswell Park Memorial Institute (Cell Growth Medium)
S	Indicative of Plural, i.e. Tlrs
SANAS	South African National Accreditation System
SCF	Stem Cell Factor
SD	Standard Deviation
SLE	Systemic Lupus Erythematosus
SOP	Standard Operating Procedure
Ss	Single-Stranded (Nucleic Acid)
SSC	Side Scatter
TB	Tuberculosis
TD	Thymus Dependent (Antigen)
TI	Thymus Independent (Antigen)
TLMem	Tissue-Like Memory B-cells (Also TLM)
TLR	Toll-Like Receptor
TNF	Tumor Necrosis Factor
TR	Transitional B-cells
UNAIDS	Joint United Nations Programme on HIV/AIDS
V	Variable (Gene Segment)
VIP	Vasoactive Intestinal Peptide

VPAC1/2	VIPR1/2 or VIP Receptor 1 or 2

Introduction

Immune activation is one of the driving forces behind disease progression in untreated, chronic HIV-1 infection, where multiple immune cell types are affected including both T-cells and B-cells (Moir & Fauci, 2008). Numerous studies have shown that B-cells are hyperactivated and display poor functional responses in acute HIV-1 infection, and that these defects begin to emerge before the quantitative decline in $CD4^+$ T-cells (Shen & Tomaras, 2011). While their primary function is that of antibody secretion, of particular interest in acute HIV-1 infection is the B-cell response to T-independent antigens, which engage mainly the toll-like receptor pathways and bypass isotype switching and affinity maturation. In doing so, the B-cell response to these innate stimuli is much faster, albeit not broadly-specific, resulting in secretion of IgM specific to the TLR-inducing antigens (Moir & Fauci, 2013). Furthermore in chronic, untreated HIV-1 infection, there is an abundance of both viral and bacterial antigen stimuli available to B-cells. Viral antigenemia is due to both ongoing HIV replication plus other chronic viral infections (e.g. CMV). Bacterial antigens are present due to destruction of gut-associated lymphoid tissue (GALT), leading to translocation of gut microbial antigens into the systemic circulation, providing an important source for ongoing activation of the immune system. Both bacterial components (e.g. lipopolysaccharide) and viral components (single-stranded RNA) antigens are considered to be T-independent antigens, capable of triggering toll-like receptors (TLR4 by LPS and TLR7/8 by ssRNA), both during acute and chronic HIV-1 infection (Lester, *et al.,* 2008; Baenziger, *et al.,* 2009).

Immune exhaustion is another cardinal feature of untreated chronic HIV-1 infection, where effector cell responses are severely impaired as a result of persistent antigenic stimulation and immune activation (Klatt, *et al.,* 2013). Decreases in cell function are correlated with increased presence of inhibitory receptors. On T-cells these include PD-1, Tim-3 and others; while on B-cells, receptors such as CD305, CD300a, CD85j, and CD72 confer inhibitory function (Kardava, *et al.,* 2011). These receptors down-regulate activation signals, via immunoreceptor tyrosine-based inhibitory motifs (ITIMs) in their cytoplasmic tails, which, when ligated, either block or significantly decrease the activity of cell signaling molecules. It is thought that expression of these receptors is up-regulated in response to persistent activation signals, and the cells are in fact trying to limit activation and the adverse pathological consequences thereof. However, in doing so, the cell compartments gradually lose their function (due to the fact that the activation signals, both viral and GIT-associated antigens, are never cleared), and HIV-1 disease progresses towards AIDS (Boasso & Shearer, 2008).

Current protocols for treatment of HIV-1 infection in South Africa are combination antiretroviral therapy (ART), with initiation of treatment being based on having a CD4 less than 350 cells/µl (Evans, 2013). ART is a potent inhibitor of viral replication; however, immune activation is not always adequately managed in those on treatment, and indeed the ongoing antigenic stimulation remains, mainly from bacterial antigens due to microbial translocation across the GIT. The GALT is a storage zone for multiple memory $CD4^+$ T-cell populations specific for various antigens, and are the earliest cell population to be depleted in HIV infection due to apoptosis (following direct infection and/or activation-associated cell death). This process is thus a consequence of and a contributor to immune activation in primary infection (Klatt, Funderberg, & Brenchley, 2010). A useful adjunct to ART would be regimens which down-regulate activation signals without affecting the integrity of the immune response. The immuno-modulatory neuropeptide, vasoactive intestinal peptide (VIP) is known to selectively down-regulate activation signals on activated $CD4^+$ T-cells (via the VPAC2 receptor), while not significantly affecting resting $CD4^+$ T-cells (Ipp, *et al.,* 2014). Of particular importance is the ability of VIP to downregulate Fas, thus protecting activated CD4 T cells from activation-associated cell death. Little is known of the action of VIP, or its receptor VPAC2, on peripheral blood B-cells, and to our knowledge, this is the first study to investigate this.

Our research question investigated whether B-cell responses of HIV-infected individuals to innate antigens (TLR ligands), such as bacterial LPS, and single-stranded viral RNA resulted in significantly altered expression of markers of activation, inhibition or exhaustion, and apoptosis signaling, as compared with HIV-uninfected controls. We also compared the expression of these B-cell markers with standard disease correlates such as CD4 count, HIV viral load, and CD38 expression on $CD8^+$ T-cells. Once the responses to stimulation were established, we further investigated whether the novel immuno-modulatory neuropeptide, VIP could down-regulate TLR-induced expression of markers of activation, inhibition, and apoptosis signaling.

Chapter 1: Literature Review

1.1 B-cell Physiology in Healthy Individuals

1.1.1 Introduction

B-cells play an important role in the mediation of humoral immunity via antibody secretion. The antibodies that are secreted in response to an infection bind to specific antigens on the pathogen's surface, and in doing so aid in effective clearance of infection. Distinct antibody isotypes exist, each of which occupies a specific niche allowing specialised elimination of pathogens.

1.1.2 Antigen independent B-cell development and maturation

B-cells originate from common lymphoid progenitor cells, which in turn are derived from hematopoietic stem cells (Murphy, *et al.*, 2008). Stromal cells within the bone marrow provide signals that stimulate early B-cell development, in the form of both membrane-bound and soluble chemokines and cytokines; and cellular interactions between cell-adhesion molecules and their respective ligands (Melchers, *et al., 2000*). The earliest cell of B-cell lineage is known as an early pro-B-cell (CD34+CD10+CD19+CD20-), where Ig gene rearrangement begins (Sanz, *et al.*, 2010).

The Ig heavy chain (IgH) gene recombination begins with the joining of the Diverse (D) and Joining (J) regions (see Fig 1.1), resulting in a unique D-J sequence where the cell becomes a late pro-B-cell (Melchers, *et al., 2000*). A second recombination then follows, in which the recombined Variable (V) region is joined to the D-J sequence, resulting in a unique intact μ heavy chain gene (Melchers, 2005). The resulting VDJ sequence is then joined to the Constant (C) region, and heavy chain gene recombination ceases to occur, and the cell becomes a Pre-B-cell. Approximately 45% of Pro-B-cells do not produce μ heavy chains, and are eliminated at this stage of their development (Melchers, *et al., 2000*). The newly-produced heavy chains are tested for functionality by the formation of a pre-B-cell receptor (Pre-BCR), where the heavy chain combines with light chain-like proteins to signal to the pro-B-cell whether the VDJ recombination has been successful or not (Hardy & Hayakawa, 2001). At this point the pre-B-cell is induced to proliferate, and recombination of the light chain genes occurs. An important concept in the gene recombination process is allelic exclusion, which is the process whereby only one of the two alleles of a diploid cell is expressed, due to the fact that successful recombination of the heavy chain of both alleles has the potential to produce two separate receptors with different antigen specificities, on the

same cell. This occurs in both heavy and light chain recombination (Pieper, Grimbacher, & Eibel, 2013).

Light chain genes lack D segments, therefore only V and J rejoins to create a functional light chain protein (Fig. 1.1), and in the same manner as heavy chain recombination, the light chain genes are recombined on one chromosome, before proceeding to the other chromosome (Murphy, *et al.*, 2008: pp.267). The light chain locus on human chromosomes can either be of the κ or λ type. Isotypic exclusion is the process whereby only one form of the light chain gene can be expressed, and in humans, the κ light chain locus has a tendency to recombine before the λ locus. If the eventual light chain gene recombination has been successful, the pre-B-cell is stimulated to proliferate, the resulting progeny being termed immature B-cells, which then express IgM (Melchers, 2005)

Before the immature B-cell leaves the bone marrow (Fig. 1.2), its antigen receptor is tested for auto-reactivity in a process known as central tolerance (Kyewski & Klein, 2006). B-cells that cross-link strongly with a self-antigen in the bone marrow, will not mature, and undergo apoptosis or clonal deletion; or, will be induced into a state of immunological ignorance, or become permanently unresponsive (anergic). The immunologically ignorant cells however, can be activated in certain conditions, such as inflammation or with high levels of self-antigen (Pieper, Grimbacher, & Eibel, 2013). Once the immature B-cells leave the bone marrow, they home towards the spleen, where they undergo further maturation and selection (Fig. 1.2).

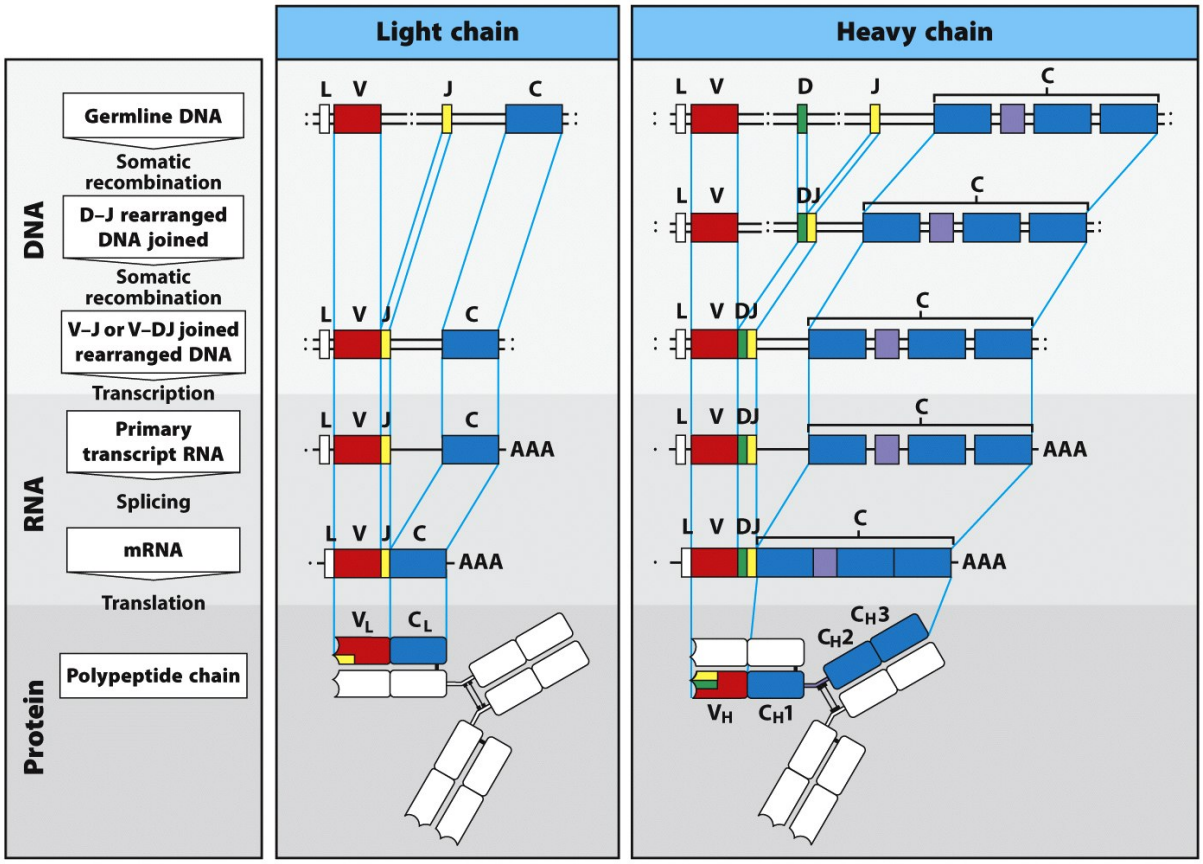


Figure 1.1 Schematic overview of Heavy and Light Chain gene rearrangement.

Early pro-B-cells first undergo joining of the D and J gene segments, which is followed by joining of the V region to the D-J sequence, which then joins the C region, resulting in an intact μ Heavy chain gene on a Pre-B-cell. Light chain genes lack the D region, soon the V and J sequences are joined to create a functional light chain. Adapted from Murphy, et al. (2008).

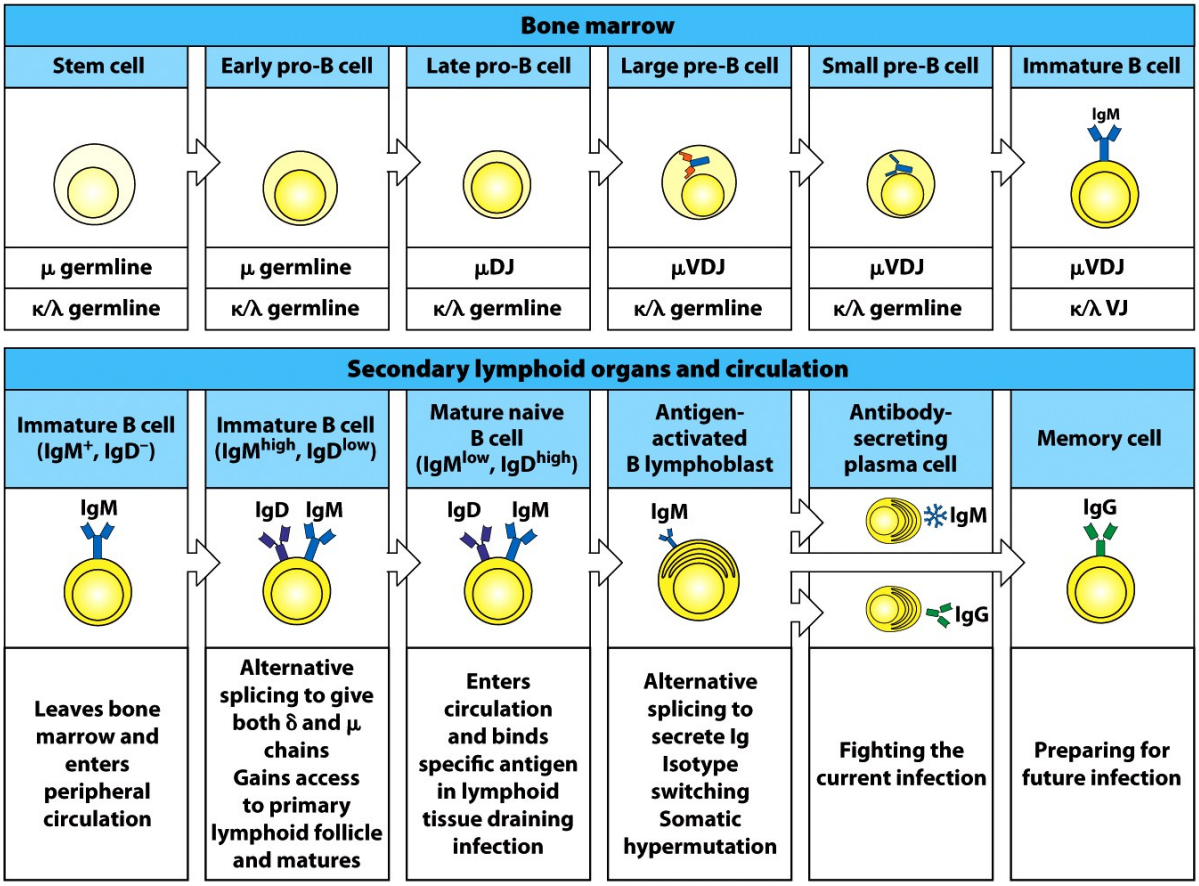


Figure 6.25 The Immune System, 3ed. (© Garland Science 2009)

Figure 1.2 Schematic overview of B-cell development from haematopoetic stem cell, to memory B-cell.
Adapted from Parham (2009).

,

1.1.3 Antigen dependent B-cell development and maturation

1.1.3.1 Antigen Specific Activation: T-cell Independent Antigens

T-cell independent antigens are non-proteinaceous (i.e. polysaccharides or nucleic acids), and can be further broken down into thymus-independent (TI) type 1, or TI type 2 antigens (Lanzavecchia & Sallusto, 2007; Fig. 1.3). As the name suggests, these antigens stimulate a B-cell response without the help of CD4+ T-cells, as APCs do not normally process and present these types of antigen via the major histocompatibility complex (MHC) to naïve CD4 T cells, which is required for promoting B-cell help (Rawlings, *et al.*, 2012). TI type 1 antigens comprise TLR-ligands such as LPS or viral RNA and result in the formation of memory B-cells, in contrast to TI type 2 antigens, which are multivalent, consisting mainly of repeating epitopes such as bacterial capsular polysaccharides or viral capsid proteins, which do not result in formation of memory B-cells (Vinuesa & Chang 2013). TI type 2 antigens cross-link the BCR upon ligation, leading to activation. The alternate complement pathway activates B-cells in a similar fashion, where antigen-bound C3d leads to BCR signaling via recruitment of CD21, and C4-binding protein can provide a $CD4^+$ T-cell-like signal via CD40 engagement (Lanzavecchia & Sallusto, 2007). Antigen-presenting cells (APCs) such as macrophages and dendritic cells (DCs) produce pro-inflammatory soluble mediators such as the cytokines interleukin (IL) -2, IL-4, interferon (IFN) –α, among others, that stimulate the BAFF (B-cell activating factor) family of receptors that provide additional signals for B-cell activation (Rawlings, *et al*., 2012). During the acute phase of HIV-1 infection, pattern recognition receptors (PRRs) such as TLRs are among the first means of identifying the invading virus, chief among which is TLR7, recognizing ssRNA in endosomal vesicles (He, *et al.,* 2013). Indeed, TLR signaling has been described as pivotal in both an initiator of and contributor to the ongoing immune activation in HIV infection (Siewe, *et al.,* 2013).

1.1.3.2 Antigen-Specific Activation: T-cell-Dependent Antigen Presentation

APCs reside in areas of environmental contact within the body, such as the skin, and mucosae. APCs such as DCs and macrophages can be considered the ‘sentinels’ of the immune system, in that they constantly survey the extracellular milieu of these sites for both foreign and self-antigens. DCs are of particular interest due to their migratory capacity, and the fact that they are prime stimulators of naïve T-cells in lymphoid tissues. Once a DC has encountered Ag, in the presence of pro-inflammatory cytokines such as IL-1, IL-6 and TNF–α, it up-regulates co-stimulatory molecules (CD40, CD80, CD86) and cytokines (IL-1, IL-12, TNF-α) and migrates to T-cell areas of peripheral lymphoid tissue (Steinbrink, *et al*. 2009).

DCs then activate naïve CD4+ T-cells which then become reactive towards the specific Ag if it is foreign or pathogenic, whereas if the Ag is that of the body (self-Ag), the CD4+ T-cell becomes nonreactive or tolerant towards the self-Ag.

The CD4+ T-cells then proceed to alter surface molecule expression by up-regulating CD40 ligand (CD40L) and altering chemokine receptor expression (increased CXCR5 and decreased CCR7 expression). These CD4+ T-cells (also known as helper T-cells) migrate towards the lymphoid follicle down a CXCL13 chemokine gradient (secreted by follicular DCs). At the same time, naïve B-cells are activated when they encounter antigen via the BCR and also TLRs in peripheral environmental contact sites. On encountering antigen, they down-regulate CXCR5 and up-regulate CCR7 expression, and subsequently migrate out of the follicles and meet the activated CD4+T-cells. CD40, which is constitutively expressed by B-cells, interacts with CD40L on the activated helper T-cells and induces the transcription factors NF-κB and AP-1. The activation of these transcription factors allows genes crucial to the germinal centre reaction to be transcribed (Tarlinton, 2006).

1.1.3.3 The Germinal Centre Reaction

The germinal centre is an area of the lymphoid follicle that Ag-activated B-cells migrate to in order for rapid B-cell proliferation to occur (Klein, *et al.*, 2003; Fig. 1.3). The germinal centre can be divided into two areas based on appearance in microscopic section, namely the dark and light zones consisting of rapidly proliferating B-cells (known as centroblasts), and B-cells undergoing selection, respectively (Tarlinton, 2006). The B-cells in the dark zone undergo somatic hypermutation, which is the process whereby point mutations occur within the V, D and J immunoglobulin (Ig) genes to create more diversity in Ag-recognizing capacity. This process is not to be confused with V(D)J recombination, which occurs in the bone marrow as part of B-cell development. Once somatic hypermutation has occurred, the B-cells then proceed towards the light zone of the germinal centre, where their Ag-recognising ability is tested by follicular dendritic cells (Tarlinton, 2006). B-cells with high-affinity Ig receptors are selected to survive and mature further, while those with low-affinity or non-binding Ig receptors are selected for death. This process ensures that only B-cells with highly-specific Ig receptors towards a particular Ag are involved in the further immune response (Phan, *et al*, 2006).

1.1.3.4 Class-Switching

Class switching refers to the process of Ig receptor isotype alterations in response to specific types of Ag, resulting in either IgM, IgG, IgE, or IgA production (McMichael, *et al.*, 2010). The mechanism whereby class switching occurs is known as switch recombination, and during this process the rearranged VDJ genes recombine with a downstream C region gene. CD40 and cytokine stimulation induces transcription via the C region gene (known as germline

transcription) resulting in different Ig isotype production in response to different types of Ag, through the CD4+ T-cell interaction. Many non-protein innate Ag's such as LPS are not recognized by T-cells, and subsequently CD4+ T-cell help does not occur, with the B-cells retaining their IgM isotype. Viral and bacterial protein Ag activates T_H1 T helper cells, which produce IFN-γ, a key inducer of IgG isotype switching (Siegrist & Aspinall, 2009)

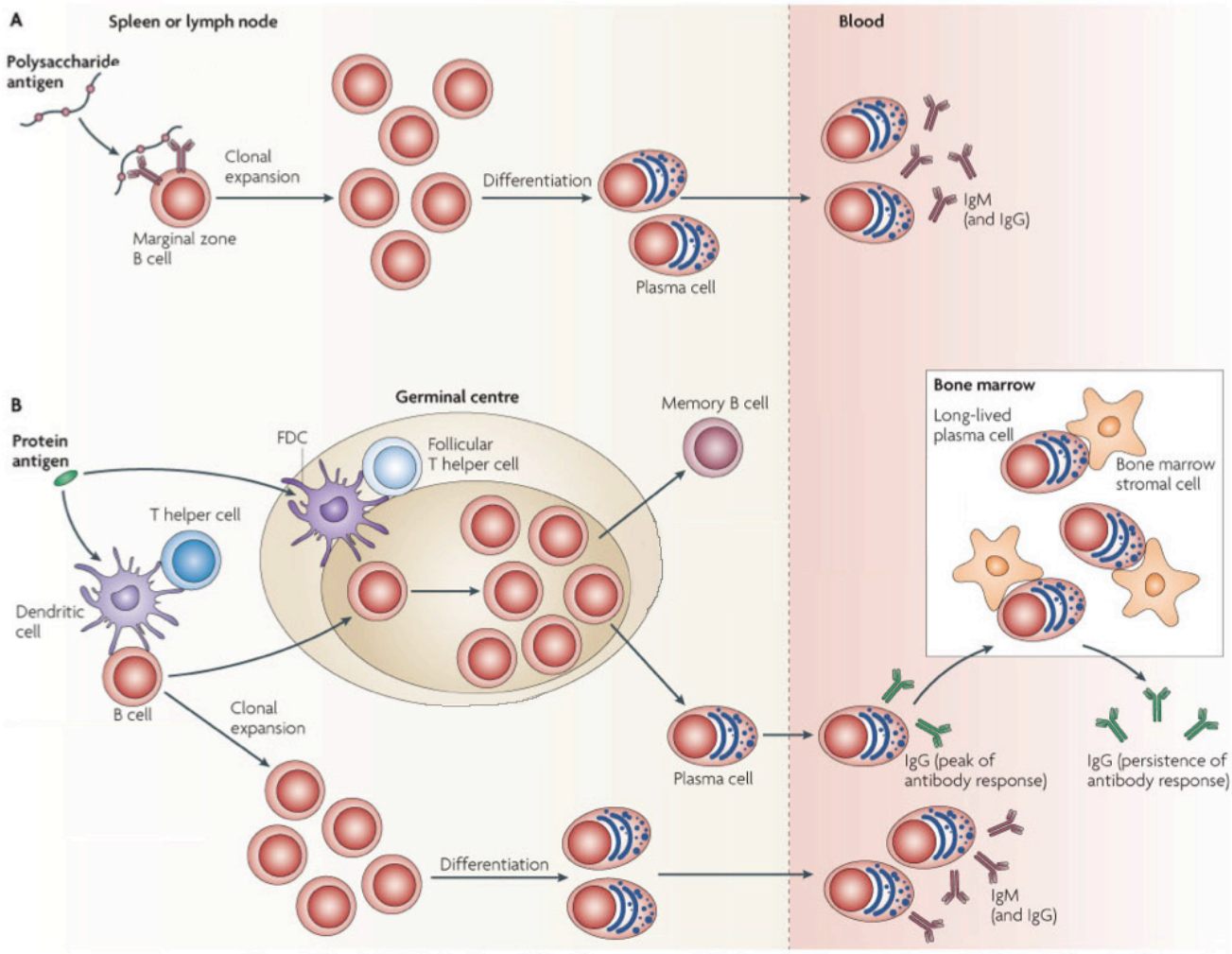


Fig 1.3: Schematic overview of the B-cell antigen response to either polysaccharide Ag (T-independent), or protein Ag (T-dependent). ***A)*** *Polysaccharide antigens reach the marginal zone of lymphoid tissues and bind to marginal zone B-cells, thereby driving their differentiation into short-lived plasma cells in the absence of T-cell help.* ***B)*** *Protein antigens arrive at the B-cell zone in the lymph nodes either by diffusion (triggering the T-independent response), or by DC trafficking and presentation in the T-cell zone (triggering T-dependent responses). This results in the generation of both short-, and long-live plasma cells; as well as long-lived memory B-cells. Adapted from Siegrist & Aspinall (2009).*

1.1.3.5 Affinity Maturation

Affinity maturation is a process, which leads to increased antibody affinity (i.e. binding strength) towards the specified Ag. Affinity maturation also only occurs when protein Ag is encountered (that is, CD4+ T-cell-dependent antigen), due to the CD40-CD40L interaction, which is a prerequisite for this process (therefore affinity maturation does not occur in the presence of non-protein Ag with innate stimulation) (McMichael, *et al*., 2010). As mentioned above, B-cells with non- or poorly-reactive Ig towards the specific Ag (due to somatic hypermutation) are selected to undergo apoptosis by the follicular DCs. Therefore the B-cells that survive are those that can bind Ag, and due to the nature of somatic hypermutation, B-cells with increasingly strongly-binding Ig are produced. Therefore, the crux of affinity maturation is that the constantly rearranging Ig-genes produce B-cells with Ig that have increasing binding strength towards the defined Ag (Pulendran & Ahmed, 2011).

1.1.3.6 B-cell Memory

The cells that have been selected for survival by the follicular DCs then exit the germinal centre and proceed to differentiate into either memory B-cells, or plasma cells highly-specific for the Ag in question (Tangye & Tarlinton, 2009). Memory cells are long-lived cells, which recognize and rapidly respond to previously-encountered Ag. Memory cells in peripheral circulation and in lymph nodes become Ag-specific plasma cells in response to recall Ag (i.e. Ag that has already been encountered); this secondary or repeat Ag response is much more rapid than the primary or initial Ag response (Tarlinton, 2006).

Plasma cells are terminally differentiated B-cells with a distinctive morphology that produce large amounts of Ag-specific antibodies. They are found mainly in the extra-follicular sites of the lymphoid organs (i.e. the lymph node medulla and splenic red pulp) and consist of two distinct types, namely the long- and short-lived plasma cell (Siegrist & Aspinall, 2009). As mentioned above, CD40 and cytokine stimulation (such as IL-2, IL-4 and IL-6) result in B-cell differentiation into plasma cells, which is mediated by the transcription factor BLIMP-1 (also known as PR domain zinc finger protein 1). Short-lived plasma cells are generated in response to thymus-independent Ag, which do not induce a T-cell response, while long-lived plasma cells home to the bone marrow and are sustained by BCMA (B-cell Maturation Antigen, also known as TNFR superfamily 17) receptor stimulation. These long-lived plasma cells continue to produce antibodies for years, even in the absence of antigenic stimulation, with the bone marrow becoming the primary site of antibody production (Tangye & Tarlinton, 2009).

1.1.4 B-cell Subsets

B-cells inhabit many niches within the human immune system, and have surface markers characteristic to each niche. As mentioned above, B-cells develop in the bone marrow from

haematopoietic stem cells (HSC), once they reach the immature B-cell stage of development; they migrate out of the bone marrow into the blood stream, towards secondary lymphoid organs, where further maturation and development takes place.

B-cells can be identified primarily by their expression of both CD19 and CD20, also known as pan-B markers. CD19 is expressed on the surface of pro-B-cells in the bone marrow, and lost on long-lived plasma cells, while expression is maintained on long-lived memory B-cells (Hardy & Hayakawa, 2001). CD20 expression is gained on the B-cell surface at the pre-B-cell stage of development and is lost in plasmablasts (Eisenberg & Looney, 2005). CD19 expression is essential for normal antibody responses, and defects in CD19 expression cause delays in maturation, proliferation and antibody production (Wang & Carter, 2005). CD20 is highly expressed on the cell surface, and is a therapeutic target in B-cell lymphomas, where the anti-CD20 drug Rituximab has been shown to effectively deplete peripheral blood B-cells *in vivo* (Eisenberg & Looney, 2005).

1.1.4.1 Peripheral Blood Subsets

The five major peripheral B-cell subsets to be discussed and which are the focus of this study are as follows: Transitional (immature) B-cells, mature naïve B-cells, activated memory B-cells, resting memory B-cells, and plasmablasts. All of the above-listed subsets express CD19, while plasmablasts and transitional B-cells express variable levels of CD20.

Transitional/Immature B-cells are CD10+ B-cells that were first described in patients with Systemic Lupus Erythematosus (SLE) who underwent B-cell depletion therapy with Rituximab, and they have also been reported in chronic HIV infection, as well as other states of immunodeficiency such as Common Variable Immunodeficiency (CVID) and Idiopathic CD4+ Lymphocytopenia (ICL) (Moir, *et al., 2010*). Healthy individuals have low circulating number of these cells, which increases in frequency in diseases such as chronic HIV infection. This is due to increased serum levels of IL-7, which is produced as a homeostatic compensation mechanism to the CD4 lymphopenia of HIV infection (Ho, *et al*., 2006). Human transitional B-cells can be subdivided into separate populations based on the expression of CD24 (an undefined sialoglycoprotein) and CD38 (cyclic ADP ribose hydrolase). T1 B-cells ($CD10^{+}CD24^{hi}CD38^{hi}$) are found primarily in peripheral blood, and T2 B-cells ($CD10^{+}CD24^{hi}CD38^{lo}$) are mostly restricted to the spleen (Sims, *et al*., 2005). The T1 population bears a striking phenotypic resemblance to that of hematogones, which are non-malignant B-cell precursor cells (Agarwal, *et al.*, 2010). There appears to be conflicting information with regard to whether transitional B-cells are indeed hematogones, Sportès, *et al* (2010) for example refers to transitional B-cells and hematogones as two distinct entities.

Mature naïve B-cells constitute the largest portion of peripheral blood B-cells. They are antigen-naïve cells, characterized by high surface expression of complement receptor 2 (CR2) also known as CD21 and negativity for CD27 (a member of the TNFR superfamily). These cells are the mature form of transitional B-cells, and are short lived (Ettinger, *et al*., 2005). In fact, approximately 50% of these cells die every 3 days in the steady state, when not exposed to Ag (Murphy, *et al.*, 2008: pp.304).

Memory B-cells are classified according to their expression of CD27 and CD21, and can be either activated, or resting. CD27 positivity distinguishes both activated- and resting memory cells from naïve cells, while CD21 expression represents the ability to internalise immune complexes, therefore cells with $CD21^{-}$ or $CD21^{lo}$ expression have a decreased ability to recognize complement-bound Ag (Das, *et al.*, 2011). Erdei, *et al.* (2009) found that survival of memory B-cells was impaired when CD21 was not expressed. $CD21^{lo}CD27^{hi}$ expression characterises memory B-cells with an activated phenotype, that is, memory B-cells that are proliferating and in the process of differentiating into plasma cells.

Plasmablasts are the immature, actively dividing form of plasma cells, which exist in circulation for a few days before they cease to divide and either undergo apoptosis, or further differentiate into plasma cells and migrate towards lymph nodes, or the bone marrow (Fairfax, *et al.,* 2008). Plasmablasts can be defined by the high expression of CD38, and absence of CD138 (Syndecan 1), and have the following immunophenotype: $CD20^{-}$ $CD19^{+}CD27^{hi}CD38^{hi}CD138^{-}$ (Llinàs, *et al*., 2011). Plasmablasts lose CD19 and CD20, and gain CD138 expression as they mature into plasma cells and subsequently migrate to the bone marrow (Pinto, *et al.,* 2013).

1.2 Human Immunodeficiency virus

1.2.1 HIV epidemiology in South Africa

A report by the Joint United Nations Program on HIV/AIDS estimated that as of 2013, there are more than 35 million people living with HIV world-wide (UNAIDS, 2014). At least 24.7 million of these people live in Sub-Saharan Africa, representing approximately 70.6% of the global HIV burden. 6.3 million HIV-infected individuals live in South Africa; with the largest population of HIV-infected individuals in the world. This number corresponds to approximately 10% of the South African population (UNAIDS, 2014).

Since the rollout of antiretroviral therapy (ART) in 2004, there has been a dramatic decrease in the number of new infections in South Africa (Evans, 2013). Approximately 86% of people

with known HIV status in Sub-Saharan are receiving treatment, 76% of which have achieved viral suppression. South Africa has an HIV treatment coverage of more than 72% (UNAIDS, 2014). It is estimated that, since 1995, ART has prevented more than 4.8 million deaths in sub-Saharan Africa (UNAIDS, 2014).

1.2.2 Immunopathogenesis of HIV-1 infection

A hallmark feature of HIV-1 infection is the loss of CD4+ T-cells. Both direct infection and "bystander" death are implicated in this CD4+ T-cell loss. CD4+ T-cells are the main targets for HIV-1 infection via the interactions of gp120 membrane glycoprotein on the HIV-1 virion with the CD4 receptor. In addition, entry requires interaction with either the CCR5 or CXCR4 chemokine co-receptors on the CD4+ T-cell (Haase, 2010). Once gp120 has bound to CD4 and its co-receptors (mediated by the HIV-1 membrane glycoprotein gp41) fusion of the two entities occurs, which allows entry of viral core and subsequent release of viral RNA and reverse transcriptase (RT) into the CD4+ T-cell cytoplasm. Thereafter, RT-mediated reverse transcription of viral RNA into cDNA occurs, which is then integrated into the host's genome via the virally-encoded HIV-integrase enzyme. The integrated provirus can then be replicated (in activated CD4+ T-cells) and encode for viral proteins which allows for assembly of new virions which bud off at the CD4 T-cell membrane (Klatt, *et al*., 2013).

The most common route of HIV transmission in Sub-Saharan Africa is via heterosexual intercourse, and therefore it is important to understand the dynamics of HIV transmission at mucosal sites (UNAIDS, 2012). Experiments using a non-human primate model of mucosal HIV transmission have demonstrated that HIV can cross the mucosal barrier within hours, subsequently producing a so-called 'founder population' of infected resting CD4 T-cells (Haase, 2010). The next 10 or so days following primary infection is known as the 'eclipse' phase, where there is no detectable viral RNA in the plasma. The founder population then expands sufficiently to disseminate throughout the secondary lymphoid organs and establishes a self-propagating infection in what is known as acute HIV syndrome (Fig. 1.4, Fauci, 1996). This stage of HIV-1 infection is characterized by several pathophysiological events, the most profound being a rapid increase in HIV-viraemia. While HIV directly infects only 20% of the total CD4 population in the GALT, an additional 60% undergo apoptosis in response to activation signals (including pro-apoptotic cytokines and death receptor ligands such as TNF-α, TRAIL, and FasL) from the infected cells. Altogether, about 80% of GALT $CD4^{+}$T-cells are depleted within the first 3 weeks of HIV-1 infection (McMichael, *et al*., 2010). B-cell dysfunction is evident at this early stage despite the lack of direct infection of these cells by the virus. This dysfunction is related to the loss of cell populations crucial to the formation of germinal centers. Approximately 50% of germinal centers in the GALT are lost in the acute phase of HIV-1 infection (McMichael, *et al.*, 2010).

Peak plasma viraemia occurs towards the end of the acute phase of HIV-1 infection, and begins to decrease to a more stable level known as the viral set point, which is maintained by a homeostasis between viral turnover and the adaptive immune system (Ostrowski, 2010). The chronic phase of HIV-1 infection (referred to as 'clinical latency' in Fig. 1.4) that follows can last between weeks and years before the onset of the clinical symptoms of AIDS. At this stage of infection, inflammation caused by the initial viral infection remains unresolved, thereby resulting in chronic stimulation of the immune system resulting in immune activation (Fig. 1.4 below), which further drives systemic inflammation (Desai & Landay, 2010).

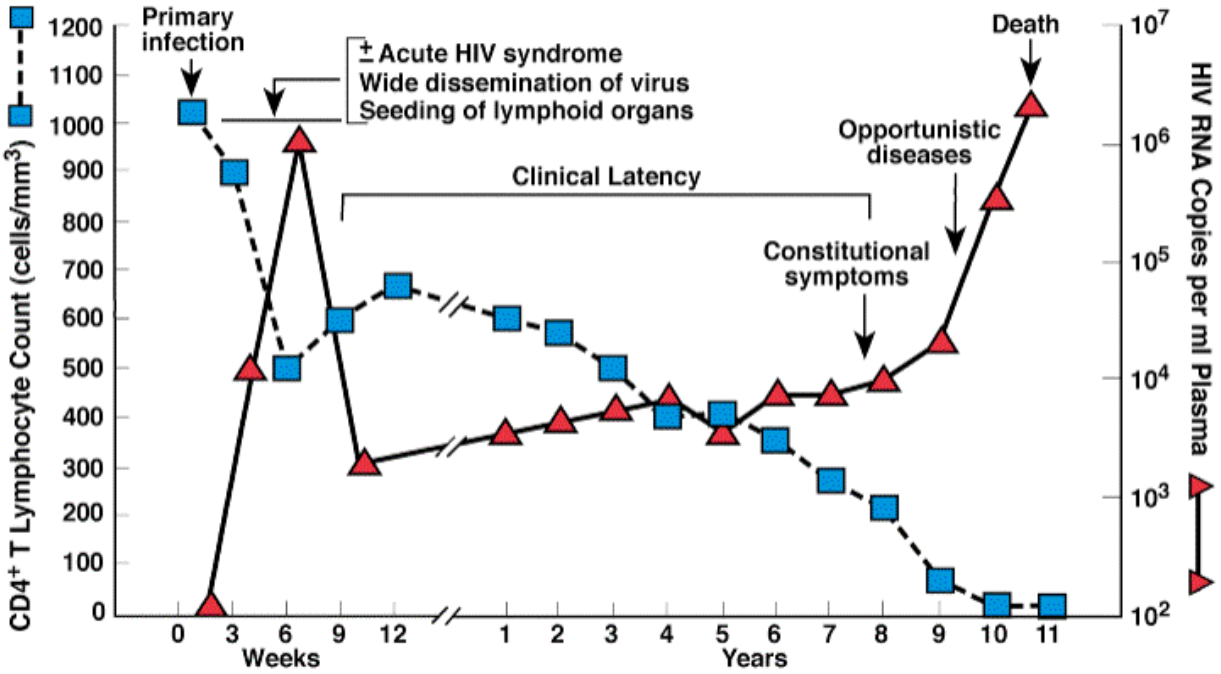


Fig. 1.4 Typical course of HIV infection. Adapted from Fauci (1996)

1.3 HIV-related inflammation & Immune Activation, and B-cell Dysfunction

1.3.1 General Features of Inflammation and Immune Activation in HIV

Immune activation is defined by Appay & Sauce (2009) as "A rather broad expression that covers a large range of events or observations involved in active molecular or cellular processes…and their consequences". Immune activation in HIV-1 infection has been linked to direct infection by HIV-1, increased pro-inflammatory cytokine production by innate cells (which further drives both bystander, and direct cellular activation), increased systemic LPS and other microbial products due to microbial translocation, other co-infections, and loss of regulatory T-cells (McMichael, *et* al., 2010). HIV-associated immune activation has been linked to HIV-viremia, lymphopenia, and microbial translocation in the GIT (Ruffin, *et al.*, 2012). Ongoing immune activation is one of the characteristic features of chronic HIV infection, which occurs while viraemia is still relatively well-controlled. Predictive markers of disease progression vary, although both CD4 count and plasma HIV-1 viral load are used to monitor immune status, and viral burden (Siliciano & Siliciano, 2010). The well-described marker of immune activation: CD38 expression on $CD8^+$T-cells is an increasingly-relevant means of assessing HIV-1 disease progression (Appay & Sauce, 2009)

Immune activation results in an increased expression and up-regulation of biomarkers associated with activation (both soluble and membrane-bound), increased cellular proliferation and apoptosis, and an increase in the secretion of inflammatory biomarkers such as growth factors, chemokines, and cytokines (Kiazyk & Fowke, 2008). Markers of B-cell activation, demonstrated to be elevated as a consequence of immune activation during chronic HIV infection include CD70, CD71, CD80, CD86, and CD23 (Moir, *et al*., 2008). Recent studies report an increase in IL-21R expression (Ruffin, *et al.*, 2012), as well as increased expression of PD-1 and decreased BTLA (B and T-Lymphocyte Attenuator) expression on B-cells, the implications of which are similar to that of increased CD38 and PD-1 on $CD8^+$T-cells in chronic HIV infection (Boliar, *et al.*, 2012). In addition, the loss of CD28 (the receptor for CD80 and CD86) and increased CD57 (human natural killer-1, an adhesion molecule) expression have been described (Desai & Landay, 2010). Activated memory B-cells in chronic (untreated) HIV infection are also more prone to apoptosis, with increased expression of Fas (CD95) (Moir, *et al.*, 2010).

1.3.2 Direct Interactions Between HIV and the B-cell Compartment

Immune activation has been associated with persistent antigenic exposure resulting in the immune system constantly being in an activated state against the perpetual presence of viral, (translocated) GIT products, and opportunistic/chronic non-HIV infection antigens (Badley, *et al.,* 2000). Immune activation is a normal response to acute infection; is usually short-lived and decreases as the foreign Ag is cleared. In untreated HIV, however, the Ag is never cleared, and its constant presence results in a state of chronic immune activation (Malaspina, *et al.* 2003). In this manner, the direct interactions between HIV and the immune system cause several abnormalities in the B-cell compartment.

HIV virions opsonised by complement and HIV envelope proteins such as gp120 and nef are involved in the direct interaction between HIV and B-cells, via complement receptors and the BCR respectively (Moir & Fauci, 2008). Gp120 binds to C-type lectins (such as DC-SIGN, also known as CD209) on the B-cell membrane, and in so doing, induces immunoglobulin class switching, and increased levels of its related enzyme, activation-induced cytidinedeaminase (AID) (Moir & Fauci, 2009). However, Moir and Fauci in 2008 reported seemingly conflicting data, where, in an *in vitro* model, nef had been found to accumulate within endosomes in B-cells and interfere with CD40L-mediated signaling, thereby inhibiting immunoglobulin class switching and activation.

TLR signaling by TLR ligands is a separate pathway to that of BCR-mediated signaling, and also serves as a means of direct innate stimulation of B-cells by HIV. TLR 7 and 9 are both viral sensors that recognize ss-RNA and CpGDNA sequences, respectively (Haas, *et al*., 2011). Constitutive TLR signaling in B-cells in humans is restricted to the memory compartment, and upregulation of TLRs on naïve B-cells requires BCR-mediated signaling, usually via type 1 IFNs (Lanzavecchia & Sallusto, 2007). An indirect means of *in vivo* B-cell stimulation by TLRs (to be discussed further on) is via TLR4 signaling, which recognizes bacterial LPS from the GIT (Mauri & Bosma, 2012).

1.3.3 Indirect Interaction between HIV and the B-cell Compartment

In addition to the direct effect of HIV on the immune system, there is an indirect effect of the infection on immune activation. Pro-inflammatory cytokines and growth factors such as IFN-α, TNF-α, IL-6, IL-10, CD40L (Appay & Sauce, 2009) and BAFF (Shen & Tomaras, 2011) are thought to contribute towards the aberrant activation of B-cells in HIV-viremic individuals, due to their increased serum levels during HIV-infection (Moir, *et al*, 2011). These soluble mediators are produced by cells of the innate immune system in response to HIV infection. Plasmacytoid dendritic cells (pDC) play an important role in this so-called ‘first line of defense’ against viruses, via production of type 1 interferons (such as IFN-α, which inhibits

viral replication) and induction of IDO (indoleamine 2,3-dyoxigenase), which plays a role in immune tolerance and the suppression of T-cells under normal physiological conditions (Boasso & Shearer, 2008). Other important secretors of inflammatory mediators are macrophages, which are known to secrete proinflammatory cytokines such as TNF-α, IL-1β, and IL12 in response to CD4+ T-cell apoptosis (Ostrowski, 2010)

1.3.4 Chronic Innate Immune System Activation

The innate immune system is an important first line of immunological defense as it is the first cellular compartment to respond to pathogenic stimulation (Haase, 2010). Antigen-presenting cells have high concentrations of pattern-recognition receptors on their cell surface, which recognize several highly-conserved classes of molecular structures associated with groups of micro-organisms, known as pathogen-associated molecular patterns (PAMPs) (Kanzler, *et al*, 2007). When APCs encounter PAMPs such as bacterial LPS (or host-derived damage-associated molecular patterns, known as DAMPs), they induce an inflammatory response that results in recruitment of cells of both the innate and adaptive immune systems to sites of infection. The APCs (primarily DCs), having engulfed the antigen, proceed to migrate to secondary lymphoid organs and present the processed antigen to cells of the adaptive immune system, and initiate an adaptive immune response (Gonzalez, *et al*., 2011).

The chronic stage of HIV infection can be characterized by sustained activation of the innate immune system due to the presence of viral antigen (both HIV and other chronic reactivated viruses e.g. CMV) and ongoing exposure of innate immune cells to gut microbes, or their products, due to the ‘leaky gut’ phenomenon (Appay & Sauce, 2009). The ’leaky gut’ phenomenon refers to the damage inflicted on the GIT following massive localized HIV infection and depletion of CD4+ T-cells from the GALT (during acute HIV-1 infection). As a result of this massive loss of CD4+ T-cells, the integrity of the mucosal epithelium is disrupted and this surface in effect becomes permeabilized, leading to what’s known as HIV enteropathy (Brenchly & Douek, 2008 [1]). The subsequent epithelial permeability creates the scenario of microbial translocation, whereby bacteria, other microorganisms and their products enter the circulation and cause further immune activation at the GALT sites and systemically. The presence of these microorganisms in the setting of increasing HIV-related immunosuppression leads to an additional mechanism for opportunistic infections to occur, and enhance the progression to AIDS (Brenchly & Douek, 2008 [2]).

Chronic immune activation ultimately manifests as the inability of the immune system to mount an appropriate response to antigenic stimulation. pDCs initiate the first anti-viral response within the first few hours of infection in the form of IFN-α, which limits viral

replication in the short-term while an adaptive, antigen-specific immune response is developed (Boasso & Shearer, 2008). Markers of pDC activation (Type 1 IFN and IDO) normally decrease after the acute phase of infection when the adaptive immune response is initiated, but in the case of HIV, the levels of these markers remain elevated, despite the initiation of the adaptive immune response. The inability of the adaptive immune response to clear the infection, as well as the immune down-regulatory effect of IDO (which further inhibits the adaptive immune response) contributes towards the persistence of, and shift towards the chronic stage of HIV infection (Boasso & Shearer, 2008).

An additional contributing factor to chronic immune activation occurs within the germinal centre, via follicular dendritic cells (fDC) antigen presentation. These APCs present antigen to naïve B- and T-lymphocytes in the lymphoid germinal centers during lymphocyte development. However, continuous viral replication results in persistent exposure of fDCs to virions. These virions are then continuously presented to the naïve lymphoid cells, which persistently stimulates them, as well as exposing CD4+ T-cells to infection (Fauci, 1996). Similarly, myeloid DCs (also known as conventional DCs) in the body's periphery also play a role in persistent stimulation by capturing and processing viral antigens in the periphery, and then migrating towards the lymphoid follicles to present Ag to B- and T-cells (Steinman & Banchereau, 2007).

This poses a vicious cycle of the immune activation in chronic HIV infection, whereby APCs unwittingly contribute to immune activation, further viral replication, as well as continuous immune suppression.

1.3.5 Chronic Adaptive Immune System Activation

1.3.5.1 T-cell Response to HIV

Although multiple immune cell types are affected by HIV-1 infection, as mentioned previously, the CD4+ T-cell population is perhaps the most affected immune compartment. The CD4+ molecule present on the 'helper' T-cell population is the primary target of the HIV, and is a membrane-bound receptor that forms part of the T-cell receptor complex. It plays a role in T-cell activation, which occurs when it interacts with MHC class II on APCs (Haase, 2010).

There exists a detrimental interplay between T-cell activation and viral replication, in that HIV infects and replicates primarily in activated T-cells, subsequently resulting in an increased plasma viral load and propensity towards T-cell apoptosis, but also an increased level of T-cell activation. Therefore, CD4+ T-cells inadvertently contribute towards their depletion by responding to (HIV) antigen (Brenchley & Douek, 2007). Direct T-cell infection by HIV is not

a primary driver of T-cell apoptosis, due to the number of directly-infected cells being significantly fewer than the total number of cells undergoing apoptosis (Hazenberg, *et al*. 2003). The incessant T-cell decline, in spite of a steady viral load, can therefore be attributed to more indirect means, such as that induced by immune activation and inflammation which result in a process known as 'bystander cell death' of uninfected CD4+ T cells (Kiazyk & Fowke, 2008).

1.3.5.2 B-cell Hyperactivity

HIV infection, and its subsequent immune activation cause a number of disturbances in the B-cell compartment, namely hypergammaglobulinemia, polyclonal B-cell activation, and impaired reactivity to immunization and *in vitro* activation signals (De Milito, 2004). Other notable abnormalities include increased cell turnover, activation marker expression, and autoantibody expression, as well as an increase in the frequency of HIV-related B-cell malignancies (Moir & Fauci, 2009).

Hypergammaglobulinemia refers to the raised presence of gammaglobulins in circulation, which are predominantly IgM, however other antibody classes are also involved. These antibodies comprise both of HIV- and non-HIV antibodies, the latter of which consists of low-affinity- and auto-antibodies (that is, self-reactive antibodies), whose presence is indicative of dysregulated CD4 T-cell help (De Milito, 2004). In the context of HIV infection, the pro-inflammatory state that exists is conducive to B-cell activation and class switching from the IgM to IgG isotypes (De Milito, *et al.,* 2004).

In the acute phase of HIV infection, complement proteins bound to HIV virions bind to the CD21 (complement) receptor, which induces polyclonal activation and terminal differentiation of B-cells (Shen & Tomaras, 2011). This results in, among other things, the generation of antibody-secreting plasma cells, whose presence, in the form of antibody-virion immune complexes is first detectable at around 19 days post-infection (Levesque, *et al.* 2009). Furthermore the same authors reported that functionally active neutralizing antibodies only appeared in serum around 12 weeks after infection, compared to priming immunizations with non-pathogenic, non-HIV-1 viruses, which elicited a peak neutralizing antibody titer at around 14 days post-infection. HIV thus appears to evade the initial adaptive immune response. The constant presence of HIV-antigens in serum during chronic HIV infection can be said to be a major contributor towards HIV-associated immune activation and de-regulation, including B-cell dysregulation (Appay & Sauce, 2009).

Memory B-cell numbers are significantly decreased in the peripheral blood of individuals with chronic HIV infection, and are prone to die by apoptosis (Titanji*et al.* 2006). However, Moir and Fauci (2008) found this to be a somewhat contradictory finding due to the fact that

memory B-cells are characterized by the expression of CD27, which is also a marker of terminal differentiation and activation. As these two features are significantly over-represented in chronic HIV infection, CD27 alone does not reflect true memory B-cells. The authors suggest that CD21, CD20 and CD19 can be used to distinguish between memory B-cells and aberrantly activated B-cells. As a result of a defective memory B-cell compartment, response to both new and recall antigens is severely defective in individuals with chronic HIV infection (De Milito, 2004). Serum antibodies against both viral and bacterial antigens such as those of measles and *S. pneumonia,* respectively, have been found to be decreased in chronically HIV-infected patients, and did not increase significantly (compared to controls) with antiretroviral therapy (ART) (Titanji, *et al*, 2006). The same authors proposed that the impairment of serologic memory could be due to the immense viral replication that takes place during primary HIV infection, which may be responsible for the depletion of antigen-specific memory B-cells through altered TNFR expression and apoptosis. Such defective immune memory may play an important role in the development of opportunistic infections during chronic HIV infection.

1.3.5.3 B-cell Malignancies Associated with Hyperactivity and Exhaustion

Several malignancies, such as Kaposi sarcoma, non-Hodgkin lymphoma (NHL), and cervical cancer, have been associated with HIV infection, and as such have been classified as AIDS-defining malignancies (ADMs) by the Centers for Disease Control and Prevention (Dauby, *et al.*, 2011). However, with the advent of HAART, there has been both a decrease in ADM's, as well as a significant increase in non-AIDS defining malignancies (NADM) such as Hodgkin's lymphoma, anal, lung and liver cancers (Dauby, *et al.*, 2011). HIV-related NHL represents the largest fraction of HIV-associated malignancies, and it is most often comprised of a single malignant clone, which has frequent involvement of extranodal sites, and is indicative of poor prognosis (Capello, *et al.*, 2009). A proposed means of lymphomagenesis is via B-cell activation, due to the fact that many B-cell stimulatory factors are elevated in serum, such as IL-6, IL-10, BLyS, and CXCL13 (Thapa, *et al*., 2012). The same authors found that miR-21 levels (a B-cell oncogene and marker of poor prognosis) levels were higher in activated B-cells from lymphoid organs (i.e. sites of activation) compared to peripheral blood subsets (Thapa *et al.,* 2012).

'Exhaustion' of immune cells is a concept that arose in T-cell immunology, and refers to a stage of differentiation where there is increased expression of multiple inhibitory receptors, a reduced expression of activation markers, and a decrease in functional capacity of a specific T-cell (Küppers, 2008). Exhausted B-cells are characterized by elevated expression of the inhibitory receptors programmed cell death receptor 1 (PD1) and cytotoxic T-lymphocyte antigen 4 (CTLA4). In addition elevated CD20 expression, low expression of CD21 and

CD27, and expression of Fc-receptor-like 4 (FCRL4) has been observed (Moir & Fauci, 2008). These B-cells, also known as Tissue-like memory B-cells, are similar to exhausted T-cells due to their loss of proliferative, effector, and homing properties (Moir & Fauci, 2008).

1.3.6 The Impact of Antiretroviral Therapy

1.3.6.1 Antiretroviral Therapy in South Africa

Since its inception in 1996 (and rollout in 2004 in South Africa), HAART has resulted in a considerable and prolonged decrease in the progression of HIV to full-blown AIDS disease (Lichtenstein, 2009; Evans, 2013). HAART consists of a regimen of different classes of ARVs, usually consisting of reverse transcriptase-, integrase-, and protease inhibitors, whose primary mechanism of action is to inhibit viral replication, thereby decreasing plasma viraemia to undetectable levels and usually also the associated systemic immune activation (Lichtenstein, 2009).

ART results in a dramatic restoration of CD4 count to near normal (pre-HIV) levels , however there exists a lower-limit threshold of CD4 count, below which the benefits of ART are vastly reduced (Jain & Deeks, 2010). The current CD4 count threshold as set by the WHO, is 350 cells/µl, despite there being a dramatic reduction of non-AIDS conditions (such as cardiovascular, liver, and kidney disease) when ART is initiated at even higher CD4 counts (Jain & Deeks, 2010). There is a reconstitution of both naïve, and to a lesser extent, memory T-cell subsets with the initiation of HAART, which occurs concurrently with a dramatic reduction in immune activation (Cotugno, *et al.*, 2012).

1.3.6.2 Impact of Antiretroviral Therapy on B-cell Function and Distribution

HAART has been found to reduce HIV-related hypergammaglobulinemia, as well as to restore absolute B-cell count and to shift B-cell subset distribution back to pre-HIV status. Moir *et al.* (2008) found that the relative percentages of activated memory, and immature transitional B-cells decreased, while those of the resting memory and mature naïve increased, 12 months after initiation of HAART, which was similar to the subpopulation distribution of HIV-uninfected controls. The same study also found a reduction of apoptosis-prone activated memory cells. Current data however does indicate that the B-cell compartment remains functionally impaired, in spite of HAART (Pensleroso, *et al.*, 2009).

Protective Ab titers have been found to be deficient, even with successful HAART regimens, such that humoral immune responses are severely deficient in such individuals (Cotugno, *et al.*, 2012). In spite of the normalization of B-cell subpopulations, there still exists suboptimal functionality of B-cells in patients on HAART, especially when there is a delay in its initiation (Pensleroso, *et al.*, 2009). The same authors found that when HAART was initiated in acute

HIV infection, both the T- and B-cell populations were normalized in terms of both function and number compared to matched controls.

The most notable and important function of B-cells is Ab secretion, one of the key factors in humoral immunity. When The B-cell compartment is compromised, which begins to occur early in HIV infection before the qualitative and quantitative defects of T-cells are evident, there is evidence of defective humoral defense mechanisms (Moir & Fauci, 2013). In this sense, if HAART administration is delayed, a state of impaired development occurs, resulting in gradual loss of protective Ab titers. With this in mind, especially in vertically-infected children, a novel approach in vaccination strategy is needed to maintain protective Ab responses in these individuals, although prevention of mother to child transmission (PMTCT) programs have effectively eliminated vertical transmission (Pensieroso, *et al.*, 2009; Cotugno, *et al.*, 2012).

Antibodies with the ability to effectively neutralize HIV-1 virions are known to develop at a slow pace in most individuals, taking approximately 12 weeks post infection, or longer to appear, and even, so only about 20% of infected individuals go on to produce broadly neutralizing antibodies 20 to 30 months after initial infection (McMichael*, et al*., 2010). McMichael, *et al.* (2010) have hypothesized that the delay in broadly neutralizing antibody production is due to the profound loss of cell populations crucial to germinal center formation, and there is therefore a significant impairment in the capacity for affinity maturation, and somatic hypermutation.

1.3.7. Novel Markers of B-cell Dysregulation and Potential Therapeutic Targets in HIV-1 Infection

In a few recent studies, a large group of inhibitory receptors was found to be expressed on B-cell peripheral blood populations, especially so on the tissue-like memory subset (exhausted B cells) (Llinàs, *et al*., 2011). Silva *et al.* (2011) found that there was significantly altered expression of receptors containing ITIM cytoplasmic domains, which are postulated to down-regulate BCR-mediated signals. The same authors found that there was a significant increase in expression of ITIM-containing receptors, such as FcRL4 (Fc-Receptor Like 4, CD307d) and CD85j (Leukocyte Immunoglobulin-like Receptor Subfamily B Member 1) with untreated HIV-1 infection (Silva *et al.,* 2011).

Moir, *et al.* (2008) also identified several potential inhibitory receptors that were expressed on tissue-like memory B-cells in HIV-1 infected individuals, which in addition to CD85j and FcRL4, included CD72, and CD305, which are also ITIM-containing inhibitory receptors. The exact function of many of these markers is unknown, however they are thought to contribute towards the exhausted phenotype that exists with chronic HIV-1 infection. These markers represent putative therapeutic targets in which it may be possible to down-regulate the inhibitory phenotype of the effector cells in chronic HIV-1 infection analogous to PD-1 blockade in T-cells.

Therapeutic targets are varied in HIV-1 infection, and for the sake of this thesis we will focus on those affecting the B-cell compartment. Vaccination has been established for more than 200 years, and although most successful vaccines were made without much immunological insight, vaccine strategies against HIV-1 have been largely unsuccessful to date (Pulendran & Ahmed, 2011). Early efforts in HIV vaccine development were crude, and although antibody responses were developed, most strains of HIV were not effectively neutralized (Mascola & Montefiori, 2010). The AIDSVAX gp120 vaccine stimulated the production of non-neutralising Ab towards HIV Env proteins; while the STEP vaccine was able to stimulate a $CD8^+$response, it elicited non-protective effect against the virus; however a recent trial in Thailand which utilized a canary pox viral vector expressing gp120, *Gag*, and *Pol*, followed by AIDSVAX resulted in 30% fewer vaccine-recipients becoming infected with HIV-1 (McMichales, *et al*., 2011). Broadly-neutralising antibodies (bNAbs) against HIV have been the long-sought after ‘holy grail’ of HIV vaccine research. These bNAbs have been shown to neutralise greater than 90% of HIV-1 isolates regardless of their clade (McGuire, *et al.*, 2014). The main target of these bNAbs is the CD4-binding site (CD4-BS) on HIV gp 120 Env glycoprotein, which is normally protected from humoral identification by conformational masking and glycan (Zhou, *et al*., 2010). The most successful of these bNAbs is VRC01,

which achieves strong neutralisation of Env in a manner different from that of CD4, where there is no conformational changing of Env upon binding (which would hinder further neutralisation) (Li, *et al*., 2011). The main failing point in bNAb generation upon vaccination is due to the fact that the vast majority of Env immunogens do not predictably activate B-cells expressing germline BCRs, a possible reason why only approximately 30% of HIV-1 infected individuals generate BNAbs (McGuire, *et al*., 2014). It is clear from this that understanding B-cell function, and dysfunction in HIV is crucial to furthering vaccine research.

ART has been shown to decrease systemic hyperactivation concomitantly with suppressed viral load, although despite this viral suppression immunologic activation remains higher than in uninfected individuals (Cadogan & Dalgleish, 2008). Therefore, intervention strategies that limit immune activation, to be used in conjunction with ART are of major importance.

In the current study a novel potential immunotherapeutic approach of dampening B-cell activation and/or inhibiting B-cell apoptosis via the action of VIP, a neuropeptide, was also investigated (discussed below).

1.3.7 Neural-immune Communication and Immunomodulation

The immune system is organized in a manner, such that different aspects of the immune response are controlled from the various organs of the immune system, namely, cellular immunity via the thymus and lymph nodes; humoral immunity via the bone marrow and spleen; and cells of the innate immune system at environmental contact sites such as the skin and mucosa (Thayer & Sternberg, 2010). These anatomical sites are further divided into specialized zones and regions where the growth and differentiation of immune cells are regulated.

These anatomical sites, however, do not operate in absolute isolation due to the presence of neural pathways that innervate the organs of the immune system (Pozo & Delgado, 2004; Fig. 1.5). More specifically, both the autonomic and central nervous (via the hypothalamic pituitary-adrenal system) systems play a role in regulation of the immune system, and in turn through secretion of soluble mediators like cytokines, feedback loops between the two are established (Fig. 1.5, Pozo & Delgado, 2004).

Vasoactive intestinal peptide (VIP) and pituitary adenylatecyclase-activating polypeptide (PACAP) are neuropeptides belonging to the glucagon/secretin family, the former of which was initially discovered in the mid-1980s, where it was shown to up-regulate adenylatecyclase in a number of tissues (Wiik, *et al*., 1985). VIP is released both by lymphoid innervation (i.e. neural cells in the lymphoid tissue; Fig. 1.5) and directly by T-

helper type 2 (Th2) cells and regulatory T-cells (Tregs), and thereby promoting Th2 or anti-inflammatory type response (Pozo & Degado, 2010). The Th2 response results in the release of Il-4, IL-5, and IL-10, which are important for IgG1, and IgE synthesis, and inhibition of macrophage activation (which leads to a down-regulated cellular immune response) respectively (Pozo & Delgado, 2010). VIP is a linear 28 residue neuropeptide that acts on two receptors, VPAC1, and VPAC2, both of which belong to the B1 family of G-protein coupled receptors (GPCR) (Tan, *et al*., 2014).

VIP has been shown to down-regulate immune responses and skew responsiveness toward a Th2 predominance. It has also been proven to have a cyto-protective effect whereby VIP scavenges oxygen radicals (Pozo, *et al.*, 2000). VIP therefore has the potential to modulate the chronic inflammation and immune activation that is associated with HIV infection (Pozo, *et al*., 2000). Furthermore, Branch (2011) has shown that the VPAC2 agonist, Helodermin (in a dose of 10^{-9}M) was able to inhibit HIV infection via prevention of HIV viral cDNA integration into the host DNA. Similarly, Bokaei, *et* al. (2007) found that a strong resistance to HIV-1 infection was conferred among Jurkat cells when incubated concurrently with VPAC2-specific agonists. These authors also found that VPAC2 specific agonists were able to directly inhibit the ability of HIV cDNA integration into host DNA.

Ligation of VPAC2 by VIP has been shown to be a potent inhibitor of activation-induced apoptosis in CD4+ T-cells (Delgado & Ganaea, 2006). VIP reduces Fas-Fas ligand expression on activated $CD4^+$ T-cells via VPAC2 signaling (Filippatos, *et al.,* 2001). In doing so, the authors propose that VPAC2 inhibition of T-cell apoptosis may result in the preservation of a pool of memory T-cells with the ability to fight the infection (Filippatos, *et al.,* 2001).

Expression of the VIP receptors, VPAC1 and VPAC2 has been extensively studied in $CD4^+$ T-cells using molecular techniques for mRNA expression (Pozo, *et al*., 2000; Ganea & Delgado, 2002; Pozo & Delgado, 2004). T-cells express both VPAC1, and VPAC2, however it appears that VPAC1, although constitutively activated, is down-regulated upon stimulation, while VPAC2 is expressed at low levels at baseline, and up-regulated with stimulation (Ganea & Delgado, 2002). VIP signaling through the VPAC2 receptor has been found to have anti-inflammatory downstream effects, and has been implicated in the management of both autoimmune, and chronic inflammatory disorders (such as HIV-1 infection) (Pozo & Delgado, 2004). Furthermore, VPAC2 expression in untreated HIV infection was found both to be upregulated, and positively correlated with Fas-L expression (Ipp, *et al.,* 2014) This indicates that therapy targeting VPAC2 would only affect activated cells, and therefore be a selective means of inducing an anti-inflammatory state on activated $CD4^+$ T-cells, and further

diminishing their propensity towards Fas-mediated apoptosis. Mature B- and T-cells traffic to, and reside in the lymph nodes, where sympathetic innervation is present, effectively linking the nervous system with the immune system (Huang, *et al.,* 2013). Therefore, if $CD4^+$ T-cells have receptors for neurotransmitters such as VIP, it is conceivable that other lymphocyte populations, such as $CD8^+$ T-cells, or B-cells would have similar receptor expression patterns. To our knowledge, the current study is the first to investigate both VPAC2 expression on B-cells, and the effect of VIP on down-regulating aberrant marker expression on peripheral blood B-cell subsets.

The ongoing problems on immune activation associated with both treated, and untreated HIV infection has resulted in a large gap in current treatment protocols. The impact of B-cell populations with HIV infection has been known for some time, although as mentioned earlier, treatment with HAART does not result in complete restoration in their function. Therefore, therapeutic approaches (adjuvant to current protocols) that could limit the effects of immune activation on both T-cell and B-cell function would be greatly beneficial.

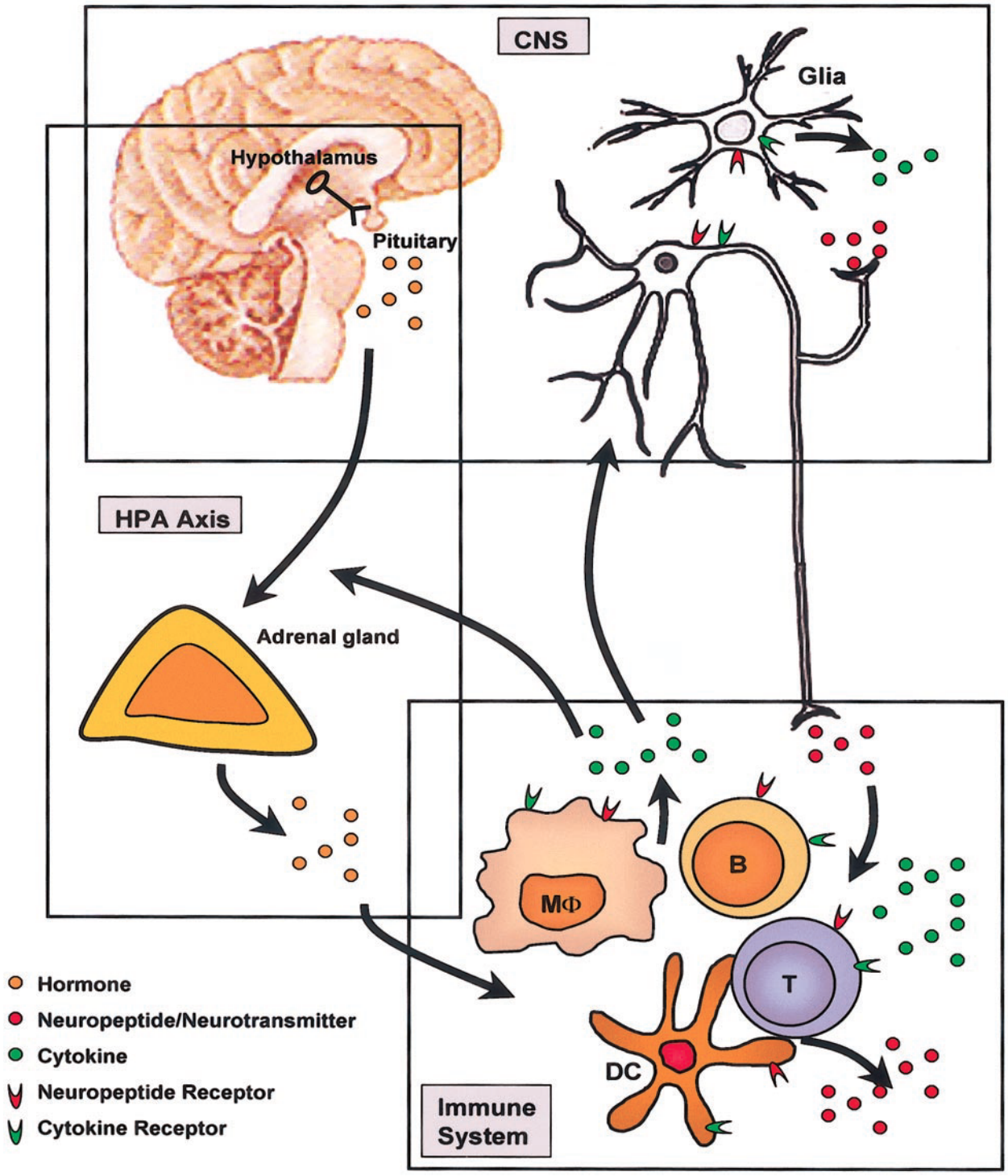


Fig. 1.5. The bidirectional interactions between the immune, and neuroendocrine systems. *Soluble mediators are released and recognized by both systems, and immune cells and neuroendocrine cells share common receptors and even have overlapping functions (Pozo & Delgado, 2004)*

1.4 Aims and Objectives

1.4.1 Aims

The primary Aim of this study was to investigate B cell phenotype, subset distribution, and activation / exhaustion status in South African untreated chronically HIV-infected individuals and uninfected controls, and to relate these findings to clinical markers of disease progression. Secondary Aims were: to investigate the expression of the VIP receptor VPAC-2 on the B-cell population and to evaluate B cell responsiveness *in vitro* following TLR stimulation and, and assess whether VIP can modulate activation-associated marker expression.

1.4.2 Objectives

The objectives of the study were to:

- Assess the impact of chronic, untreated HIV on B cell subset distribution (transitional, naïve, effector, memory) and status (resting, activated, exhausted) and relationship with CD4 count, viral load and immune activation (as measured by classic readout such as CD38 expression on CD8 T cells)
- Evaluate expression of traditional markers of B cell activation and inhibition/exhaustion (CD70, CD72, CD86, CD38) as well as a selection of less well-described markers, (CD305, CD300a, CD85j & FCRL4) together with standard markers for B cell identification (CD10, CD19, CD20, CD21, CD27)
- Assess *in vitro* B cell function following stimulation with TLR ligands (LPS and R848) by determining changes in expression of markers of activation (CD70, CD86, CD38), and inhibition/exhaustion (CD72, CD85j, CD300a, CD305, FCRL4)
- Determine expression of VIP receptor VPAC-2 on B cells (*ex vivo* and following *in vitro* stimulation)
- Assess ability of VIP to limit B cell activation or apoptosis signaling as measured by CD95 expression

Chapter 2: Methods

2.1 Patient Cohort

Twenty-one patients and 18 controls were recruited from the Emavundleni primary health care HIV prevention and testing (HPT) clinic in Crossroads, Cape Town (affiliated with the Desmond Tutu HIV Centre). The criteria for recruitment of HIV+^{ve} individuals were: age - 21 years or older; a CD4 count of > 200 cells/µl; ART naïve; and asymptomatic for TB. HIV-^{ve} individuals were also 21 years or older, and sourced from the same demographic region, thus representative of the racial and socio-economic status of the study subjects. Subjects were excluded if they displayed any clinical symptoms of any other infectious-, or non-communicable diseases. The study was approved by the Human Research Ethics Committee (HREC) of Stellenbosch University (HREC N07/09/197). Informed consent was obtained from each participant prior to sampling. Blood was drawn from the patients and controls into two 10ml Heparin, and one 5ml EDTA, BD Vacutainer® tubes with BD Hemogard® lids (Becton Dickenson (BD) Biosciences, San Jose, CA, USA). Samples were then couriered to the Division of Medical Virology, Faculty of Medicine and Health Sciences, Stellenbosch University within two hours of collection. As this study constituted a sub-study of the larger HIV Activation and Inflammation Group (HAIG), there was considerable collaboration and cooperation between researchers utilizing the collected blood specimens.

2.2 CD4 T-cell and CD19 B-cell BD TruCount™ Assays

CD4 T-cell and CD19 B-cell counts were performed using the EDTA blood sample via standard stain-lyse-no wash BD Multitest™ and TriTest™ protocols. Multitest™ CD3-FITC/CD8-PE/CD45-PerCP/CD4-APC reagent was used for the CD4 T-cell counts, and TriTest™CD3-FITC/CD19-PE/CD45-PerCP reagent was used for the CD19 count (both Becton Dickenson (BD) Biosciences). BD Trucount tubes (BD Biosciences) were used in both assays. Trucount tubes were labeled according to HIV group and number (Control HIV-^{ve}:“N100” or HIV+^{ve}:“P100”, etc.). Twenty µl of Multitest antibody cocktail (for CD4 Trucount) or Tritest (for CD19 Trucount) was then pipetted into the bottom of the tube above the steel retainer and bead pellet. Fifty µl EDTA blood was then added to the tube which was subsequently vortexed, and then incubated for 15 minutes in the dark at room temperature. After incubation, 450µL of 1:10 diluted FACSlyse™ solution (BD Biosciences) was added and the tubes vortexed again, and allowed to incubate for 15 minutes in the dark at room temperature. The samples were then analyzed on the flow cytometer (BD FACSCalibur with MultiSET™ software). This CD4 count method is the manufacturer-recommended SOP used in the Division of Medical Virology, Faculty of Health Sciences, Stellenbosch University, and is South African national accreditation system (SANAS)-accredited.

2.3 HIV Viral Load

Two ml of EDTA blood was centrifuged at 20°C at 300g (Jouan CR422, Thermo Fisher Scientific Inc., Waltham, MA, USA) for 12 minutes at room temperature with the brake off. Approximately 1ml of plasma was then decanted into a 1.7ml Axygen microcentrifuge tube (Axygen, Inc., Union City, CA, USA) and the sample was sent for viral load testing using the NucliSensEasyQ® HIV-1 v1.2 Viral Load Test (BioMerieux Inc., Boxtel, Netherlands). This is an HIV-1 RNA quantitative assay that monitors viral load by applying NASBA technology combined with detection of real-time molecular beacons, and has a detection range of 350 to 10^6 copies/ml. This is the routine method for viral load testing in the Division of Medical Virology, Faculty of Health Sciences, Stellenbosch University, and is SANAS-accredited.

2.4 Four Colour Flow Cytometry for CD38 Expression on $CD8^+$ T-cells

A 50µl aliquot of EDTA whole blood underwent a stain-lyse-wash protocol for determination of CD38 expression on $CD8^+$ T-cells via flow cytometry (BD FACSCalibur). Pre-optimised volumes of CD3-FITC, CD8-PerCP, CD38-APC (BD Biosciences), and PD-1-PE (Biolegend) were added to a 5ml falcon tube (BD Biosciences) containing 50µl EDTA whole blood and allowed to incubate for 15 min at room temperature in the dark. Following this, 500µl 10x FACSLyse™ solution (BD Biosciences) was added to the tube and allowed to incubate for a further 15 minutes in the same conditions as before. Thereafter, 500µl of phosphate-buffered saline (PBS) containg 2% heat inactivated foetal calf serum (FCS, Gibco®, Life Technologies, Carlsbad CA, USA) was added to the tube, which was subsequently centrifuged at 300g for 5 minutes at room temperature (Jouan CR422). The supernatant was discarded, and the pellet resuspended in 500µl PBS containing 1% paraformaldehyde fixative. The tubes were kept at 4°C in the dark until acquired within 24 hours on a BD FACSCalibur flow cytometer and analysed using CellQuest Pro software.

A dotplot of forward scatter (FSC) vs. side scatter (SSC) was drawn, and a gate was set around the lymphocyte region (R2, Fig. 2.1 A). A plot of CD3-FITC vs. SSC was used to gate on 10 000 T-cells (R3, Fig. 2.1 B). Secondary plots of CD38-APC vs. CD8-PerCP, and PD-1-PE vs. CD8-PerCP were drawn using the CD3 gate (R3) to assess the $CD3^+CD8^+CD38^+$ (Fig. 2.1 C), and $CD3^+CD8^+PD\text{-}1^+$ (Fig. 2.1 D) populations.

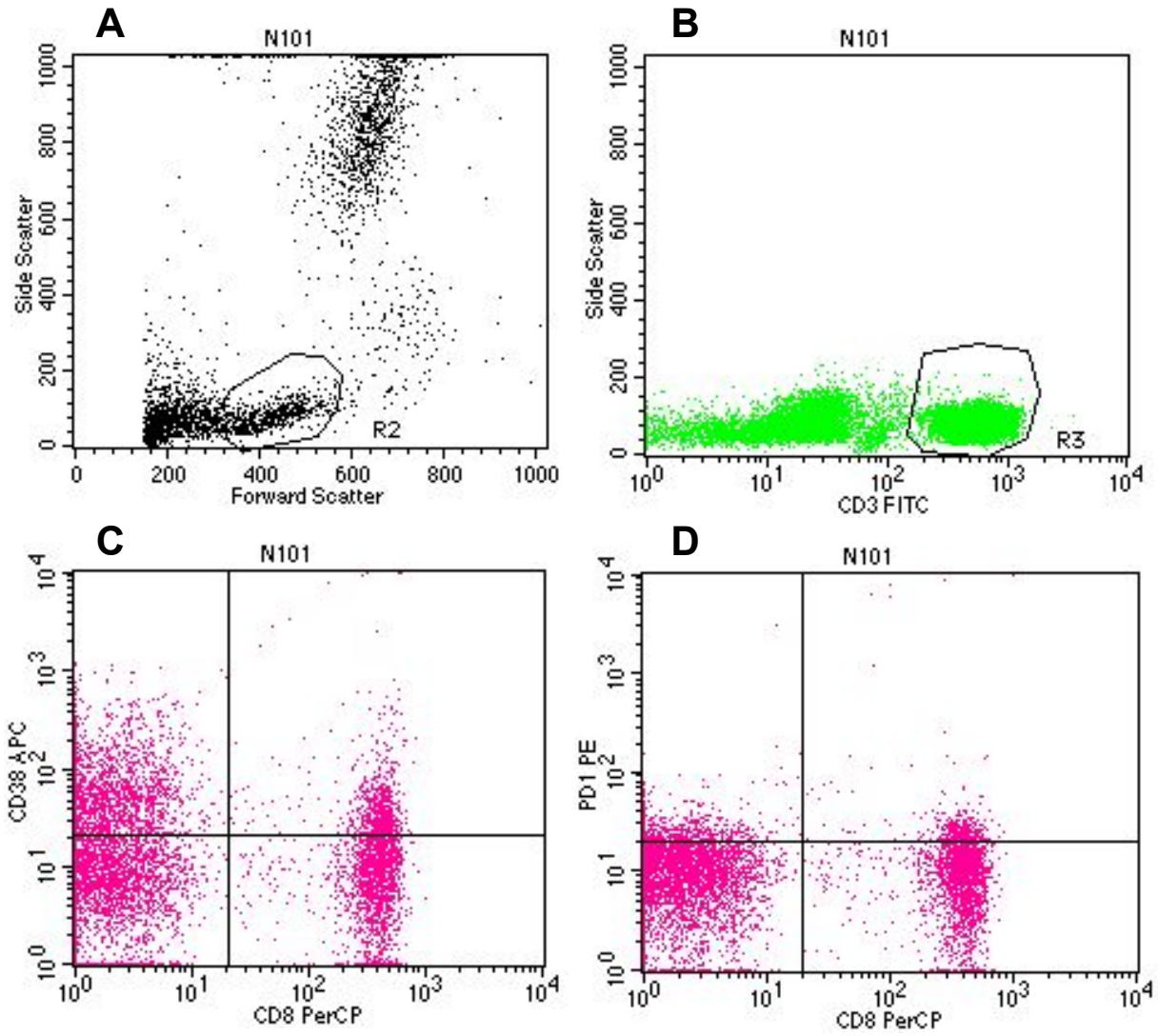


Fig. 2.1 Gating strategy for CD38 expression on CD8 T-cells. *A) A gate was drawn around the lymphocyte region on a dotplot of FSC vs. SSC (R2). B) These cells were then interrogated on a dotplot of CD3 vs. SSC, where a gate was drawn around the CD3+ population (R3), of which 10000 events were acquired. C) A plot of CD38 vs. CD8 was drawn, and displayed only the CD3+ events. D) Similarly a plot of PD-1 vs. CD8 was drawn which displayed only the CD3+ events.*

2.5 B-cell Enrichment for functional studies

B-cells were isolated for culture and stimulation using RosetteSep™ Human B-cell enrichment cocktail and Ficoll-Paque™ density gradient centrifugation (Stemcell technologies, Vancouver, British Columbia, Canada). 8ml of Heparinised whole blood was added to a 50ml centrifuge tube (Falcon, BD Biosciences) containing 400µl RosetteSep™ Human B-cell enrichment cocktail (Stemcell technologies) and allowed to incubate for 20 min at room temperature in the dark. After incubation, the whole blood solution was diluted with an equal quantity of PBS containing 2% heat inactivated FCS (Gibco®), and the resulting mixture was layered on top of 15ml Ficoll-Paque™ (Stemcell technologies) in a 50ml centrifuge tube (Falcon, BD Bioscience). The tube was then centrifuged at 1200g for 20 minutes at room temperature with the brake off (Jouan CR422), resulting in the enrichment of the B-cell population at the plasma-Ficoll interface. These cells were carefully aspirated off the plasma-Ficoll-Paque™ interface and placed in a 50ml centrifuge tube (BD Bioscience), to which an equal volume of RPMI 1640 (Gibco®, Life Technologies) containing 5% FCS (Gibco®, Life Technologies), was added. This tube was then centrifuged at 300g for 5 minutes at room temperature with the brake off. Following centrifugation, the supernatant was discarded, and the cells were re-suspended in 1ml RPMI-1640 (Gibco®, Life Technologies) containing 5% FCS (Gibco®, Life Technologies).

Cell count was performed by use of a Neubauer Haemocytometer (Hausser Scientific, Horsham, PA, USA). A 50µl sliquot of cell suspension was added to 50µl of Turk's solution (Sigma-Aldrich, St. Louis, MO, USA) and allowed to stand for 1 minute. A glass slide cover was placed on the haemocytometer and approximatedly 10µl of stained cell suspension was pipetted under the slide cover. Cells were counted, after which the volume of the 1ml cell suspension was adjusted to a final concentration of $5x10^5$ cells/ml. Purity was measured via flow cytometry (panel 6, see table 2.1), to determine whether any significant amounts of contaminating T-cells ($CD45^+CD3^+$) were remaining after enrichment.

Table 2.1: List of Antibody Panels used in this study.

*The optimal volume of each antibody was calculated by means of titration experiments, and calculated staining index. Backbone antibodies are CD19-AmCyan, CD21-APC, CD27-PerCp-Cy5.5, and CD10-APC-Cy7. Intracellular fluorochromes are indicated by *. Panels 1 - 6 were run on a BD FACSCanto II, and panel 7 was run on a BD FACSCalibur.*

Panel #	FITC	PE	APC	PerCP-Cy5.5	PE-Cy7	APC-Cy7	Pacific Blue	AmCyan
1: Activation	CD70	CD126	CD21	CD27	CD38	CD10	CD25	CD19
2: Activation/Inhibition	CD72	CD305	CD21	CD27	CD38	CD10	CD86	CD19
3: Inhibition/Apoptosis	CD85j	CD300a	CD21	CD27	CD307d	CD10	CD95	CD19
4: TLR/Exhaustion	CD287*	CD284	CD21	CD27	CD307d	CD10	CD86	CD19
5: VPAC		VPAC 1	CD21	VPAC2				CD19
6:Purity	CD3	CD19		CD45				
7: CD38/8	CD3	PD-1	CD38	CD8 (PerCP)				

2.6 B-cell Culture

Ninety-six-well plates were set up as per Fig. 2.2: 1 well per stimulant (including unstimulated), per flow cytometry staining panel (1 to 5, Table 2.1), per patient, and a single well per patient of panel 6 (Purity). The cells were cultured in an incubator for 18 hours in the following conditions: 37°C, 95% humidity, 5% CO_2.(Nuaire). Isolated cells were assessed for purity directly after enrichment, via flow cytometry. Panel 6 (Purity pane, Table 2.1l) contained CD3-FITC, CD19-PE and CD45-PerCP, which allows for detection of presence of contaminating T-cells based on both CD3 and CD45 expression. The negative selection/enrichment procedure employed resulted in an average of 80% purity, consistent with the product literature (StemCell Technologies). Cells were also assessed for viability before and after culture for 18 hours using Trypan Blue solution (Sigma-Aldrich) with the TLR ligands/agonists and/or VIP. There was also an unstimulated control to which the stimulant conditions were compared. Less than 1% of cells stained positive for Trypan blue before and after incubation for 18 hours.

2.6.1 B-cell Stimulation with LPS and R848

A total of 5 x10^4 enriched B-cells (i.e. 100µl of adjusted cell preparation) were added to a well of a round bottomed tissue culture 96-well plate (Falcon, BD Bioscience). Into each well,

Gibco® RPMI-1640 (Life Technologies) containing 5% FCS (Gibco®, Life Technologies) and either 0.1µg/ml LPS (TLR4 ligand) or 0.1µg/ml R848 (an imidazoquinoline compound, which stimulates TLR7/8) (both Invivogen, San Diego, CA, USA), was added (final volume of each well was adjusted to 200µl). RPMI-1640 (Gibco® Life Technologies) containing 5% FCS (Gibco®, Life Technologies) in the same volume as the TLR stimulant was used in the unstimulated wells. The optimal concentration of both LPS and R848 was based on the product literature, and further confirmed via a dose- and time-optimising assay performed on isolated B-cells of healthy donors incubated at 37°C, 95% humidity, 5% CO_2 for a specified time period (Nuaire, Plymouth, MN, USA). Time-dose combinations included 18h, 24h, 48h, and 0.01µg/ml, 0.1µg/ml, and 1.0µg/ml). Responsiveness to stimulation was assessed via flow cytometric evaluation of expression of the activation markers in panel 1 (Table 2.1) relative to the unstimulated state. Unstimulated matched controls were run at each time point.

2.6.2 B-cell inhibition with VIP

Twelve samples each of the HIV+^{ve} and HIV-^{ve} groups underwent inhibition with VIP, however 4 HIV+^{ve} samples were excluded due to low CD4 counts (<200 cells/µL) and undetectable viral loads, leaving the total number of VIP-inhibited samples at 8 HIV+^{ve} and 12 HIV-^{ve}. Cells were co-incubated (hereafter known as 'inhibited') with 10^{-8}M VIP and 0.1µg/ml of either R848 or LPS per $5x10^4$ cells (See Fig. 2.2). The optimal concentration of VIP was based on the findings of Bokaei, *et al*. (2007), and also confirmed by other members of our laboratory on $CD4^+$ lymphocytes and monocytes. Time- and dose- (18h, 24h, 48h, and 10^{-7}M, 10^{-8}M, 10^{-9}M) optimizing assays were also performed on isolated B-cells of healthy donors to determine the optimal concentration of VIP and time of culture for B-cells. Culture response to VIP inhibition was measured via flow cytometry for VPAC-2 expression relative to baseline (i.e. uninhibited) controls, run at each time point. This data was used concurrently with viability determination via Trypan Blue (Sigma-Aldrich) staining at each time point, and live/dead discrimination under light microscopy (Olympus) on a Neubauer Haemocytometer (Hausser Scientific). The viability of the inhibited cells at each time point was compared to that of the control cells, where viability was consistently ≥ 90%. Viability of the inhibited cells did not differ from that of the controls by more than 5%.

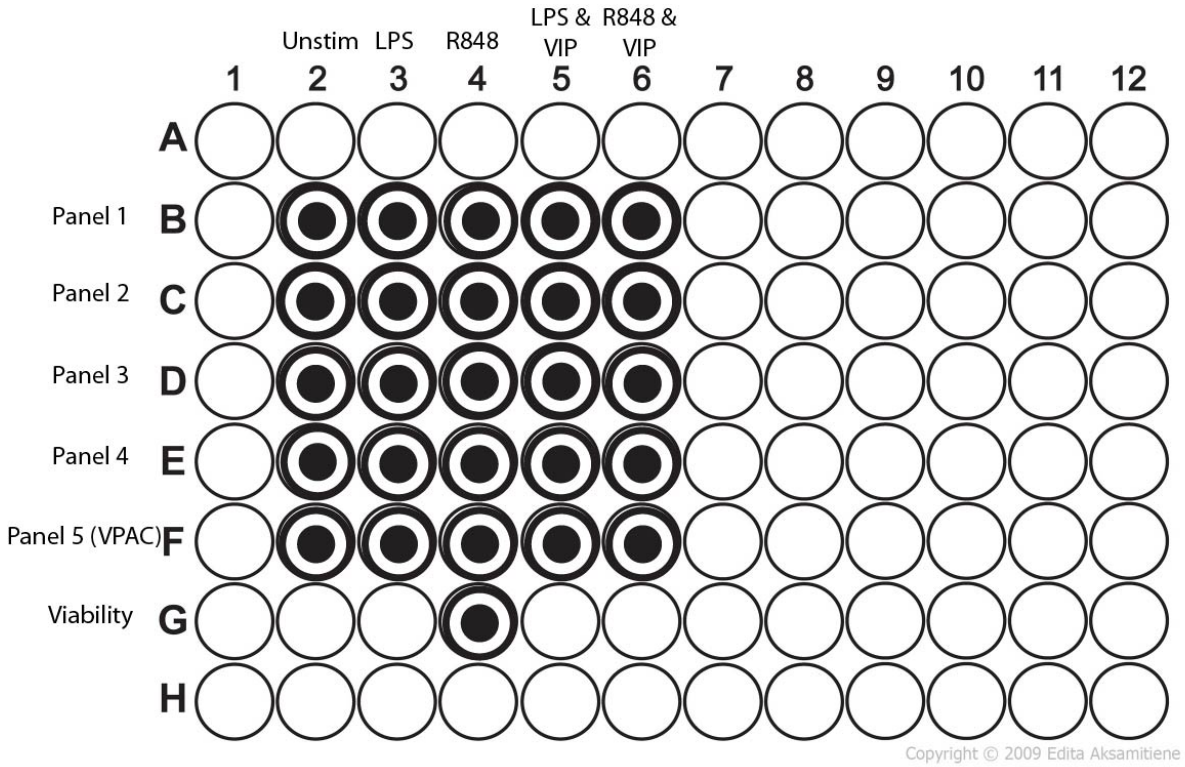


Fig.2.2. Schematic of 96-well plate showing the layout of the unstimulated, stimulated, and VIP-inhibited cells respectively*. Each well represents a separate culture condition and panel. The 'viability' well (G4) was unstimulated and underwent Trypan Blue staining to determine the number of cells that may have died during the 18h of culture.*

2.7 Flow Cytometry Assay Optimisation

2.7.1 Antibody titrations

Antibody titrations were performed in order to determine the most efficient quantity of each antibody for optimal staining, with minimal wastage of unbound antibody (Lamoreaux, Roederer, & Koup, 2006). Staining index (see below) was determined for each antibody in isolation and was performed whenever a different batch of antibody was used for the first time in a panel. The recommended amount of antibody was added to a 5ml Falcon® tube (BD Biosciences), followed by 50µl of whole blood, and allowed to incubate at room temperature for 15 minutes in the dark. Following this, 500µl of 10x diluted FACSLyse (BD Biosciences) was added to each tube, which was then allowed to incubate for a further 15 minutes in the dark at room temperature. Following this, 500µl of PBS containing 2% FCS was added to each tube, which was then centrifuged at 300g for 5 minutes at room temperature (Jouan CR422). The supernatant was then discarded, and the pellet was resuspended in 350µl of PBS containing 2% FCS. The same procedure was performed using doubling dilution titrations of the recommended antibody volume (i.e. 1/2 recommended volume, 1/4 recommended volume, 1/8 recommended volume).

Staining index is a metric that is calculated by the separation of a positively stained and negatively stained population, and is determined by taking the mean fluorescence intensity (MFI) of the negative population (MFI_{Neg}, Fig. 2.3a), and dividing it by the difference in MFIs between the two populations (D, Fig. 2.3a). Alternatively, one can also calculate the staining index by dividing MFI_{Pos} by MFI_{Neg}, which will give similar results. The optimal concentration/volume of monoclonal antibody was determined by the saturation point (Fig. 2.3b), which is the point where there was the most efficient separation of the positive and negative populations.

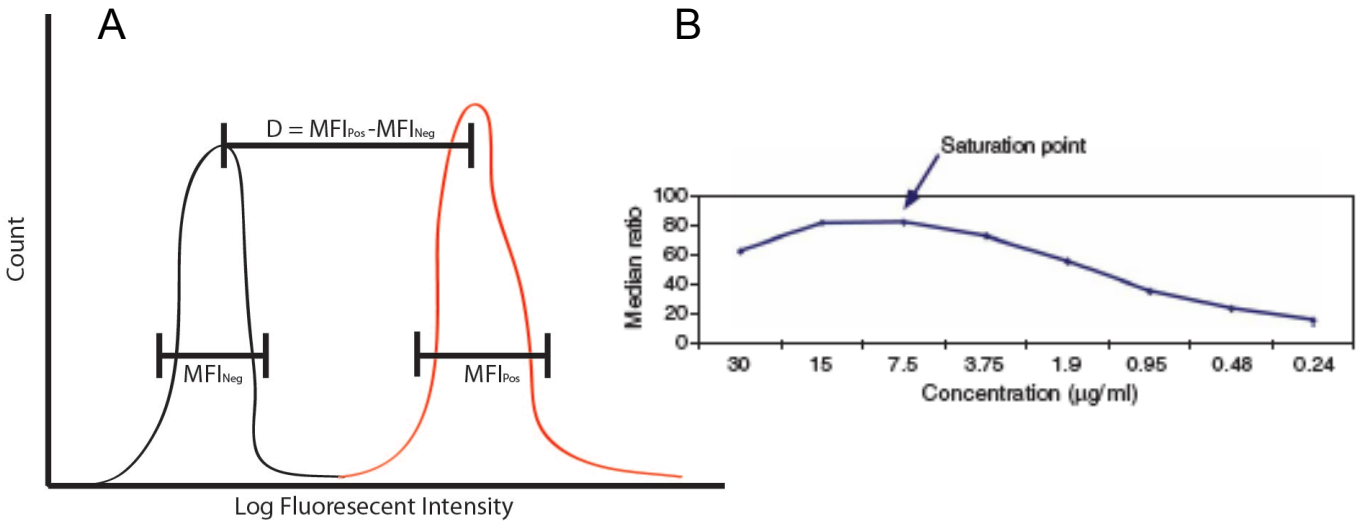


Fig. 2.3. Schematic overview of Staining Index *.A) D refers to the difference in mean fluorescent intensity of the negative population (MFI_{Neg}in black), and the positive population (MFI_{Pos} in red). Staining index may either be calculated as MFI_{Neg}/D, or MFI_{Pos}/MFI_{Neg}. B) Graph of staining indices for titrated concentrations of monoclonal antibody. The saturation point is indicated on the graph, which is the point at which there is optimal separation of the positive and negative populations. There is a rise in the curve due to the increase in signal-to-background ratio, which is a decrease in "D", and a bright negative population. Adapted from Lamoreaux, Roederer, &Koup, (2006).*

2.7.2 Compensation for fluorochrome spillover between detectors

Spectral overlap is a phenomenon that occurs in flow cytometry, whereby the fluorescence emitted by a fluorochrome (e,g. PE) is picked-up by a detector other than the appropriate one (e.g. the FITC detector). This is due the spectral emission of certain fluorochromes overlapping. Compensation was therefore performed on a monthly basis to adjust for spectral overlap between fluorochromes, and performed according to the manufacturer's specifications (BD Biosciences, 2013). The titrated quantity of monoclonal antibody was added to a 5ml Falcon™ tube, followed by 1 drop of the BD Positive CompBeads. The antibody CompBead mix was then gently vortexed, and allowed to incubate for 15 minutes at room temperature in the dark. 1ml of PBS containing 5% FCS (Gibco, Life Technologies) was then added to the sample, which was then centrifuged at 200g for 5 minutes. The supernatant was discarded, and then 1 drop of the BD Negative CompBeads was added to the tube, followed by 1ml of PBS containing 5% FCS (Gibco, Life Technologies), and gently vortexed. Compensation was performed using a semi-automated procedure on the BD FACSCanto II (BD Biosciences), according to manufacturer's specifications. Compensation settings were copied across for each experiment for the month, and only re-run if the QC checks (section 2.7.4) failed despite having followed troubleshooting steps.

2.7.3 Determination of true population negativity via FMO

Fluorescence minus one (FMO) is a means of determining the cutoff for positivity in flow cytometry. The principle of the test is to have a tube set up as if you were running an experiment, except that the tube contains all except for one fluorochrome (Macker & Trotter, 2006). This is especially useful for markers whose expression is unknown. It is also of value when comparing separate conditions in stimulation assays. FMOs are also useful to ensure minimal spillover and to eliminate autofluorescence. FMOs, and compensation controls were run each time the flow cytometer was serviced or if a new batch of antibody was used (section 2.7.2.).

2.7.4 Daily and weekly QC of the FACSCanto II

Experimental voltages were set according to compensation controls; however, these voltages were adjusted according to the daily and monthly voltage baseline QC (quality control) checks. These checks were performed in order to optimize the colour compensation settings, and were specifically designed for use with tandem dye conjugates. Daily and monthly checks were performed through the Cytometer Setup & Tracking (CS&T) application (part of the FACSCanto II software, FACSDiva), using BD CS&T beads (BD Biosciences). The CS&T application also monitors instrument performance over time, via Levey-Jennings plots, and alerts the user when any of the detectors are performing outside their desired

range. “CS&T Performance” was run daily, while “CS&T Baseline” was run on a monthly basis. These QC checks allowed the FACSCanto II settings to be standardized to a certain level/norm, across different days, as well as across different FACSCanto IIs. It is important to note that if the CS&T application passed, then the compensation settings were likely to have been unaffected. It is only when the baseline CS&T application was run, that compensation controls were re-run, or if the daily CS&T performance failed repeatedly, despite having followed troubleshooting steps. Daily experiments were, however run on the cytometer settings from the compensation protocol, while the CS&T application was merely used to determine cytometer reliability.

***Table 2.2: List of Antibody manufacturers and clones**. All antibodies were mouse anti-human.*

	Function	Vol	Fluorochrome	Clone	Isotype	Manufacturer
Tritest	CD3/CD19/CD45 for enumerating B-cells	20µl	FITC	SK7/SJ25 C1/2D1	IgG1, κ	BD Biosciences
CD3	T-cell receptor	10µl	FITC	SK7	IgG1, κ	BD Biosceinces
CD8	T-cell subset marker	10µl	PerCP	SK1	IgG1, κ	BD Biosciences
CD10	Neprilysin, expressed on B-cell precurors	5µl	APC-Cy7	HI10a	IgG1, κ	Biolegend
CD19	Pan B-cell marker	5µl	AmCyan	SJ25C1	IgG1, κ	BD Biosciences
CD21	Complement receptor 2	5µl	APC	B-Ly4	IgG1, κ	BD Biosciences
CD25	IL-2 receptor α-chain	5µl	Pacific Blue	BC96	IgG1, κ	Biolegend
CD27	TNF superfamily receptor, marker of B-cell memory	2.5µl	PerCP-Cy5.5	O323	IgG1, κ	Biolegend
CD38	Cyclic ADP ribose hydrolase	5µl	PE-Cy7|APC	LS198-4-3	IgG1, κ	Beckman Coulter |BD
CD70	Activation marker, ligand for CD27	5µl	FITC	Ki-24	IgG3, κ	BD Biosciences
CD72	Inhibitory receptor, ligand for CD5	5µl	FITC	J4-117	IgG2b, κ	BD Biosciences
CD85j	Inhibitory receptor which down-regulates activation of APCs	5µl	FITC	GH1/75	IgG2b, κ	BD Biosciences
CD86	B7.2, upregulated upon activation, ligand for CD28	2.5µl	Pacific Blue	IT2.2	IgG2b, κ	Biolegend
CD95	FAS receptor, indicative of apoptosis signaling	2.5µl	Pacific Blue	DX2	IgG1, κ	Biolegend
CD126	IL-6 receptor	5µl	PE	M5	IgG1, κ	BD Biosciences
CD279	Programmed cell death 1 (PD-1)	5µl	PE	EH12.2H7	IgG1, κ	Biolegend
CD284	Toll-Like Receptor 4, recognizes LPS	5µl	PE	HTA125	IgG2a, κ	Biolegend
CD287	Toll-Like Receptor 7, intracellular receptor, recognizes ssRNA	2.5µl	FITC	533707	IgG2a, κ	R&D Systems
CD300a	Inhibitory receptor, implicated as a receptor for phosphatidylserine	5µl	PE	E59.126	IgG1, κ	Beckman Coulter
CD305	LAIR-1, an inhibitory Ig-like receptor, implicated as a collagen receptor	5µl	PE	DX26	IgG1, κ	BD Biosciences
CD307d	FcRL4, an inhibitory Fc-like receptor found on exhausted B-cells	5µl	FITC	413D12	IgG2b, κ	Biolegend
VPAC1	Receptor for VIP, constitutively expressed	10µl	FITC	SP234	IgG1, κ	Santa Cruz
VPAC2	Receptor for VIP, upreg upon activation	10µl	PerCP	SP235	IgG1, κ	Conjug. in house

2.8 Multi-parameter Flow Cytometry of Whole Blood for *Ex Vivo* Phenotyping (Characterisation of Markers of Activation, Inhibition And Exhaustion)

2.8.1 Sample Preparation

One-hundred µl of Heparinised whole blood was added to a 5ml falcon tube (BD Biosciences) containing a cocktail of up to 8 monoclonal antibodies, each conjugated to a different fluorochrome (The same panels were used for both the whole blood, and isolated cell assays Table 2.1). The tubes were allowed to incubate for 15 minutes in the dark at room temperature, after which, 1ml of 10x diluted FACSLyse (BD Biosciences) was added to each tube, which was then allowed to incubate for a further 15 minutes in the dark at room temperature. Following this, 500µl of PBS containing 2% FCS was added to each tube, which was then centrifuged (Jouan CR422) at 300g for 5 minutes at room temperature. The supernatant was then discarded, and the pellet was resuspended in 350µl of PBS containing 2% FCS. The tubes were stored in the dark at 4°C until they were read on a FACSCanto II flow cytometer (BD Biosciences). This same procedure was followed for the determining CD38 expression on $CD8^+$ T-cells (CD3-FITC, CD8-PerCP, CD38-APC, PD-1-PE), except these samples were acquired on a BD FACSCalibur (BD Biosciences).

2.8.2 Gating Strategy

All cells were initially gated on FSC-area versus FSC-height to discriminate between the single cells populations, and doublets (Fig. 2.5a). Cells were then gated on CD19-AmCyan (BD Biosciences) versus SSC-area (Fig. 2.5b) to elucidate the CD19+ population. A dotplot of FSC versus SSC (Fig. 2.5c) was drawn to see whether the cells in P2 fell into the lymphocyte area. [This is known as back-gating, and it is used as a cross-check mechanism in multi-parameter flow cytometry, allowing cell populations to be visualized in multiple different gated populations]. A maximum of 10 000 $CD19^+$ events were acquired in the B-cell gate, or the sample was run for 5 minutes.

The B-cells were further subdivided into their respective sub-populations (Fig. 2.5d) in a dotplot of CD21-APC (BD Biosciences) versus CD27 PerCP-Cy5.5 (Biolegend), namely resting memory (RMem), activated memory (AMem), naïve (N), and Tissue-Like Memory (TLMem). Transitional (TR) B-cells were gated on CD10-APC-Cy7 (Biolegend) versus CD19-AmCyan. Plasmablasts were elucidated in panels 1 and 2 in a dotplot of CD27-PerCP-Cy5.5 versus CD38 PE-Cy7 (Beckman Coulter) (Fig. 2.5e). Each subset was then investigated for expression of the activation, inhibitory, or exhaustive markers, per B-cell subset, excluding TR B-cells, which were only enumerated. Positive expression cut-off of each marker was determined by FMO (See section 2.7.3).

2.9 Multi-parameter Flow Cytometry of Isolated B-cells for Functional Characterisation of Markers of Activation, Inhibition, and Exhaustion

2.9.1 Sample Preparation

Following 18 hours of culture, the 96-well plates were centrifuged at 300g for 5 minutes at room temperature (Jouan CR422). The supernatant was discarded and 200µl PBS containing 2% FCS was added to each well, after which the pellet was gently agitated. The plates were centrifuged again in the same conditions, after which the supernatant was discarded and 200µl PBS containing 2% FCS was added to each well, and the pellet was gently agitated. The content of each well was then transferred to separate 5ml Falcon tubes (BD Biosciences) to which the appropriate antibody cocktail was added (See section 2.7.1 for details on Ab titrations). This was allowed to incubate for 15 minutes at room temperature in the dark. Thereafter, 200µl PBS containing 2% FCS was added to each of the tubes, which were then centrifuged at 300g for 5 minutes at room temperature. The supernatant was then discarded, and the cells were resuspended in 350µl PBS containing 2% FCS, and the stored in the dark at 4°C for a maximum of 2 hours until they were read on a FACSCanto II flow cytometer (BD Biosciences). If the FACSCanto II was unavailable for direct analysis, samples were fixed using BD Stabilising Fixative (BD Bioscience), which preserves cells and prevents tandem dyes (such as APC-Cy7) from degrading.

Following this, panel 4 of each sample then underwent intracellular staining for CD287. Here, 250µl BD CytofixCytoperm™ (BD Biosciences) was added to each tube in panel 4 instead of 350µl PBS containing 2% FCS, and the sample was incubated in the dark at 4°C for 30 minutes. Thereafter, 500µl 10x diluted BD PermWash™ was added to the sample, which was then centrifuged at 300g for 5 minutes at 4°C. The supernatant was subsequently discarded, and a further 300µl 10x diluted BD PermWash™ was added to the sample to resuspend the cell pellet. The appropriate quantity of intracellular fluorochrome (Table 2.1) was added to the tubes, which were further incubated at 4°C for 20 minutes. The tubes were then centrifuged at 300g for 5 minutes at 4°C, following which their supernatant was discarded. The cells were then resuspended in 350µl PBS containing 2% FCS and stored in the dark at 4°C until they were read on a FACSCanto II flow cytometer (BD Biosciences).

2.9.2 Gating Strategy

Isolated cells were initially gated on FSC-area versus FSC-height to discriminate between the single cells populations, and doublets (Fig. 2.6a). A maximum of 10 000 B-cells were then gated on CD19-AmCyan (BD Biosciences) versus SSC-area (Fig. 2.6b) to elucidate the CD19+ population. A plot of FSC vs. SSC was used to determine whether the B-cells fell within the predictable lymphocyte area (Back gating) The B-cells were further subdivided into

their respective sub-populations (Fig. 2.6d) in a dotplot of CD21-APC (BD Biosciences) versus CD27-PerCP-Cy5.5 (Biolegend) (as shown in panel 1 dotplot D below), namely RMem, AMem, N, and TLMem. TR B-cells were gated on CD10-APC-Cy7 (Biolegend) versus CD21-APC. Plasmablasts were elucidated in panels 1 and 2 in a dotplot of CD27-PerCP-Cy5.5 versus CD38 PE-Cy7 (Beckman Coulter) (Fig. 2.6e).

2.10 Statistical Analyses

Statistical analyses were performed using GraphPad Prism software (Version 5, GraphPad Software, San Diego, California, USA). Due to the complexity of the gating strategy, which involved multiple gates and Boolean logic to elucidate all the relevant subpopulations, some subpopulation events were not excluded from the rest resulting in combined subset %s adding up to a total percentage > 100% (maximum was 105). We corrected for this by calculating the ratio out of 100, i.e. where the sum of the subpopulations was 105, and the % MN was 52.5%, then we multiplied the %MN by the correction factor (correction factor: 100/105=0.9523). Therefore, the corrected %MN was 50%. Where % increase or decrease was described, the value from the second condition was subtracted from that of the first, and the result divided by the value of the first condition, which was then multiplied by 100. Therefore if expression was 20% at baseline, and 60% with stimulation, then % increase was (60-20)/20, which is a 200% increase.

Where two parameters were compared with one another, an unpaired T-test was used due to the fact that most of the data was normally distributed (as calculated by the Shapiro-Wilk test for normality), where stated a Mann-Whitney U test was performed for non-parametric data. This method was primarily used when comparing the same parameter between the experimental and control groups. For multiple comparisons within e.g. unstimulated vs. stimulated cells in the same experimental group (either HIV+, or HIV-), a repeated measures one-way Analysis of Variance (ANOVA) was used, and if the data was non-parametric, a Friedman test was performed. These analyses were then followed by either of the following posttests; a Dunn's multiple comparison test for non-parametric data, or a Bonferroni's multiple comparison test for parametric data. Multiple correlations were performed using a Spearman's rank correlation test. Data are reported as mean percentage ±SD (Standard deviation) expression unless otherwise stated. Significance levels were set at $p<0.05$, while any p-value >0.05 is reported as non-significant "ns". Percentage inhibition was calculated by dividing the difference in percentage expression before and after inhibition, by the percentage expression before inhibition. Percentage expression was determined by dividing the number of positive events by the number of events in each subset. A biostatistician was

consulted to ensure that all statistical analyses performed were appropriate to the data set. Box and whisker plots were drawn using GraphPad Prism software, where the median is represented by a vertical line through the box, and the mean is represented by a "+" within the box. The whiskers were drawn according to the Tukey method, where the upper whisker stops at the data point that is largest value less than the sum of the 75th percentile plus 1.5 times the interquartile range (IQR). Any data points above this were plotted as individual points. Similarly, the bottom whisker stops at the data point that is the lowest value greater than the 25th percentile minus 1.5 IQR, and any points less than this value were plotted as individual data points (GraphPad Software, 2014).

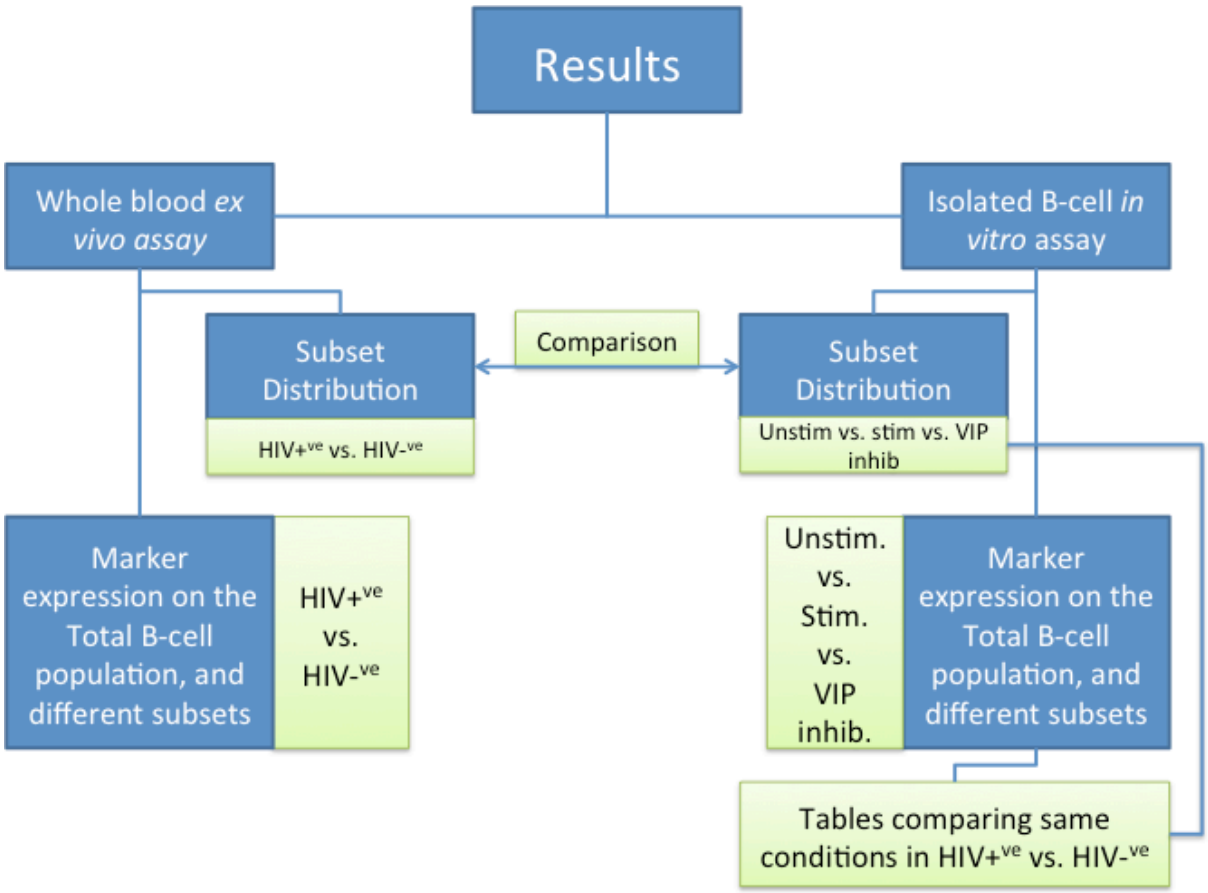


Fig. 2.4 Schematic overview of data analysis. *The results of this study were divided into two main subsections, namely the ex vivo whole blood data, and the overnight cultured isolated B-cells. We first compared subset distribution within that of the ex vivo data (i.e. between HIV+^{ve} and HIV-^{ve}), then followed by the phenotypic marker expression in the same manner. Next, the ex vivo subset distribution was compared with that of the unstimulated cultured B-cells, which was then compared with the different isolated B-cell culture states. Thereafter Phenotypic marker expression was investigated between the different culture conditions within the HIV+^{ve} group, and also between the two groups in the same condition (i.e. unstimulated CD38 on Activated Memory cells between the HIV+^{ve} and HIV-^{v} groups). See section 2.12 for details on statistical analysis*

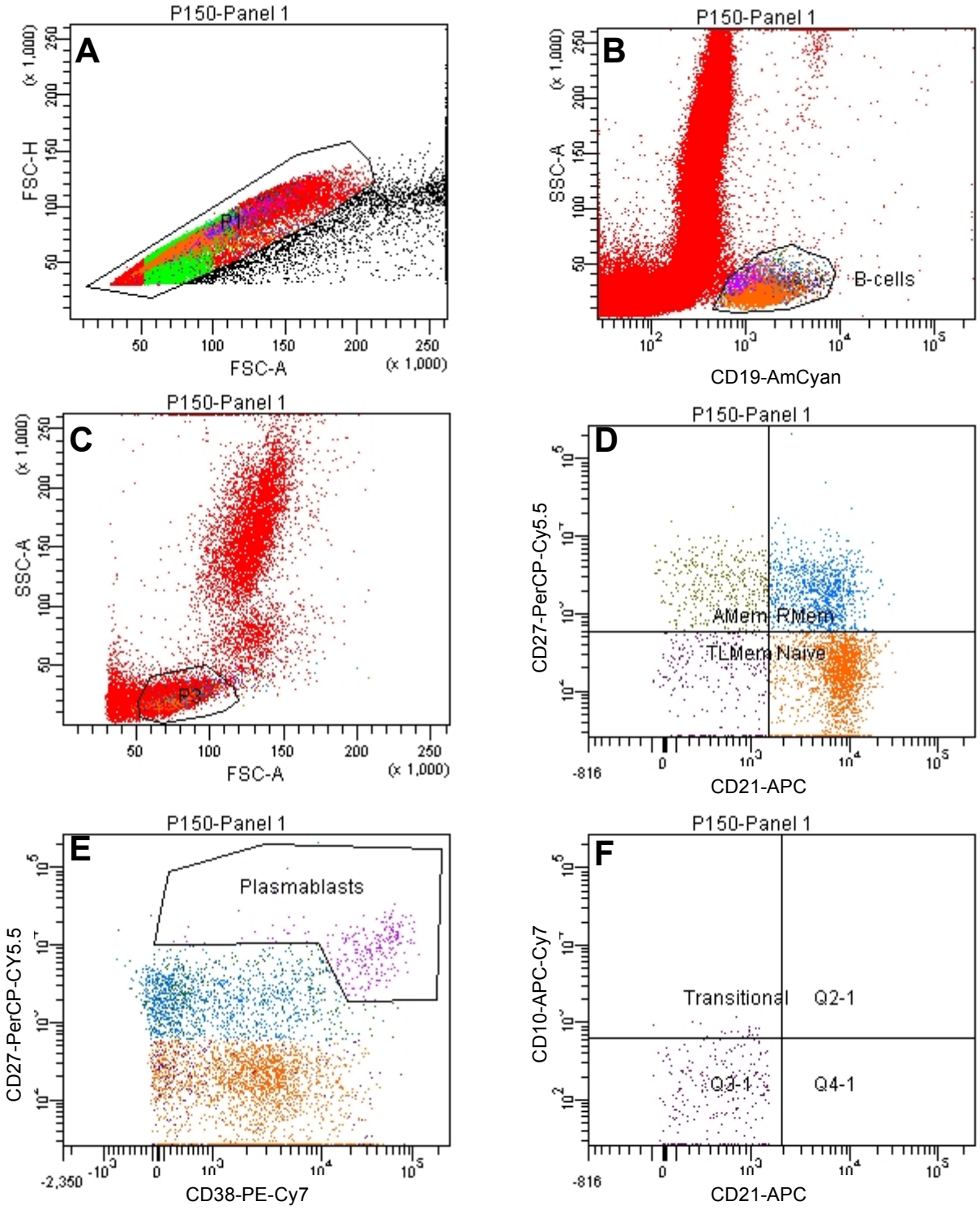


Figure 2.5: Whole blood gating strategy. *A) Cells were initially gated on FSC-A vs. FSC-H to obtain singlet and live cells in gate 'P1'. B) These cells were then gated according to CD19-AmCyan vs. SSC-A to obtain the total B-cell population in gate 'P2'. C) Gate 'P2 AND P3' was used to ensure that all cells used for further analysis were B-cells. D) Cells from 'P2 AND P3' were plotted on CD21-APC vs. CD27-PerCP-Cy5.5 to visualize the activated, resting, and tissue-like memory cells, and mature naïve B-cells. E) Plasmablasts were gated on CD38-PE-Cy7 vs. CD27-PerCP-Cy5.5, and these cells, which would otherwise show up in the 'AMem" quadrant of the previous plot, were separated via the gate 'AMem AND NOT Plasmablasts'. F) Transitional B-cells were gated on CD21-APC vs. CD10-APC-Cy7 from the 'TLMem' quadrant (i.e. CD21-CD10+. Q2-1 & Q4-1 contained no cells)*

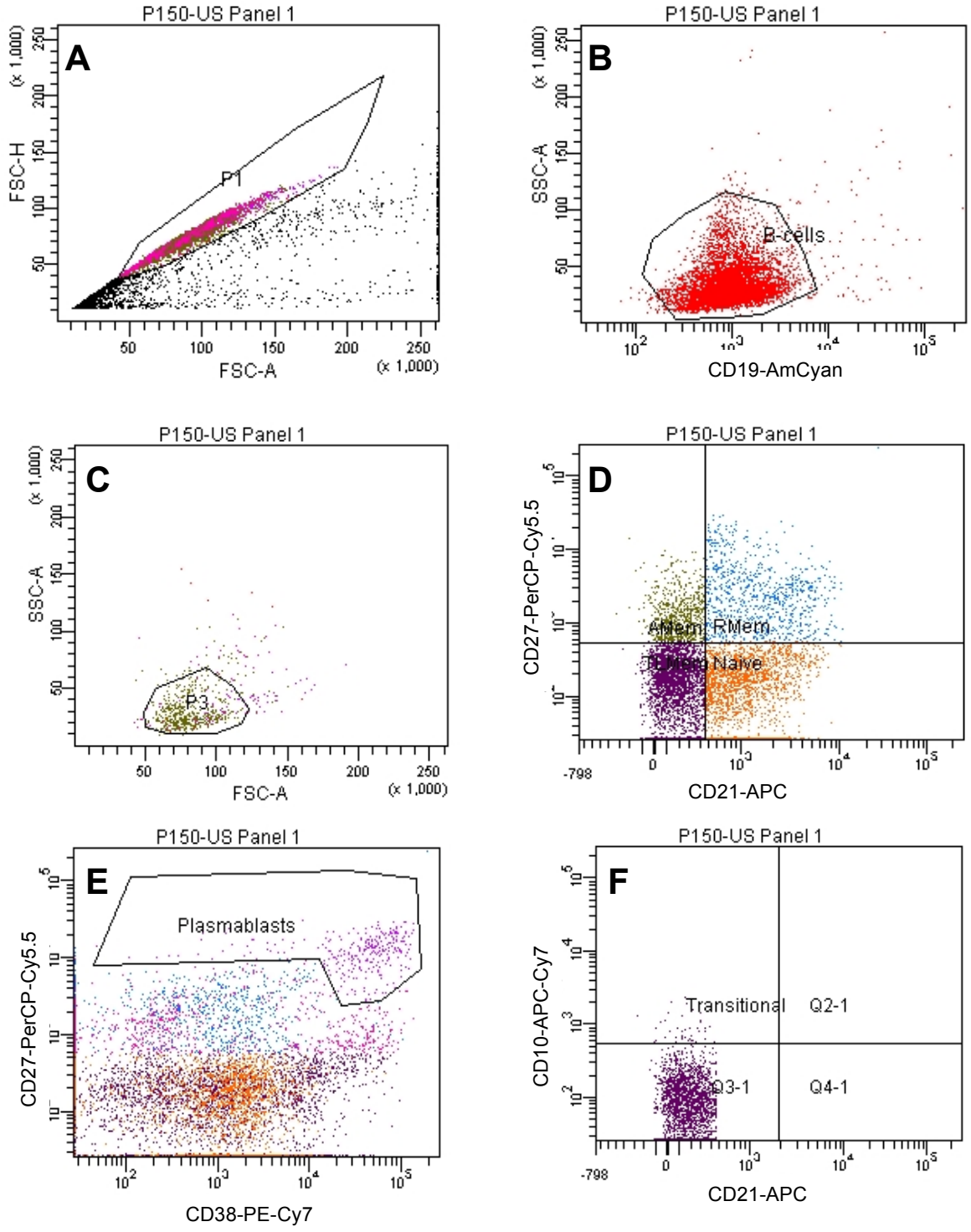


Figure 2.6: Isolated-cell gating strategy. *A) Cells were initially gated on FSC-A vs. FSC-H to obtain singlet and live cells in gate 'P1'. B) These cells were then gated according to CD19-AmCyan vs. SSC-A to obtain the total B-cell population in gate 'P2'. C) Gate 'P2 AND P3' was used to ensure that all cells used for further analysis were B-cells. D) Cells from 'P2 AND P3' were plotted on CD21-APC vs. CD27-PerCP-Cy5.5 to visualize the activated, resting, and tissue-like memory cells, and mature naïve B-cells. E) Plasmablasts were gated on CD38-PE-Cy7 vs. CD27-PerCP-Cy5.5, and these cells, which would otherwise show up in the 'AMem" quadrant of the previous plot, were separated via the gate 'AMem AND NOT Plasmablasts'. F) Transitional B-cells were gated on CD21-APC vs. CD10-APC-Cy7 from the 'TLMem' quadrant (i.e. CD21-CD10+. Q2-1 & Q4-1 contained no cells)*

Chapter 3 Results

The current study examined the phenotypic and functional properties of B cells in chronically HIV-1 infected individuals and healthy controls. The ultimate goal was to ascertain whether B cells in South African infected individuals were substantially affected by HIV-1 infection, even when CD4 count was relatively stable and participants were asymptomatic. The study assessed total B cells and B cell subsets in both study groups with regard their absolute numbers and distribution and phenotypic characteristics. A focus throughout was how immune activation (as determined by CD38 expression on CD8+ T-cells) was related to changes in the B-cell compartment. Following on from the ex vivo characterization, we also assessed functional responsiveness of B cells to TLR-mediated stimulation (LPS and R848) to ascertain whether chronic HIV-1 impacted on B-cell function. Finally we examined the VIP-VPAC ligand-receptor pathways as a potential therapeutic target for modulating aberrant B cell responses

Expression of markers of activation, inhibition, and exhaustion was measured on each B-cell subset, as well as on the total B-cell population in both study groups. For the ex vivo subset characterization and phenotyping component, single expression of each marker on each individual subset and total B cells was analysed, and data from the 21 HIV+ve samples was compared to that of the 18 HIV-ve control group samples. For the majority of the stimulatory and inhibitory data that is presented graphically, only the HIV+ve group was plotted. In the isolation and stimulation component of this study, in vitro marker expression was also assessed per subset between the HIV+ve group and HIV-ve control, in each of the stimulation conditions (LPS and R848). Stimulation experiments were performed with/without VIP to assess the modulatory capacity of VIP on B-cells. Co-expression of certain markers was investigated in this study; however the results presented below are restricted to single marker expression to avoid over-complicating a large data set. Furthermore, only significant data is presented in figures for the same reason. Twelve samples each of the HIV+ve and HIV-ve groups underwent inhibition with VIP, however 4 HIV+ve samples were excluded due to low CD4 counts (<200 cells/μL) and undetectable viral loads, leaving the total number of VIP-inhibited samples at 8 HIV+ve and 12 HIV-ve, so one might expect to find greater statistical significance with higher sample numbers.

3.1. Subset Distribution

3.1 Ex vivo subset distribution

A brief summary of the study cohort demographic is presented in Table 3.1. The mean absolute B-cell count as determined by Trucount assay (BD Biosciences) was significantly decreased in the HIV+^{ve} group (188.6 ± 181.3 vs. 276.8 ± 141.8 cells/μL, p=0.0002, Fig. 3.1 B). CD4 count was decreased with HIV (472.3 ± 237.5 vs. 819.9 ± 275.4; p<0.0001, Fig. 3.1 A) as expected, while the proportion of CD8+T-cells co-expressing CD38 was increased with HIV infection (46.25 ± 22.94 vs. 28.33 ±17.95; p<0.0001, Fig. 3.1 C).

***Table 3.1. Summary of patient demographics**. Data represented as mean ± standard deviation (SD). ns-not significant, significant data indicated in bold.*

	N	Age	% Male	% Female	CD4 count	CD19 count
HIV+^{ve}	21	32 ± 7.84	20%	80%	**472.3 ± 237.5**	**188.6 ± 181.3**
HIV-^{ve}	19	30 ± 8.64	40%	60%	**819.9 ± 275.4**	**276.8 ± 141.8**
p-value	-	ns	-	-	**<0.0001**	**0.0002**

The relative percentage of resting memory cells was significantly decreased in the HIV+^{ve} group, with a mean percentage of 17.78 ± 7.54% of total B-cells compared to 26.33 ± 8.65% in the HIV-^{ve} control (p=0.0097), while the relative percentage of the tissue-like memory cell phenotype was increased in the HIV+^{ve} group (24.57 ± 14.84% vs. 15.43 ± 10.39%, p=0.0314). The relative percentages of plasmablasts, transitional B-cells, mature naïve, and activated memory B-cells were not significantly different between the HIV+^{ve} group and the HIV-^{ve} control (Fig. 3.3).

The percentage tissue-like memory cells in the HIV+^{ve} group *ex vivo* correlated negatively with percentages of resting memory and mature naïve B-cells (r=-0.4844, p=0.026, Fig. 3.4 C; r=-0.7795, p<0.0001, Fig. 3.4 A), and also positively with the percentage of activated memory cells (r=0.6532, p=0.0013, Fig. 3.4 E). The percentage mature naïve B-cells also correlated negatively with the percentage activated memory cells (r=-0.8457, p<0.0001, Fig. 3.4 B). Although there was no significant difference in their relative proportions between the HIV+^{ve} and HIV-^{ve} groups, there was a positive correlation between the percentages of

transitional cells and plasmablasts in the HIV+^{ve} group (r=0.7219, p=0.0002). These two populations were therefore increased in the same HIV+-^{ve} patients, who generally had lower CD4 counts. The plasmablast population also correlated positively with percentage activated memory cells (r=0.4481, p=0.042).

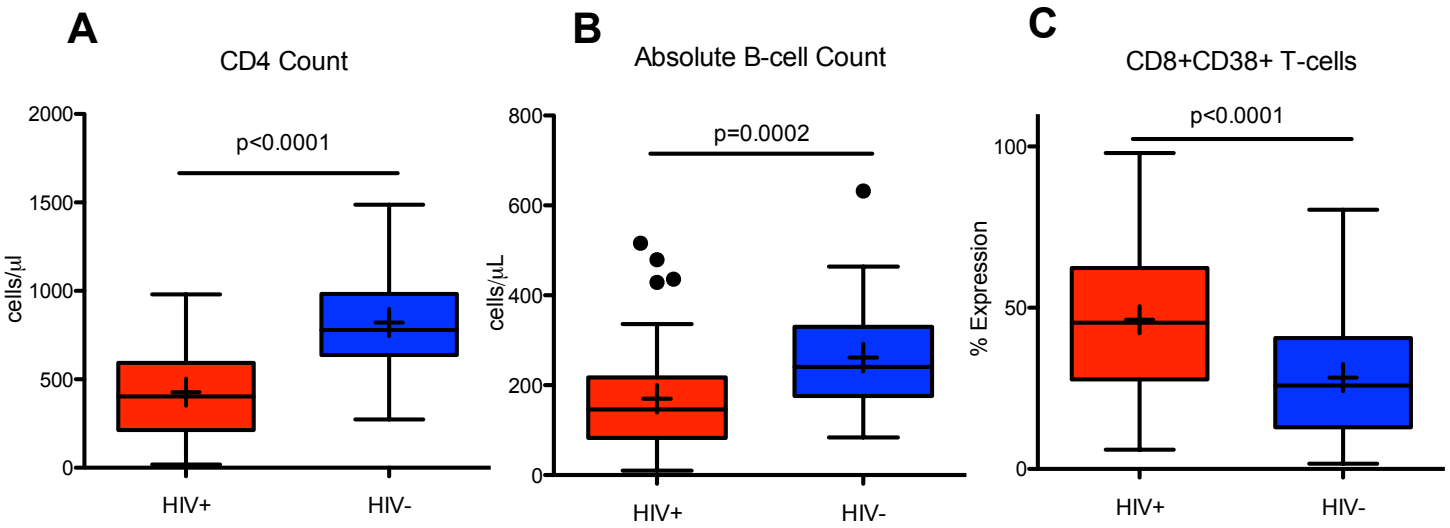


Fig. 3.1: Markers of disease progression and immune activation. *A) CD4 count was decreased with HIV (472.3 ± 237.5 vs. 819.9 ± 275.4; p<0.0001) as expected. B) Absolute B-cell count was also decreased with HIV infection (118.6 ± 181.3 vs. 276.8 ± 141.8; p=0.0002). C) The proportion of CD8$^+$T-cells co-expressing CD38 was increased with HIV infection (46.25 ± 22.94 vs. 28.33 ±17.95; p<0.0001).*

The relationship of absolute B-cell count to well-described markers of HIV disease progression and immune activation, namely CD3, CD4 and CD8 counts; HIV viral load, and CD38 & PD-1 expression on CD8$^+$ T-cells was assessed (Fig 3.3). Absolute B-cell count correlated positively with both CD3 and CD8 counts (r=0.2985, p=0,0299, Fig. 3.2 A; r=0.2755, p=0.0459, Fig. 3.2 B), but not with CD4 count (r= 0.2277, p= 0.0977, Fig. 3.2 C), however a trend towards a positive correlation was noted. As described in other studies, CD38 expression on CD8$^+$ T-cells correlated positively with viral load (r=0.5147, p=0.041), and negatively with CD4 count (r=-0.4468, p=0.042). CD4 count also correlated negatively with PD-1 expression on CD8$^+$ T-cells (r=-0.2440, p=0.0021), therefore healthy individuals with higher CD4 counts had lower expression of PD-1 on CD8$^+$ T-cells. The resting memory cell population showed a positive correlation with PD-1 on CD8$^+$ T-cells (r=-0.4481, p=0.042, Fig. 3.4 F), meaning that resting memory cells were increased in patients with higher levels of immune exhaustion. Similarly, percentage resting memory cells correlated positively with CD4 count (r=0.4533, p=0.039, Fig. 3.4 D), which indicates that this population was decreased in patients with decreased CD4 counts.

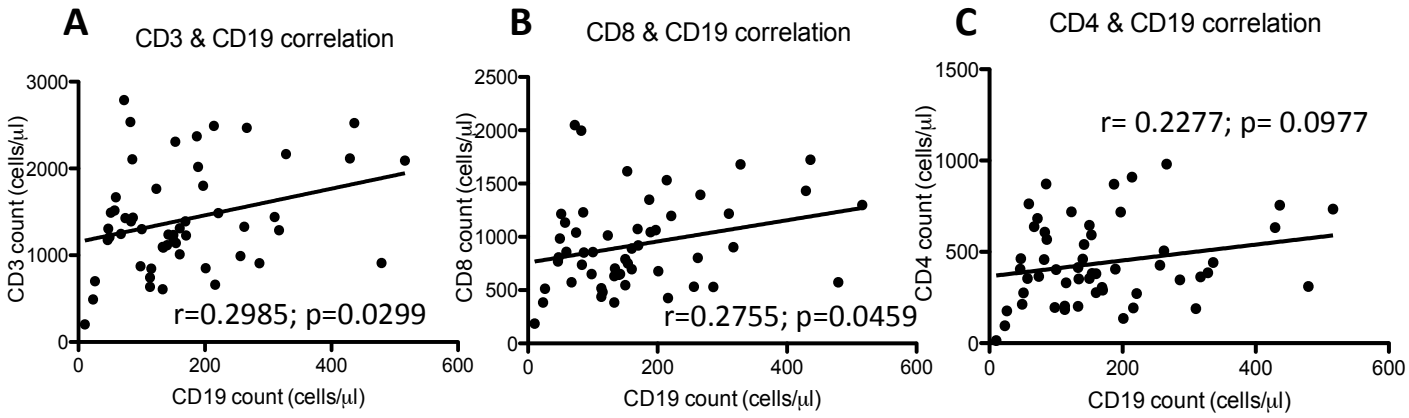
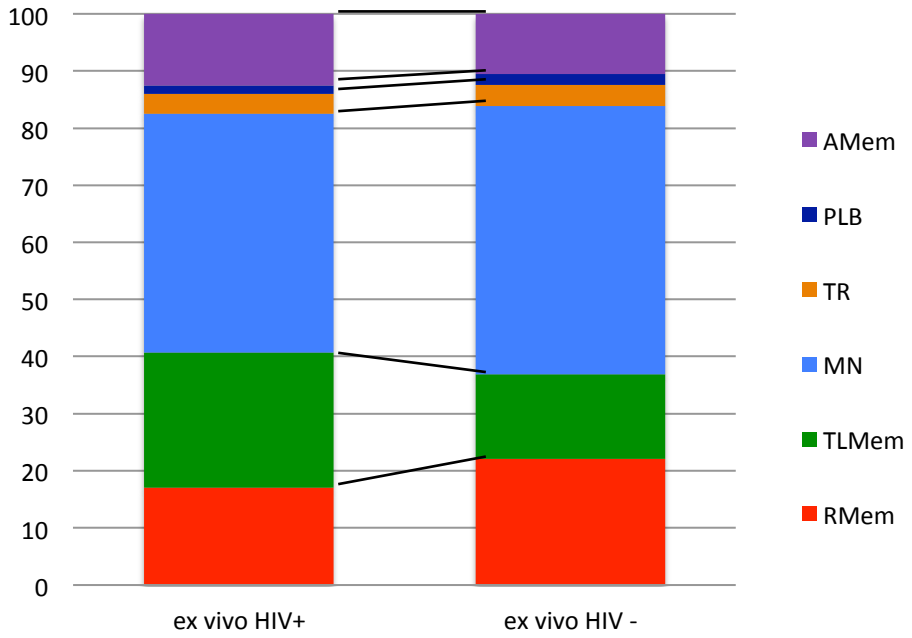


Fig. 3.2. Correlations of Absolute B-cell (CD19), T-cell (CD3), and CD4 counts. *A) CD3 count correlated positively with absolute B-cell count (r=0.2985, p=0.0299). B) CD8 count also correlated positively with absolute B-cell count (r=0.2755, p=0.0459). C) CD4 count did not correlate with absolute B-cell count, however a trend towards a positive correlation was noted, and a larger sample size may lead to significance.*



	HIV+	HIV-	p-value
AMem	12.56	10.53	ns
PLB	1.45	1.94	ns
TR	3.50	3.68	ns
MN	41.83	47.01	ns
TLMem	24.57	15.43	**0.0314**
RMem	17.78	26.33	**0.00097**

Fig. 3.3: Ex vivo subset distribution was altered in asymptomatic HIV infection. *The proportion of resting memory cells ex vivo was significantly decreased with HIV infection (p=0.0481), while the proportion of tissue-like memory cells was increased with HIV infection (p=0.0314).*

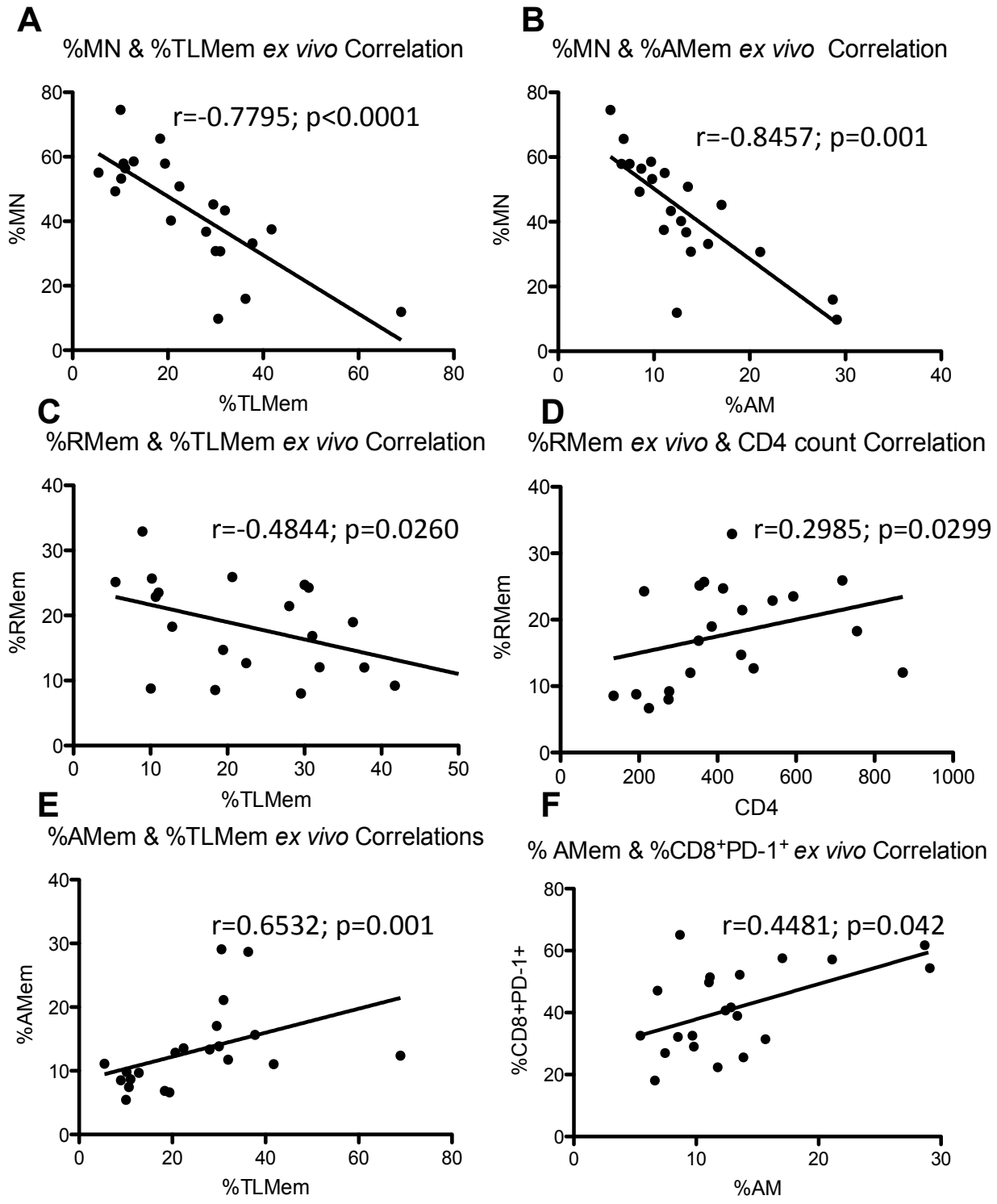


Fig. 3.4: ex vivo Subset and disease progression marker correlation graphs. *A) Relative % mature naïve B-cells correlated negatively relative % tissue-like memory B-cells (r=-0.7795, p<0.0001). B) Similarly, relative % mature naïve B-cells also correlated negatively with relative % activated memory B-cells (r=-0.8457, p=0.001). C) Relative % resting memory B-cells correlated negatively with relative % tissue-like memory B-cells (r=-0.4844, p=0.0260). D) There was a positive correlation between relative % resting memory B-cells and CD4 count (r=0.2985, p=0.0299). E) Relative % activated memory B-cells also correlated positively with relative % tissue-like memory B-cells (r=0.6532, p=0.001). F) Similarly, relative % activated memory B-cells also correlated positively with the marker of immune exhaustion, PD-1 expression on $CD8^+$ T-cells (r=0.4481, p=0.042).*

3.2 Subset distribution after 18h culture

After 18h of unstimulated *in vitro* culture, purified B-cell subset distribution of the HIV+^{ve} group, was altered when compared to that observed *ex vivo* distribution. There was a significant change after 18h culture in all subsets except for activated memory cells. There was however, no significant difference in subset distribution within the HIV-^{ve} control group after 18h unstimulated culture when compared to that of the *ex vivo* subset distribution in the same group (Fig. 3.6). Changes in the HIV+^{ve} group however are not likely to be caused by culture stress as there was not alteration in subset distribution with stimulation or inhibition relative to that of the unstimulated cells in either group. Alterations were not due to viability loss as less than 5% of cells stained positive for Trypan Blue in both the HIV+^{ve} and HIV-^{ve} groups after 18h culture with and without stimulation, furthermore, there was no difference in cell concentration before and after stimulation (data not shown). Overall population change trends following culture were similar for both groups.

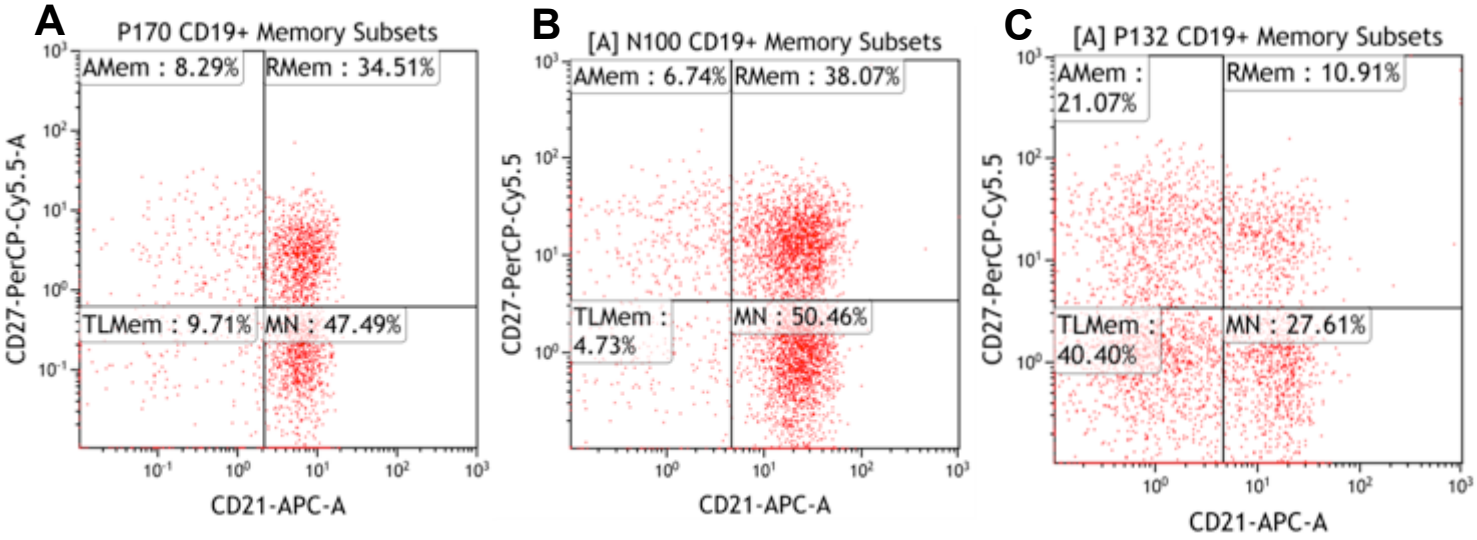
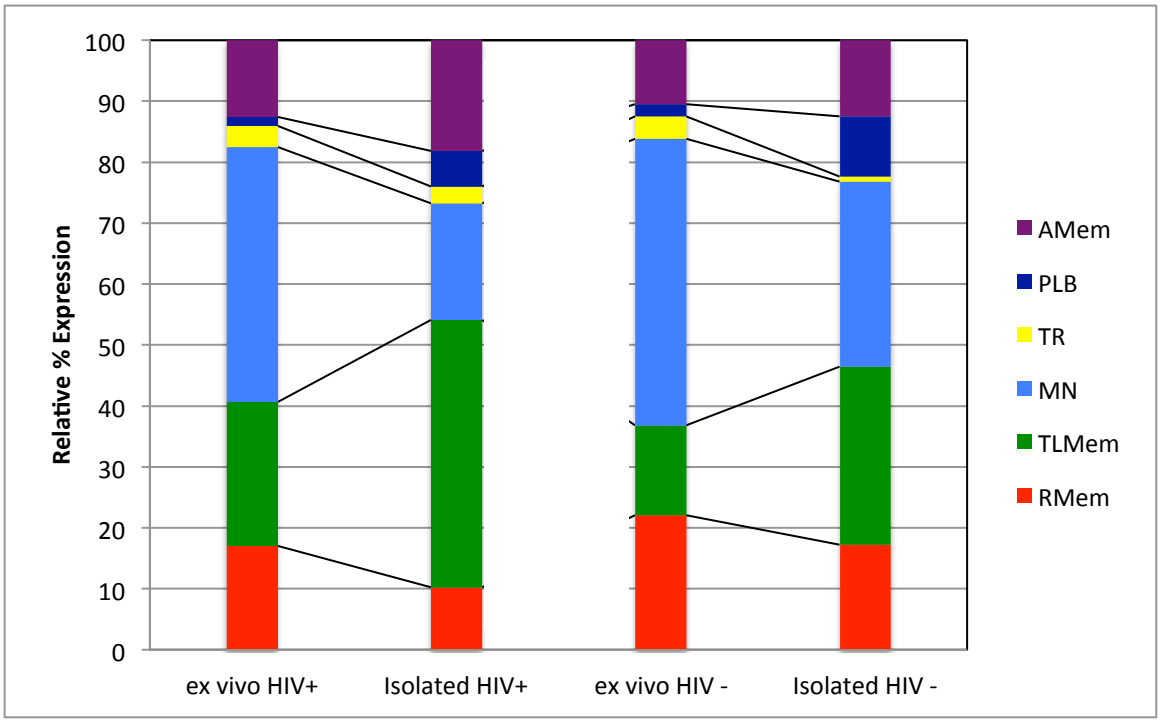


Fig. 3.5: Representative data of marker expression on two different subsets in an HIV+^{ve} patient. *Gating strategy as per chapter 3All plots represent the various peripheral blood subsets, which consist of activated memory cells (AMem), resting memory cells (RMem), tissue-like memory cells (TLM), and mature naïve (MN) B-cells. A) HIV+^{ve} patient P170 had a relatively-high CD4 count of 437 cells/µl, which correlated with higher proportion on RMem cells (r=0.4533, p=0.039, Fig. 3.4 D), meaning that individuals with higher CD4 counts had peripheral B-cell subset distributions similar to that of HIV-^{ve} individuals. B) Representative subset distribution dotplot of an HIV-^{ve} individual, with the majority of cells in the RMem, and MN compartments. C) Representative dotplot of an HIV+^{ve} individual (patient P132 with a CD4 count of 331 cells/µl), with increased proportions of AMem and TLMem populations.*



	ex vivo HIV+	Isolated HIV+	p-value		ex vivo HIV-	Isolated HIV-	p-value
AMem	12.56	17.26	ns		10.53	13.08	ns
PLB	1.45	5.51	**<0.001**		1.94	10.27	ns
TR	3.50	2.63	**<0.01**		3.68	0.86	ns
MN	41.83	18.20	**<0.001**		47.01	31.74	ns
TLMem	24.57	41.60	**<0.001**		15.43	27.16	ns
RMem	17.78	9.77	**<0.001**		26.33	18.03	ns

***Fig. 3.6: Changes in B-cell subset distributions with 18h unstimulated culture**. There was an increase in the proportions of TLMem (69.31%, p<0.001), and PLB (280%, p<0.001) in the HIV+^{ve} group with unstimulated culture. The TR, MN, and RMem populations decreased by 24.86% (p<0.01), 56.49% and 45.05% respectively (both p<0.001). The proportion of AMem was relatively constant between the two conditions. Subset distribution in the HIV-ve group was significantly unchanged with 18h unstimulated*

3.3 Phenotypic marker expression *ex vivo*

3.3.1 The total B-cell population

3.3.1.1 Expression of activation markers and toll-like receptors

Overall, there appeared to be a decrease in the percentage of B-cells expressing activation markers in the HIV+^{ve} group. For example, the activation marker CD25 was expressed on a significantly lower percentage of the total B-cell population in the HIV+^{ve} group (2.42±3.83% vs. 4.33±3.38%, p=0.0051, Fig. 3.7 B). There was also a trend towards decreased expression of co-stimulatory marker CD86 in the HIV+ group, which approached significance (7.69±12.17% vs. 12.50±11.17%, p=0.0545). There was no significant difference in the expression of CD126 and CD38 between the two groups (which are both markers of strong cellular activation). The expression of VPAC2, the VIP receptor was significantly decreased on the HIV+^{ve} total B-cell population, with 1.19±0.77% expression compared to 2.26±2.22% in the HIV-^{ve} group (p=0.0296, Fig. 3.7 A). It is possible that this generally minimal increase of activation markers *ex vivo* is due to the fact that the HIV+^{ve} patients in this cohort has relatively stable CD4 counts.

CD284 (Toll-like Receptor 4, an innate LPS receptor) expression on the total B-cell population in the HIV+^{ve} group was significantly higher than that of the HIV-^{ve} group (40.8±30.50% vs. 24.56±17.51%, p=0.0487). Total CD284 expression also correlated positively with Total CD126 expression (r=0.6139, p<0.0001), indicating that IL6 receptor expression was significantly increased with increased TLR4 expression, and therefore increased exposure to LPS. CD287 expression on the total B-cell population was proposed to be indicative of increased viral stimulation (Lanzavecchia & Sallusto, 2007), although its expression in the HIV+^{ve} group was not significantly different from that of the HIV-^{ve} group (6.40±6.71% vs. 3.34±2.63%, p=0.1101).

3.3.1.2 Inhibitory markers, and a marker associated with apoptosis signaling

Overall, there was an increase in the percentage of B-cells expressing inhibitory markers, with the highest being CD72 (an inhibitory receptor involved in the B-T cell interaction), which was significantly higher in the HIV+^{ve} group (60.98±19.50% vs. 38.49±23.68%, p=0.0038, Fig. 3.7 C). The percentage of cells expressing CD300a and CD305 (novel inhibitory receptors, which bind to phosphatidylserine and collagen respectively), were moderately (but not significantly) decreased in the HIV+ group (12.38±6.54% vs. 17.36±10.73%, p=0.151; & 78.05±13.40% vs. 83.29±7.37%, p=0.244). The percentage of cells expressing CD85j, an inhibitory receptor involved in down-regulation of APC activation, was moderately, but insignificantly increased in the HIV+^{ve} group, although this was not

significant (6.82±9.51% vs. 3.92±4.84%, p=0.317). The percentage of cells expressing CD300a correlated positively with CD4 count, even though there were no significant differences in CD300a expression between the HIV+^{ve} and HIV-^{ve} groups (r=0.4468, p=0.042).

The percentage of B-cells expressing CD307d, a novel marker of cellular exhaustion was not significantly different between the HIV+^{ve} and HIV-^{ve} groups, although there was a trend towards decreased expression in the HIV+^{ve} group (7.94±8.27% vs. 9.85±7.50%, p=0.2908). CD95 expression, which is associated with apoptosis signaling, was not significantly altered on the total B-cell population with HIV infection, however total CD95 expression correlated positively with total CD307d expression (r=0.6714, p=0.00086).

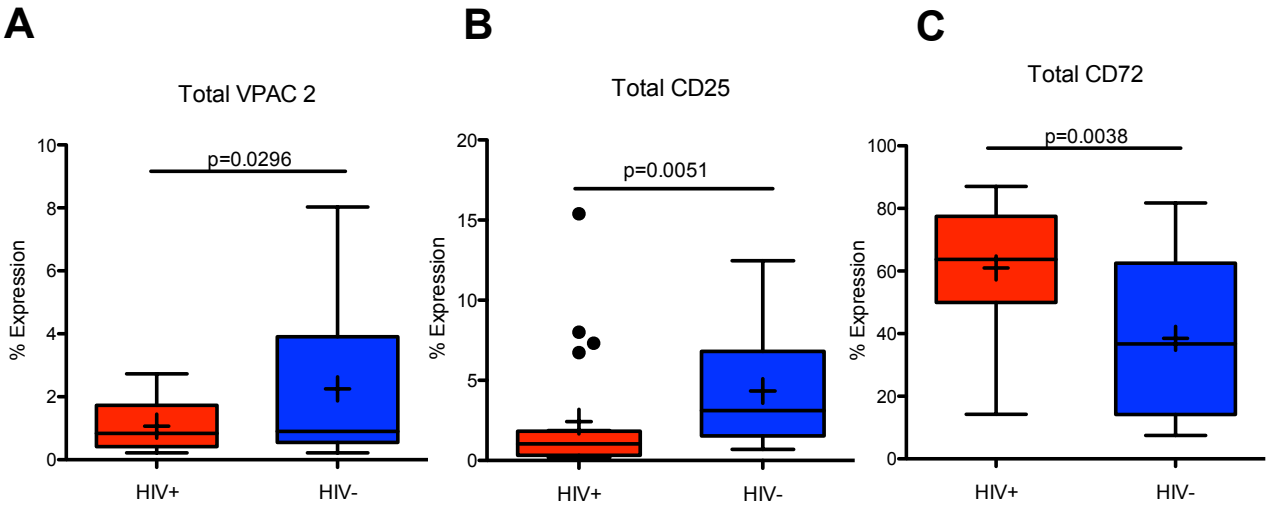


Fig. 3.7: Significantly altered ex vivo activation and inhibitory marker expression on the total B-cell population. A) *VPAC2 expression was significantly decreased on the HIV+ total B-cell population, with 1.19*±0.77% *expression compared to 2.26*±2.22% *in the HIV- group (p=0.0296) B) CD25 expression on the total B-cell population was decreased in the HIV+ group (2.42*±3.82%*) relative to that of the HIV- group (4.33*±3.38%*), p=0.0051. C) CD72 expression on the total B-cell population was increased in the HIV+ group (60.98*±19.50%*) relative to that of the HIV- group (38.49*±23.68%*), p=0.0038*

3.3.2 Activated Memory B-Cells

Activated memory B-cells are the so-called “effector” cells, which have memory against previously encountered antigens, and are the active form of resting memory B-cells. Previous work has indicated that there is an increase in the relative percentage of these cells with HIV infection (Moir & Fauci, 2013), although this study found no significant difference in the relative percentages of this subset between the HIV+^{ve} and HIV-^{ve} groups. This may be due to the fact that our small cohort had relatively high CD4 counts, and were clinically asymptomatic.

3.3.2.1 Expression of activation markers and toll-like receptors

The relative percentage of activated memory B-cells expressing CD86 was minimally (but significantly) decreased, similar to as observed in the total B-cell population, however this was a minor shift (7.09±6.24% vs. 9.46±5.23%, p=0.048, Fig. 3.8 B). The percentage activated memory B-cells expressing CD38, a marker of early cell activation, was significantly increased within the HIV+ group by a large margin (38.63±11.82% vs. 24.21±8.96%, p=0.0004, Fig. 3.8 A), even though there was no significant alteration in the total B-cell population. CD284 expression, although expressed on almost twice as many cells in the total population (p=0.0487), was not significantly altered on activated memory B-cells by HIV infection. CD287 expression on activated memory cells was consistent with that of the total B-cell population, in that expression was also not significantly altered by HIV infection.

3.3.2.2 Inhibitory markers, and a markers associated with apoptosis signaling

Consistent with the findings in the total B-cell population, CD72 expression on activated memory cells was significantly increased in the HIV+^{ve} group (33.33±20.08%, vs. 20.74±17.66% in the HIV-^{ve} group, p=0.0396, Fig. 3.9 A). Conversely, while there was no significant difference in either CD300a, or CD305 expression on the total B-cell population, expression of both on activated memory cells in the HIV+^{ve} group was decreased relative to that of the HIV-^{ve} group (19.67±10.17% vs. 29.89±13.64%, p=0.0213, Fig. 3.9 B for CD300a; and 60.91±17.88% vs. 70.74±11.26%, p=0.048, Fig. 3.9 D for CD305). Unlike that of the total B-cell population, CD307d expression on activated memory cells *ex vivo* was significantly increased in the HIV+^{ve} group, with a mean of 25.75±10.04% compared to 15.27±15.27% in the HIV- group (p=0.0015, Fig. 3.9 C). There was no significant change *ex vivo* in CD95 expression on activated memory cells with HIV infection, which is consistent with the expression found on the total B-cell population.

In summary, the activated memory B-cell subset in the HIV+^{ve} group exhibited an increase in CD38 expression (approximately 38%), which was concomitant with a decrease in CD86 expression (approximately 20%), both of which were not significantly altered in the total B-cell population. Of the inhibitory markers used in this study, only CD72 was significantly expressed at higher levels on activated memory B-cells (approximately 38% higher than that of the HIV-^{ve} group), while the marker of exhaustion, CD307d was also increased on this subset with HIV infection. CD300a and CD305 expression was decreased, and CD95 expression on this subset were not significantly altered by HIV infection.

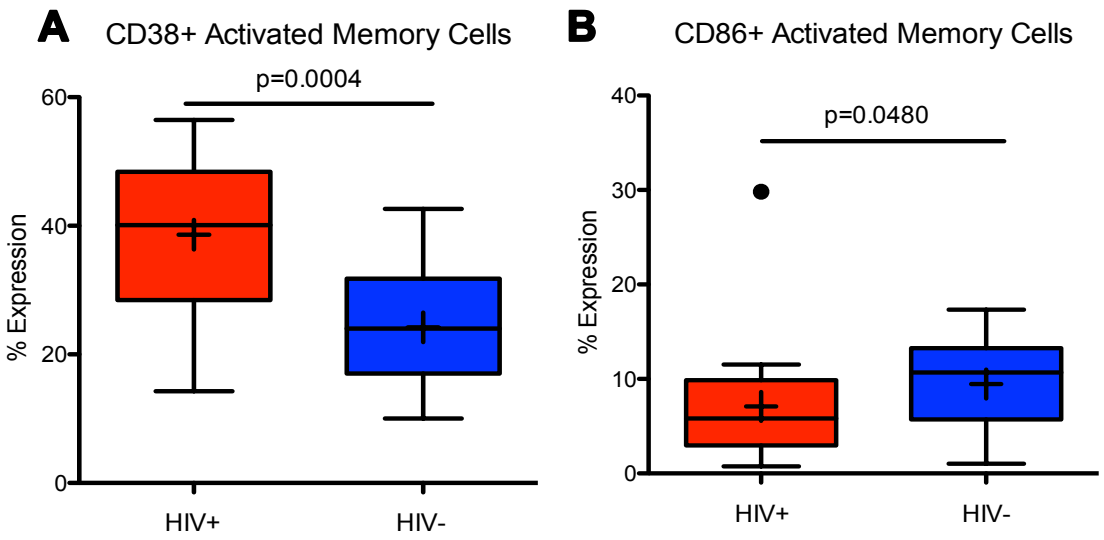


Fig. 3.8: Significantly altered ex vivo activation marker expression on activated memory cells. *A) CD38 expression was increased on HIV+ activated memory cells, with a mean of 38.63±11.82% compared to 22.40±8.96% in the HIV- group (p=0.0004). B) CD86 expression on activated memory cells was decreased in the HIV+ group, with a mean of 7.09±6.24% compared to 9.46±5.23% in the HIV- group (p=0.048).*

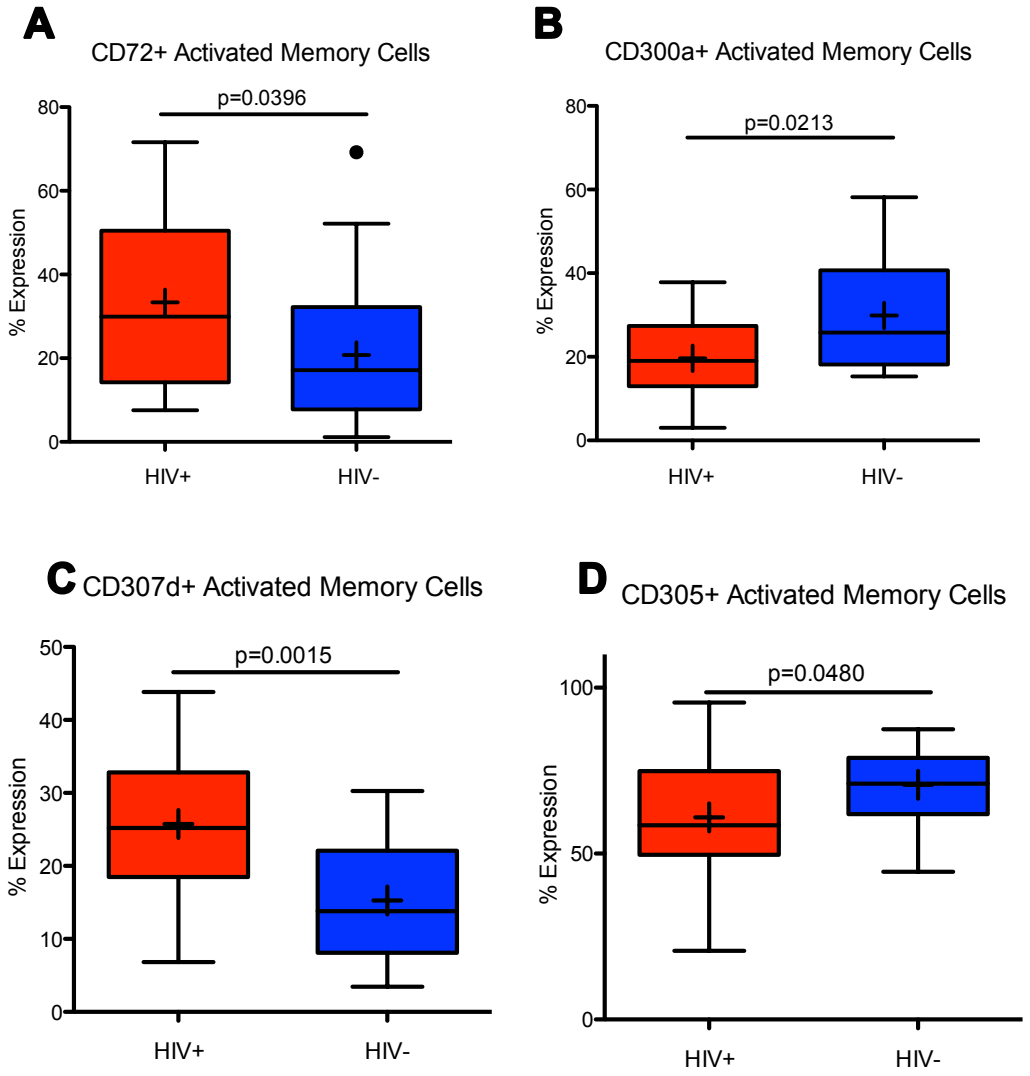


Fig. 3.9: Ex vivo inhibitory and exhaustion marker expression on activated memory cells. *A) CD72 expression on activated memory cells was increased in the HIV+ group, with a mean of 33.33±20.08% compared to 20.74±17.66% in the HIV- group (p=0.0396). B) CD300a expression was decreased on HIV+ activated memory cells, with a mean of 19.67±10.17% compared to 29.89±13.64% in the HIV- group (p=0.0213). C) CD307d expression on activated memory cells was increased in the HIV+ group, with a mean of 25.75±10.04% compared to 15.27±15.27% in the HIV- group (p=0.0015). D) CD305 expression on activated memory cells was decreased in the HIV+ group, with a mean of 60.91±17.88% compared to 70.74±11.26% in the HIV- group (p=0.0480)*

3.3.3 Resting Memory B-cells

Resting memory B-cells are the quiescent population within the memory compartment. When they encounter cognate antigen, they are re-activated and proceed to proliferate into effector (activated) memory B-cells. This compartment has been found to be decreased with HIV infection (Moir & Fauci, 2013), which has been confirmed in this study.

3.3.3.1 Expression of activation markers and toll-like receptors

CD126 expression (the IL-6 receptor) was significantly increased by roughly 60% in the HIV+^{ve} resting memory subset (39.01±25.59% vs. 14.89±9.48%, p=0.0008; Fig. 3.10 A), while its expression was not significant on either activated memory B-cells, or the total B-cell population. Relative expression of CD25 (the Il-2 receptor) was significantly decreased on resting memory cells (1.76±2.11% vs. 3.91±3.26%, p=0.0017, Fig. 3.10 B) with HIV infection, although these values were both at relatively low levels (<5% expression). Interestingly, there was no significant difference in CD25 expression on activated memory cells, however its expression was consistent with the expression pattern of CD25 on the total B-cell population (<5%).

Relative CD284 expression, while not significantly altered on either activated memory B-cells, or the total population by HIV infection, was expressed on approximately 50% more resting memory cells in the HIV+^{ve} group, with a mean of 51.43±30.12%, compared to 24.84±10.07% in the HIV-^{ve} group (p=0.0057, Fig. 3.10 D). CD287 was also expressed on a significantly larger proportion of resting memory B-cells in the HIV+^{ve} group, with a mean of 10.60±13.10%, compared to 3.44±2.61% in the HIV-^{ve} group (p=0.0199, Fig. 3.10 C). This was in contrast to the total B-cell population, and activated memory B-cell subset, on which there was no significant difference in CD287 expression.

3.3.3.2 Inhibitory markers, and a marker associated with apoptosis signaling

There was no significant difference in CD307d expression between the two groups, although there was a trend towards increased expression in the HIV+^{ve} group (10.96±6.47% vs. 8.18±2.96%, p=0.1672). There was no significant change *ex vivo* in CD72, CD85j, CD300a, CD305, or CD95 expression on resting memory cells with HIV infection.

In summary, the resting memory B-cell subset had increased expression of CD126 (approximately 60%), CD284 (approximately 50%), and CD287 (approximately 68%), while CD25 expression was decreased (although the expression of this marker on the HIV-^{ve} group was <5%). None of the inhibitory markers in this study were significantly altered on resting memory B-cells *ex vivo* by HIV infection.

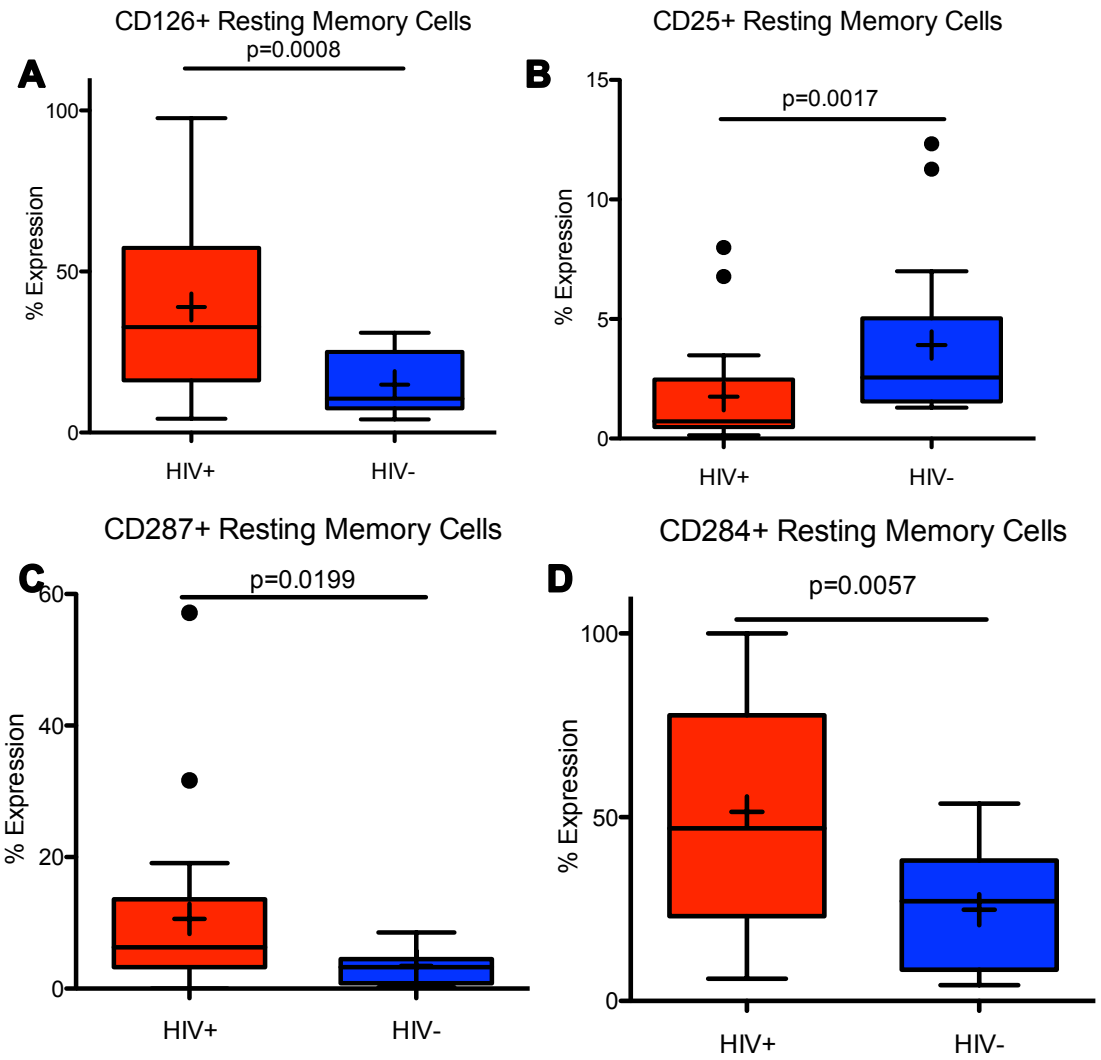


Fig. 3.10: Ex vivo activation marker and TLR expression on resting memory cells*. A) CD126 expression on resting memory cells was increased in the HIV+ group, with a mean of 39.01±25.99% compared to 14.89±9.48% in the HIV- group (p=0.0008). B) CD25 expression was decreased on HIV+ resting memory cells, with a mean of 1.76±2.11% compared to 3.91±3.26% in the HIV- group (p=0.0017). C) CD287 expression on resting memory cells was increased in the HIV+ group, with a mean of 10.60±13.10% compared to 3.44±2.61% in the HIV- group (p=0.0199). D) CD284 expression on resting memory cells was increased in the HIV+ group, with a mean of 51.43±30.12% compared to 28.84±10.07% in the HIV- group (p=0.0057).*

3.3.4 Tissue-like Memory B-cells

Tissue-like memory B-cells are the so-called “exhausted” B-cell subset, which constitutes the largest peripheral blood B-cell subset in HIV infection. These cells are derived from the memory B-cell compartment, which after activation, eventually become anergic and gain increased surface expression of inhibitory receptors, while losing activation marker expression (Moir & Fauci, 2013). In the current study this subset was found to be significantly increased (see above).

3.3.4.1 Expression of activation markers and toll-like receptors

As expected due to their loss of function (linked to loss of activation marker expression), there was no significant difference in activation marker expression on the tissue-like memory B-cell subset between the two groups, except for CD287 (TLR 7, Fig. 3.11), indicative of increased levels of viral stimulation *in vivo*. CD287 was expressed on significantly more tissue-like memory cells in the HIV+^{ve} group, with a mean of 8.28±11.39%, compared to 1.12±1.32% in the HIV-^{ve} group (p=0.0128). This level of expression was consistent with that of the activated- and resting memory B-cell subsets (approximately 10%), and slightly higher than that of the total B-cell population (approximately 6%).

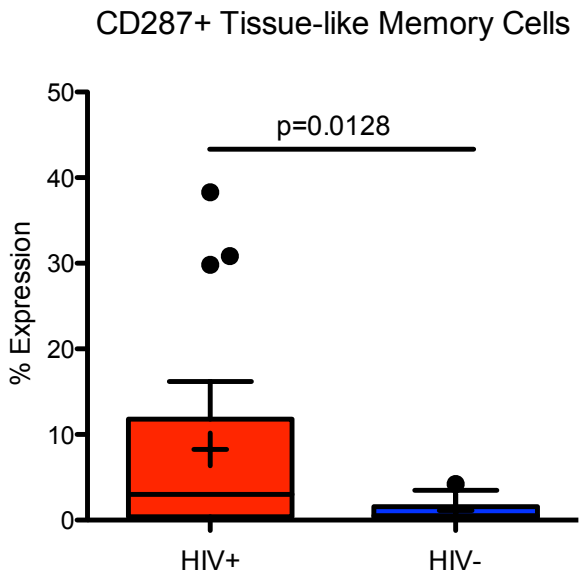


Fig. 3.11: Ex vivo TLR7 expression on tissue-like memory B-cells *was increased in the HIV+ group (8.28±11.39%) relative to that of the HIV- group (1.12±1,32%), p=0.0128*

3.3.4.2 Inhibitory markers and Fas (CD95), a marker associated with apoptosis signaling

The marker that exhibited the most dramatic increase on tissue-like memory B-cells with HIV infection was CD72, which was increased 1.45-fold compared to the HIV-^{ve} group (70.88±16.54% vs. 28.83±25.68%, $p<0.0001$; Fig. 3.12 A). This significant increase in CD72 expression on this subset is greater than that of the total B-cell population and activated memory B-cell population (both approximately 38%, Fig. 3.7 A and Fig. 3.9 A respectively). Relative CD85j expression when compared to the HIV-^{ve} group was 1.7-fold higher (19.38±23.18% vs. 7.09±10.39%, $p=0.046$, Fig. 3.12 B), and this was also the only subset in which there was a significant difference in CD85j expression with HIV infection, *ex vivo*.

CD307d expression, which is a hallmark of this subset, was significantly raised in the HIV+^{ve} group, with a mean of 12.93±10.32%, compared to 7.5±5.10% in the HIV-^{ve} group ($p=0.045$, Fig. 3.12 C). This was however; lower than that of the activated memory B-cell subset, where expression was increased by 40% to 25.75±10.04%. CD307d expression on both resting memory B-cells, and the total B-cell population was <10%, and not significantly altered with HIV infection. There was no significant change *ex vivo* in CD95 expression on tissue-like memory cells with HIV infection.

In summary, none of the activation markers used in this study were found to have significantly altered expression on the HIV+^{ve} group, except for CD287, which has similar expression levels compared to the activated- and resting memory B-cell subsets, and was slightly higher than that of the total B-cell population. The tissue-like memory B-cell subset had extremely high expression of CD72, while its expression of CD307d was lower than that of the activated memory B-cell subset. Significant expression of CD85j was noted on this subset, and on no others, while CD300a, CD305, and CD95 had expression not significantly altered by HIV infection.

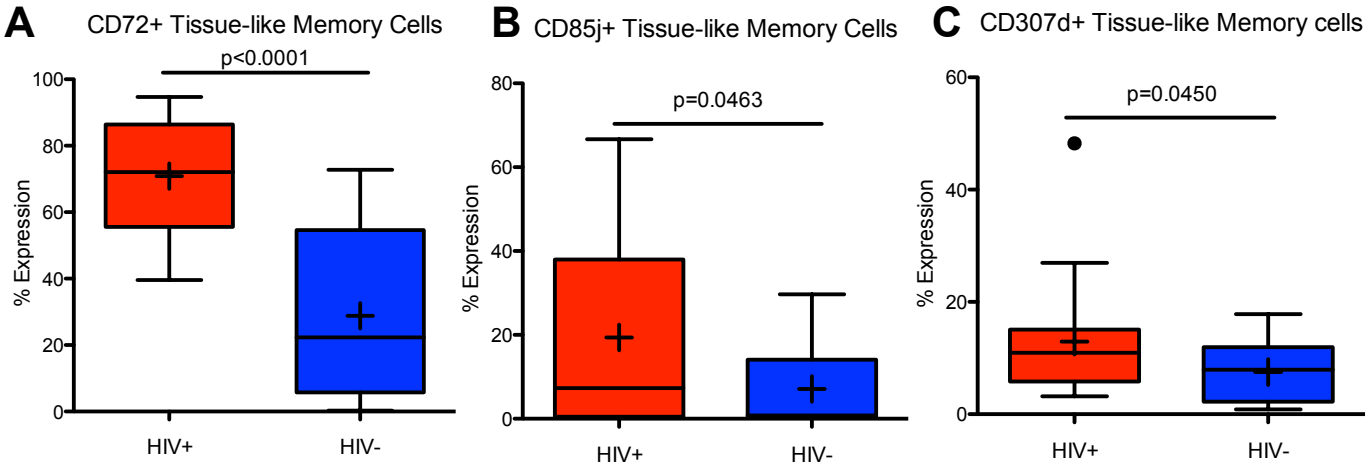


Fig. 3.12: Ex vivo Inhibitory marker expression. *A) CD72 expression was increased on tissue-like memory B-cells in the HIV+ group (70.88±16.54%) compared to the HIV- control (28.83±25.68%; p<0.0001). B) CD85j expression on tissue-like memory B-cells in the HIV+ group was increased compared to the HIV- control group (19.38±23.18% vs. 7.09±10.39%; p=0.046). C) CD307d expression on tissue-like memory B-cells was increased in the HIV+ group relative to that of the HIV- controls (12.93±10.32% vs. 7.50±5.10%; p=0.045)*

3.3.5 Mature Naïve B-cells

Mature naïve B-cells constitute the highest percentage of peripheral blood B-cells in healthy individuals, and are the mature form of the naïve B-cell as it exits the bone marrow. These cells are antigen naïve, and as such should express low levels of both activation, and inhibitory markers *ex vivo* (Moir & Fauci, 2013).

3.3.5.1 Expression of activation markers and toll-like receptors

Relative expression of CD126 on mature naïve B-cells was increased by 260% (21.56±32.25% vs. 5.91±7.83%, p=0.023; Fig. 3.13 A), which was higher than the 60% increase found on resting memory B-cells (although overall expression was higher, at 39.01±25.59%). CD126 expression was not significantly altered on the total B-cell population, or the activated- , or tissue-like memory subsets. Expression of the other markers of activation (CD70, CD38, and CD25), as well as the toll-like receptors (CD287 and CD284), used in this study were not significantly altered on the HIV+^{ve} group, compared to the HIV-^{ve} group.

3.3.5.2 Inhibitory markers, and Fas (CD95)

CD72 had the highest level of expression on this subset (77.49±23.45% vs. 45.32±36.02% in the HIV-^{ve} group, p=0.0026, Fig. 3.13 C), although there was only a difference of ~40% between the relative expressions of the two groups (compared to an approximate difference of 60% and 38% on tissue-like memory B-cells, and resting- and activated memory B-cells respectively). Mature naïve B-cells in the HIV+^{ve} group also had increased CD307d expression *ex vivo*, with a mean of 7.49±10.05%, compared to 2.58±2.27% in the HIV-^{ve}

group (p=0.0135, Fig. 3.13 B), although this was lower than that of the activated memory subset. Lastly, there was no significant change *ex vivo* in CD95 expression on mature naïve B-cells with HIV infection, which was consistent with the other subsets, as well as with the overall relative CD95 expression on the total B-cell population.

In summary, this subset expressed CD126 at levels close to that of the resting memory subset, while the other markers of activation used in this study were not significantly changed by HIV infection. Of the markers of inhibition used in this study, CD72 was consistently expressed significantly on all subsets except for resting memory B-cells, and the mature naïve B-cell subset had the highest expression of all (77.49±23.45%), although the percentage difference between the HIV+^{ve} and HIV-^{ve} groups was the second highest at 41.51% (compared to 59.33% with tissue-like memory B-cells). CD307d was expressed a low levels on this subset (<10%), although this was significantly higher than that of the HIV-^{ve} group (<5%).

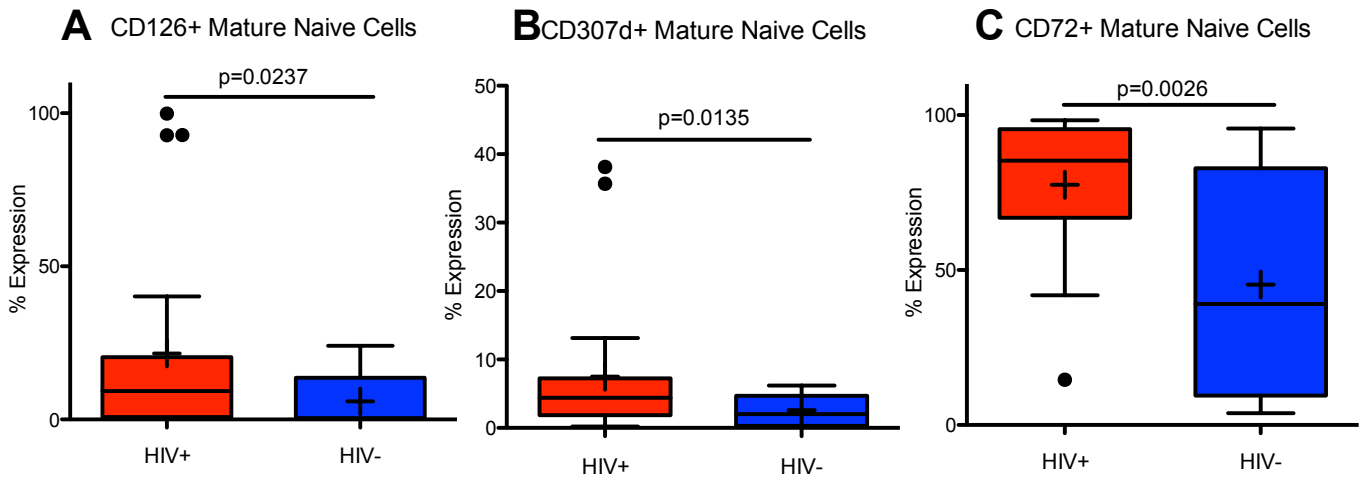


Fig 3.14: Ex vivo activation (CD126) and inhibitory (CD72 and CD307d) marker expression on mature naïve B-cells*. A) CD126 expression was increased on mature naïve B-cells in the HIV+ group (21.56±32.25%) compared to the HIV- control (5.91±7.83%; p=0.023). B) CD307d expression on mature naïve B-cells in the HIV+ group was increased compared to the HIV- control group (7.49±10.05% vs. 2.58±2.27%; p=0.0135). C) CD72 expression on tissue-like memory B-cells was increased in the HIV+ group relative to that of the HIV- controls (77.49±23.45% vs. 45.32±36.02%; p=0.0026)*

3.4 Effect of TLR Stimulation on the Total B-cell Population and Individual Subsets, and the Subsequent Impact of VIP Inhibition

This section details the results of the TLR stimulation (with either LPS, or R848), and the impact of VIP on stimulation-induced changes. These experiments were performed in order to assess whether B-cells from HIV+^{ve} and HIV-^{ve} individuals responded differently to either of the two innate stimuli as measured by expression of the above-mentioned markers of activation, inhibition, exhaustion, and apoptosis signaling. VIP was examined as a putative inhibitor of stimulation events in order to assess its potential as a selective inhibitor of cellular activation. Interestingly, even though CD284 (LPS TLR) expression was relatively high on resting memory cells *ex vivo* compared to CD287 (R848 TLR) on the same subset (Fig. 3.10 D), on the whole R848 elicited a far greater response than LPS. As a result the majority of the data described are based on R848 stimulation.

Marker expression and subset distribution *ex vivo* was compared to that of the isolated cells, both immediately after isolation (data not shown), and after 18h unstimulated culture (Appendix A, Table 1). B-cells from HIV-^{ve} individuals as a whole were not significantly altered by either isolation, or unstimulated culture, where marker expression throughout remained essentially unchanged when compared to *ex vivo* expression. B-cells from HIV+^{ve} individuals also did not have significantly altered expression post-isolation, but had some significant alterations after 18h unstimulated culture. This important finding gives an early indication that B-cells from HIV+^{ve} individuals are extremely sensitive to stimulation and may be pre-primed *in vivo*. [See Appendix A for full list of tables showing the differences in marker expression between the HIV+^{ve} and HIV-^{ve} groups for each subset in response to TLR stimulation and VIP inhibition.

Table 3.2. Summary results of response activation, inhibition, exhaustion, and apoptosis signaling markers to R848 stimulation, and to VIP inhibition. Data is represented as a significant increase (↑) or decrease (↓), or non-significant change (O). The number of arrows indicates the significance level, where one arrow - $p<0.05$; two arrows - $p<0.01$; and three arrows - $p<0.001$.

	Total B		Amem		Rmem		TLMem		MN	
	R848	VIP	R848	VIP	R848	VIP	R848	VIP	R848	VIP
Activation										
CD25	↑	O	O	O	O	O	↑↑	O	↑↑	↓↓
CD38	↑	↓	O	O	O	O	O	O	O	↓↓
CD86	↑↑	↓↓	↑↑	↓↓	↑↑↑	↓	↑↑	↓	↑	↓↓
CD126	O	O	O	O	↑	O	O	O	O	O
TLR										
CD284	O	↓	O	↓	O	O	O	O	O	↓
CD287	↑	↓	↑	O	O	O	O	↓	↑	O
Inhibition										
CD72	↑	↓↓	O	↓↓	O	O	↑	↓↓	O	O
CD85j	O	↓	O	O	O	O	O	O	↑↑↑	↓↓
CD300a	O	O	O	O	O	O	O	O	O	O
CD305	O	O	O	O	O	O	O	O	O	O
CD307d	O	↓	↑↑	O	↑↑↑	O	↑↑	O	↑↑↑	↓
Apoptosis Signaling										
CD95	↑↑↑	↓	↑	↓	↑↑↑	O	↑↑	↓↓	↑↑	↓

3.4.1 Total B-cell Population

3.4.1.1 Activation marker, VPAC2, and toll-like receptor expression

Total B-cell CD38 expression increased by 7.02% from 45.43±13.37% at baseline to 48.62±21.78% with R848 stimulation ($p<0.05$; Fig. 3.14 A), and interestingly, presence of VIP resulted in a further 21.76% decrease in total B-cell CD38 expression to that of 59.20±10.15% ($p<0.05$), which is also significantly higher than that of the baseline ($p<0.05$). CD38 expression was also not significantly altered by HIV infection on the total B-cell population *ex vivo*.

Total B-cell CD86 expression increased from 34.99±28.83% in the unstimulated control to 46.11±32.88% ($p<0.01$; Fig. 3.14 B) with R848 stimulation. Addition of VIP resulted in a 38.3% decrease ($p<0.05$) in CD86 expression to 28.45±19.37%, which was also significantly below that of the baseline ($p<0.01$). This is in stark contrast to the response of CD38 to VIP, which resulted in an increased expression. Increased CD38 expression is linked to increased movement of intracellular Ca^{2+}, while CD86 expression is involved in T-cell co-

stimulation, thereby indicating that even though both are markers of activation; they have independent downstream events to TLR stimulation.

Total B-cell CD25 expression was low *ex vivo* (<5%), but was significantly up-regulated with R848 stimulation, compared to the unstimulated control (9.65±8.57% vs. 22.17±22.69%, $p<0.05$), and although there was slight down-regulation in the presence of VIP, the results were not statistically significant. Activation marker expression of the HIV-^{ve} total B-cell population was not significantly changed by either TLR stimulation, or VIP inhibition.

Neither LPS, nor R848 stimulation significantly altered CD126, or CD70 expression on the total B-cell population within the HIV+ group. CD70 expression remained non-significantly altered in all subsets in all conditions.

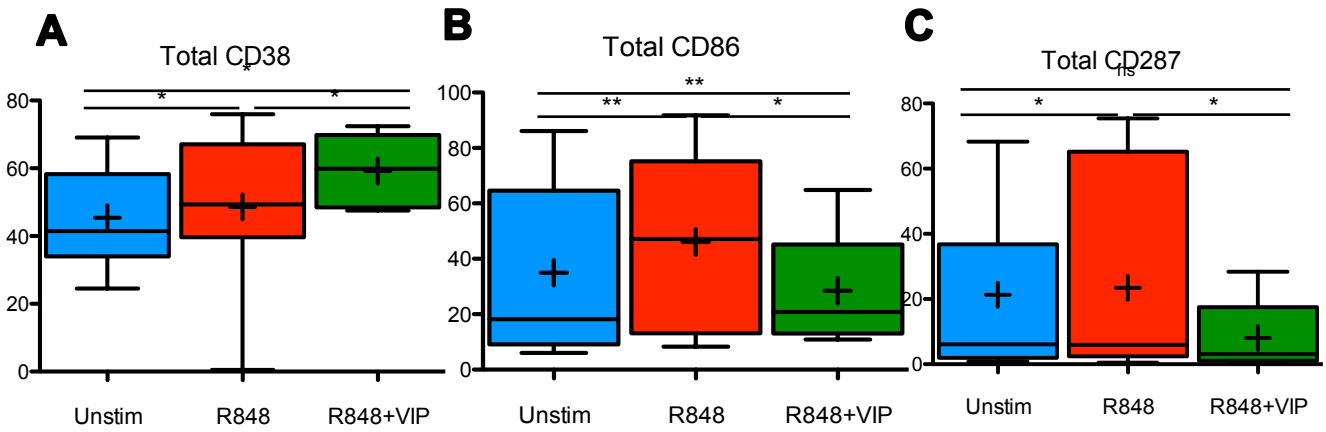


Fig. 3.14: Total B-cell activation marker (CD38 and CD86), and TLR 7 expression after R848 stimulation, and the impact of VIP *A) Total B-cell CD38 expression was raised upon R848 stimulation ($p<0.05$), further up-regulated with VIP inhibition ($p<0.05$). B) Total B-cell expression of CD86 was increased upon R848 stimulation ($p<0.01$), and significantly decreased upon VIP inhibition ($p<0.05$). C) Total B-cell expression of CD287 was increased upon R848 stimulation ($p<0.05$), and significantly decreased upon VIP inhibition ($p<0.05$).*

CD287 expression on the total B-cell population was raised significantly from 21.35±25.79% at baseline, to 23.43±31.43% with R848 stimulation ($p<0.05$), while there was no significant change with LPS stimulation. VIP was an effective inhibitor of CD287 expression on the total population, where inhibition induced a down-regulation of CD287 expression by 59.23% (to a mean of 8.06±11.59%; $p<0.05$, Fig. 3.14 C).

There was no significant change in CD284 expression on the total B-cell population with TLR stimulation. However, similar to CD287 expression, VIP was also an effective inhibitor of enhanced CD284 expression by R848. Expression decreased from 71.56±33.52% to 48.45±33.24% ($p<0.05$)

VPAC2 expression was only measured on the total B-cell population, due to limitations in fluorochrome-conjugated antibody availability. There was no significant change in VPAC2 expression with TLR stimulation, and also no significant VIP inhibition of VPAC2 expression. There was also no significant difference in VPAC2 expression on the total B-cell population between the HIV+ and HIV- groups in any of the stimulation or inhibition conditions.

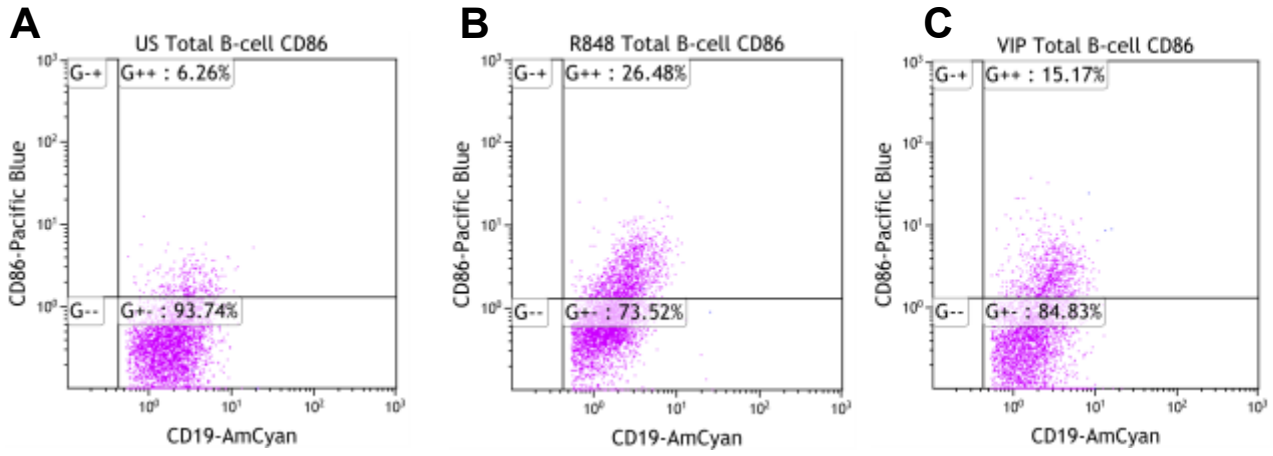


Fig. 3.15. Representative flow cytometry dotplots showing total CD86 expression at baseline, with R848 stimulation, and upon VIP inhibition. *A) Total CD86 expression on HIV+^{ve} patient P168 at unstimulated (US) baseline (6.26%, quadrant G++). B) Total CD86 expression on the same patient was 26.48% with R848 stimulation (G++). C). CD86 expression on the same patient after VIP inhibition was 15.17% (G++).*

3.4.1.2 Inhibitory receptors (CD72, CD85j, CD305, CD300a, CD307d), and a marker associated with apoptosis (CD95)

Total B-cell expression of CD72 was raised in the HIV+^{ve} group *ex vivo,* interestingly, it was significantly down-regulated by ~ 17% with TLR (only LPS) stimulation (from 21.81±15.07% to 18.10±14.33%, $p<0.05$), and underwent a further 59.03% decrease in presence of VIP inhibition to 7.41±6.57% ($p<0.01$). VIP thus successfully inhibited CD72 expression on the total B-cell population, to levels below 10% (8.23±6.77%), which was also significantly lower than that of the baseline ($p<0.01$, Fig. 3.16 D).

Neither CD300a, nor CD305 expression on the total B-cell population were significantly altered by HIV infection *ex vivo,* nor was there a response to TLR stimulation or VIP inhibition. TLR stimulation was also unable to elicit any significant changes in CD85j expression on the total B-cell population, although it was significantly downregulated with VIP inhibition ($p<0.05$, Fig. 3.16 A).

Ex vivo expression of CD307d on the total B-cell population was not significantly altered by HIV infection, nor was it significantly altered by TLR stimulation or VIP inhibition (Fig. 3.16 C). While there were no significant differences *ex vivo*, potential sensitivity to apoptosis/apoptosis signaling, as measured by CD95 expression, was increased by 19.35% to 51.37±35.91% with R848 stimulation ($p<0.001$). Furthermore, apoptosis signaling decreased by 63.27%, to 18.87±8.19% after VIP inhibition ($p<0.05$). This was also significantly lower than the baseline of 43.04±31.45% ($p<0.05$). B-cell apoptosis signaling in the HIV-^{ve} group was also not responsive to either TLR stimulation, or VIP inhibition, although the levels of CD95 expression were consistently <14% (lower than the VIP-inhibited HIV+^{ve} group, $p<0.05$, Fig. 3.16 B). The fact that CD95 was only upregulated in HIV+^{ve} individuals (and not HIV-^{ve} controls) indicates that innate stimulation may play a very important role in driving Fas-mediated apoptosis signaling (in a manner similar to that found in CD4^{+} T-cells).

Expression of both activation, and inhibitory markers on the total B-cell population in general showed the best stimulation-induced response to R848, with the exception of CD72, which responded to LPS, and not R848. CD70 expression on the total B-cell population was increased with R848 stimulation; however these levels were below 5%. CD38, CD86, and CD25 displayed significantly increased expression after R848 simulation, however VIP inhibition was only able to significantly down-regulate CD86 expression. CD287, and not CD284 expression was significantly up-regulated by R848 stimulation where approximately 25% of cells expressed CD287. VIP, however successfully inhibited expression of both CD287, and CD284 to levels below 10% and 50% respectively.

CD72 was the only marker of inhibition that was significantly altered by TLR stimulation, by which it was decreased, while VIP inhibition further decreased CD72 expression to levels well below the baseline. Of particular interest is the response of the apoptosis-signaling marker, CD95, which displayed a significant response to TLR stimulation in comparison to that of the HIV-^{ve} group; and also its response to VIP inhibition, which resulted in expression only approximately 20% greater than the HIV-^{ve} group.

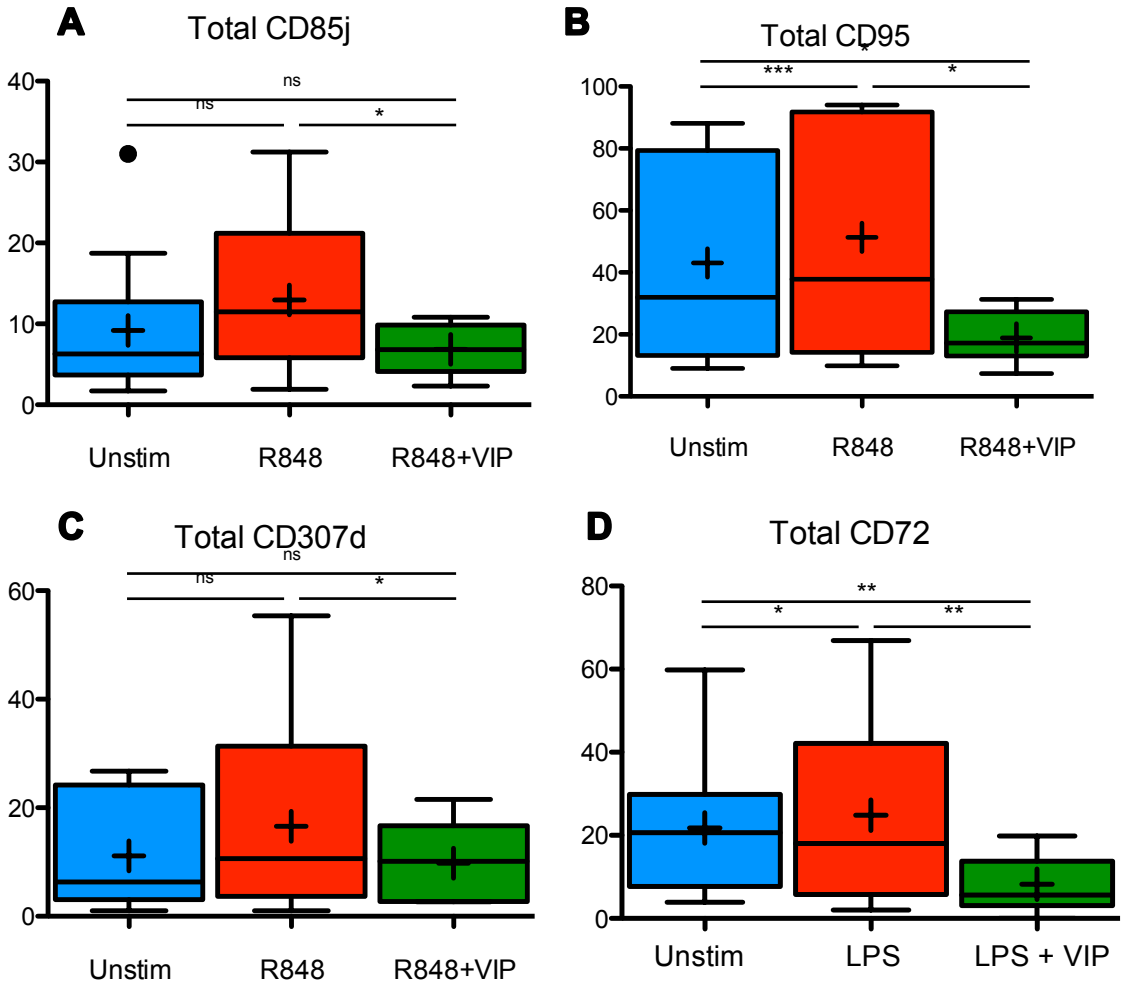


Fig. 3.16: Significantly altered expression of markers of inhibition (CD85j, CD72), exhaustion (CD307d) and apoptosis signaling (CD95) on the total HIV+^{ve} B-cell population after TLR stimulation and VIP inhibition*. A) Total B-cell CD85j expression on the total B-cell population was not significantly altered by R848 stimulation, however VIP inhibition resulted in a significant down-regulation in CD85j expression relative to the stimulated cells ($p<0.05$). B CD95 expression on the total B-cell population was significantly raised upon R848 stimulation ($p<0.001$), while VIP inhibition resulted in a near complete down-regulation of CD95 expression ($p<0.05$). C) Total B-cell CD307d expression was not significantly altered by R848 stimulation, however VIP inhibition resulted in a significant down-regulation in expression relative to the stimulated conditions ($p<0.05$). D) Total B-cell CD72 expression was raised upon R848 stimulation ($p<0.05$), and further decreased upon VIP inhibition ($p<0.01$).*

3.4.2 Activated Memory B-cells

Activated memory B-cells in the HIV+^{ve} group expressed increased levels of CD38, and decreased levels of CD86 compared to the HIV-^{ve} group *ex vivo*. There was no significant change in other markers of activation with this subset. CD72 and CD307d expression was increased relative to that of the HIV-^{ve} group, while CD300a and CD305 had decreased expression, and CD95 expression was not significantly altered by HIV infection. See below for summary table of results for AMem B-cells response to R848 stimulation, and VIP inhibition.

Table 3.3 Summary results of marker percentage change with R848 stimulation and VIP inhibition, relative to baseline unstimulated conditions on activated memory B-cells. *Negative percentage indicates percentage decrease; * = p<0.05, ** = p<0.01, ns = not significant. Significant data further indicated in bold.*

	R848-induced % change		VIP-induced Inhibition	
Activation		p value		p value
CD25	120,7%	ns	-81,2%	ns
CD38	13,57%	ns	-1,0%	ns
CD86	**42,2%**	**	**-46,4%**	**
CD126	1,3%	ns	-15,5%	ns
TLR				
CD284	3,1%	ns	**-28,6%**	*
CD287	**18,5%**	*	-62,8%	ns
Inhibition				
CD72	5,2%	ns	**-70,2%**	**
CD85j	22,9%	ns	-44,9%	ns
CD300a	9,4%	ns	-29,6%	ns
CD305	-8,4%	ns	-17,0%	ns
CD307d	**25,3%**	**	-36,6%	ns
Apoptosis Signalling				
CD95	**21,7%**	*	**-74,86%**	*

3.4.3 Resting Memory B-cells

Resting memory B-cells had an interesting expression pattern *ex vivo*, where there was significant up-regulation of activation markers with HIV infection, while inhibitory and apoptosis signaling markers were not significantly altered with HIV infection. CD126 was significantly increased by 60%, while CD284 was increased by roughly 25% on resting memory B-cells in the HIV+^{ve} group *ex vivo* (p=0.0145, and p=0.0057), respectively. CD287 expression was increased to levels above 10% with HIV infection (compared to <5%, p=0.0199). CD25 expression was decreased with HIV infection, however these levels were very low (both <5%, p=0.0017). See below for summary table of results for RMem B-cells response to R848 stimulation, and VIP inhibition.

Table 3.4 Summary results of marker percentage change with R848 stimulation and VIP inhibition, relative to baseline unstimulated conditions on resting memory B-cells. *Negative percentage indicates percentage decrease; * = p<0.05, ** = p<0.01, *** = p>0.001, ns = not significant. Significant data further indicated in bold.*

	R848-induced % change		VIP-induced Inhibition	
Activation		p value		p value
CD25	159,6%	ns	-83,7%	ns
CD38	8,8%	ns	11,1%	ns
CD86	**56,1%**	**	**-55,3%**	*
CD126	**9,6%**	*	-27,2%	ns
TLR				
CD284	-7,9%	ns	-19,9%	ns
CD287	19,3%	ns	-74,8%	ns
Inhibition				
CD72	-0,7%	ns	-62,3%	ns
CD85j	41,3%	ns	-61,4%	ns
CD300a	5,3%	ns	-19,0%	ns
CD305	2,5%	ns	-11,0%	ns
CD307d	**41,7%**	***	-14,7%	ns
Apoptosis Signalling				
CD95	**31,1%**	***	-64,0%	ns

3.4.4 Tissue-like Memory B-cells

The overall phenotypic profile of this subset *ex vivo* showed a tendency towards being inhibitory in nature, with up-regulated expression of both CD72 (1.46-fold, p<0.0001), and CD85j (1.73-fold, p=0.046). Tissue-like memory B-cells also had increased evidence of viral stimulation, due to an increase in TLR7 (CD287) expression (6.42-fold, p=0.0128), as well as a tendency towards being functionally exhausted due to up-regulated CD307d expression (72.33% increase relative to the HIV-^{ve} group, p=0.045).

3.4.4.1 Expression of activation markers (CD25, CD38, CD70, CD86, & CD126), and toll-like receptor (CD284 & CD287.) expression

CD86 expression on tissue-like memory cells in the HIV+ group increased from 27.39±23.16% to 34.84±25.56% with R848 stimulation (p<0.01), and decreased by 44.49% with VIP inhibition to 19.34±7.97% (p<0.05). CD86 expression after VIP inhibition was also significantly lower than that of baseline (p<0.01; Fig. 3.17 B). This expression pattern is in concert with that of the total B-cell population where there was an increase in expression with R848 stimulation, and a further decrease with VIP inhibition.

CD25 expression was increased on tissue-like memory cells with TLR stimulation in the HIV+ group (5.33±6.00% vs. 16.25±17.92%, p<0.01), and VIP down-regulated expression

(p<0.01) to levels similar to that of the baseline (1.98±1.63; Fig. 3.17 A). This is consistent with the pattern of CD25 expression on both the resting memory subset, as well as the total B-cell population, where although VIP inhibition did not decrease expression significantly, there was a definite trend noted.

Interestingly, CD287 expression on tissue-like memory cells was not significantly changed by TLR stimulation, however there was a significant down-regulation in its expression with VIP inhibition, from 9.69±14.32% (R848-stimulated) to essentially complete abrogation of expression (1.08±1.18%; p<0.05).

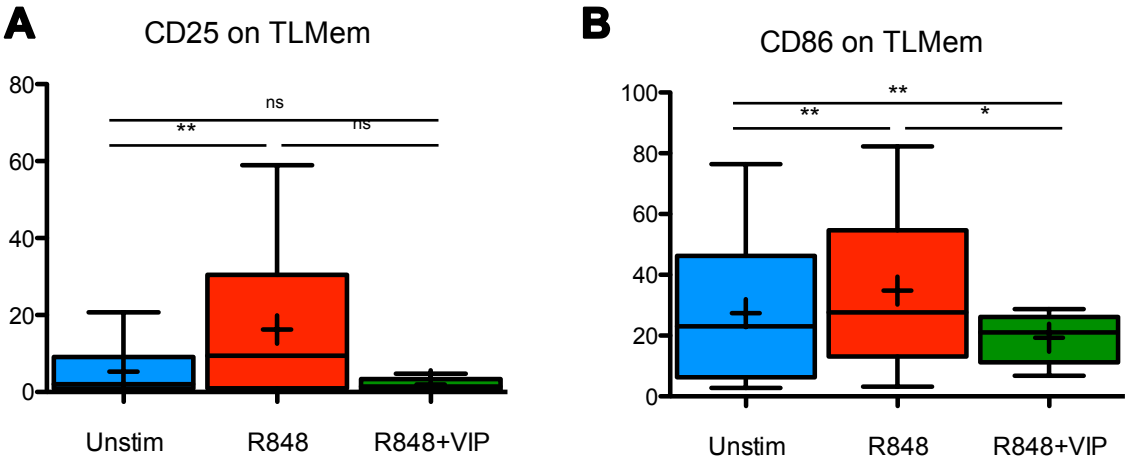


Fig. 3.17: Activation marker (CD25 and CD86) expression on tissue-like memory B-cells*. A) CD25 expression on TLMem B-cells was raised significantly by R848 stimulation (p<0.01), while VIP inhibition resulted in expression not significantly different to that of the un-stimulated baseline. B) CD86 expression was significantly raised on TLMem B-cells upon R848 stimulation (p<0.01), and subsequently decreased with VIP inhibition, to levels significantly lower than that of the un-stimulated baseline (p<0.01).*

CD126, CD38, and CD284 expression on tissue-like memory cells was slightly raised with R848 stimulation, and lowered with VIP inhibition, but these were not statistically significant. CD70 was not expressed on tissue-like memory cells, and no subsequent alterations were noted with TLR stimulation or VIP inhibition

3.4.4.2 Inhibitory markers (CD72, CD85j, CD300a, CD305, & CD307d.) and CD95

CD72 expression on tissue-like memory cells was significantly altered by R848 stimulation in the HIV+ group, where expression was raised by 51.55% from 9.54±9.35% at baseline, to 14.45±14.12% with R848 stimulation (p<0.05; Fig. 3.18 A). VIP inhibition resulted in a decrease in CD72 expression to 2.16±2.30% (p<0.01), which was also significantly lower

than baseline of 9.54±9.35% ($p<0.01$). CD72 was not expressed on tissue-like memory B-cells in the HIV-^{ve} group

In a manner consistent with the other subsets, CD95 expression on tissue-like memory cells was significantly raised with TLR stimulation, from 38.68±32.55% at baseline, to 46.98±37.26% ($p<0.01$, Fig. 3.18 B). Inhibition with VIP resulted in a mean CD95 expression that was 66.47% below baseline, and 72.39% below that of the TLR-stimulated cells (both $p<0.01$). CD95 expression on this subset in the HIV-^{ve} group was unaffected by both TLR stimulation, and VIP inhibition (Appendix A, Table 10).

CD307d expression is one of the hallmark features of this subset, and while minimal expression was noted *ex vivo* (12.93±10.32%), there was no significant change with overnight culture (11.30±6.96, Fig. 3.18 C). Furthermore, this subset had the second-lowest expression of CD307d at baseline (mature naïve B-cells had the lowest expression at baseline). There was an increase in CD307d expression upon TLR stimulation (to 14.96±8.85; $p<0.01$), however, there was no significant alteration after VIP inhibition.

CD300a, Cd305, and CD85j expression in the HIV+^{ve} group remained unchanged with both TLR stimulation and VIP inhibition and VIP inhibition.

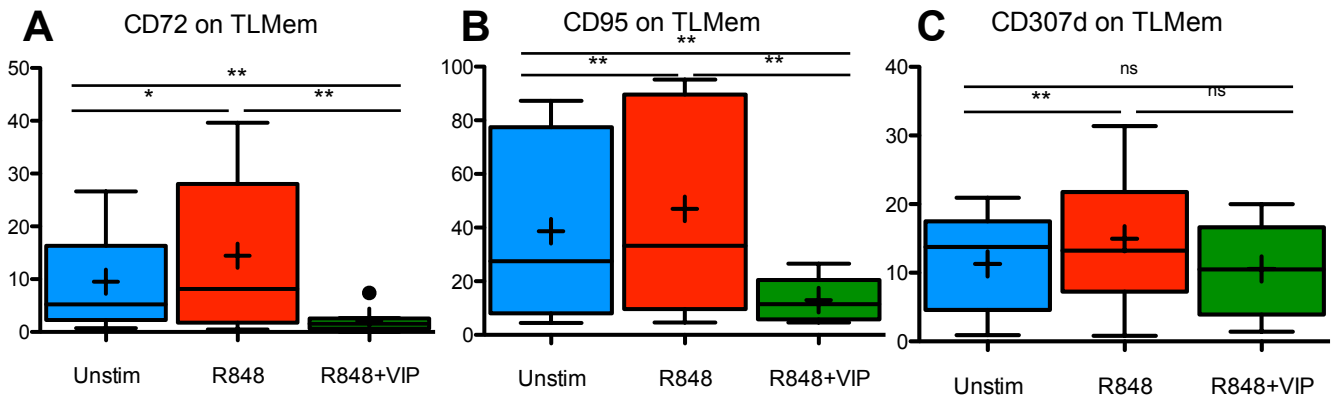


Fig. 3.18: Inhibitory (CD72), exhaustive (CD307d), and apoptosis-signaling (CD95) marker expression on tissue-like memory B-cells after R848 stimulation, and VIP inhibition*. A) CD72 expression was up-regulated upon R848 stimulation ($p=0.05$), and further down-regulated upon VIP inhibition to levels below that of the un-stimulated baseline ($p<0.01$). B) CD95 expression on TLMem B-cells was significantly raised by R848 stimulation ($p<0.01$), and further decreased to levels significantly lower than the un-stimulated baseline ($p<0.01$). C) CD307d expression was up-regulated upon R848 stimulation ($p<0.01$), while VIP inhibition resulted in expression that was not significantly different from that of the un-stimulated baseline.*

3.4.5 Mature Naïve B-cells

Mature naïve B-cells had an *ex vivo* profile that was surprisingly varied, where markers of activation, inhibition, and exhaustion were up-regulated with HIV infection. CD126, the IL-6 receptor was up-regulated 2.65-fold in the HIV+^{ve} group (p=0.0237), while CD72 was up-regulated by 70.98% with HIV infection (p=0.0026), and CD307d up-regulated 1.90-fold (p=0.0135). These cells are cognate antigen naïve, and as such should express low levels of both activation, and inhibitory markers *ex vivo*, which indicates that there is possibly due to increased innate antigenic stimulation with HIV infection.

3.4.5.1 Expression of activation markers (CD25, CD38, CD70, CD86, & CD126), and toll-like receptor (CD284 & CD287.) expression

CD86, a marker of T- cell co-stimulation, and therefore activation, had the lowest expression of all subsets on mature naïve B-cells, although there was a response to TLR stimulation, where expression increased from 13.57±14.83% at baseline, to 22.59±21.88% (p<0.05; Fig. 3.19 A). Subsequent VIP inhibition resulted in expression of 8.82±9.78%, which is significantly lower than both the TLR stimulated-, and baseline conditions (both p<0.01).

Interestingly, CD38 expression had the highest expression of all subsets, on mature naïve B-cells. While there was no significant change with TLR stimulation, VIP inhibition resulted in a significant decrease (albeit minor) in expression compared to both the TLR-stimulated (61.73±22.19% vs. 60.94±21.49%, p<0.01), and baseline conditions (63.46±22.17% vs. 60.94±21.49%, p<0.05; Fig. 3.19 B).

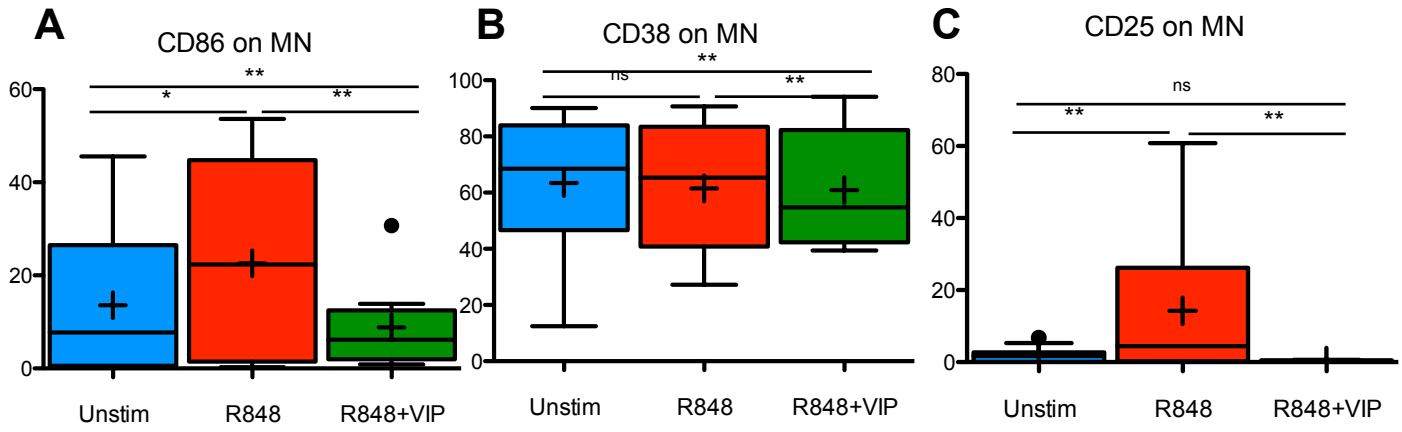


Fig. 3.19: Activation marker (CD86, CD38, CD25) expression on mature naïve B-cells after R848 stimulation, and VIP inhibition*. A) CD86 expression was raised with R848 stimulation (p<0.05), and subsequently decreased to levels below that of the un-stimulated baseline with VIP inhibition (p<0.01). B) CD38 expression on MN B-cells was not significantly raised by R848 stimulation, and decreased to levels significantly different from the un-stimulated baseline (p<0.01). C) CD25 expression was increased 14-fold R848 stimulation (p<0.01), while VIP inhibition resulted in a CD25 expression that was not significantly different to that of the un-stimulated baseline.*

CD25 was essentially not expressed at baseline, and responded significantly to TLR stimulation with an increase from 1.81±2.11% to 14.27±19.09% ($p<0.01$; Fig. 3.19 C). Similarly, mean expression decreased to 0.27±0.24% with VIP ($p<0.01$). This trend in CD25 expression was noted throughout all subsets except for activated memory B-cells, where there was no statistically significant change in CD25 expression.

Expression of CD287 was up-regulated by 53.72% on mature naïve B-cells with TLR stimulation, from baseline of 12.77±18.67%, to 19.63±27.05% ($p<0.05$), however there was no change with VIP inhibition even though a definite downward trend was noted (possibly owing to the large standard deviation indicating a vast range of data points). While CD284 expression was not significantly altered by TLR stimulation alone, it was however significantly down-regulated in both cases by VIP (with LPS) from 63.69±28.48% to 30.57±29.96% ($p<0.05$) and (with R848) 67.23±36.92% to 37.23±31.66% ($p<0.05$).

3.4.5.2 Inhibitory markers (CD72, CD85j, CD300a, CD305, & CD307d.) and CD95

In a manner similar to that noted with CD25 expression, CD85j expression on mature naïve B-cells, was extremely sensitive to TLR stimulation, with an increase from 1.81±2.42% expression at baseline, to 46.98±37.26% with R848 stimulation, representing a >24-fold increase ($p<0.001$; Fig. 3.20 A). Inhibition with VIP resulted in a significant down-regulation of expression ($p<0.01$) to levels not significantly different from that of baseline. Interestingly, CD85j expression on the HIV-^{ve} group responded in a similar manner, although with all levels <10% (Appendix A, Table 12). CD72 expression on mature naïve B-cells was not significantly altered by TLR stimulation, which is consistent with the findings in the activated memory B-cell subset. However, VIP inhibition resulted in a significant down-regulation of CD72 expression from 38.56±25.58% at baseline to 11.93±9.05% ($p<0.01$; Fig. 3.20 B).

CD307d had the lowest expression levels in the mature naïve B-cell subset, and TLR stimulation resulted in an up-regulation in CD307d expression on mature naïve B-cells from 5.95±4.50% at baseline, to 11.05±6.71% ($p<0.001$; Fig. 3.20 D), while expression was significantly down-regulated by VIP inhibition (to 7.17±4.63%, $p<0.05$).

As expected, CD95 expression was lowest on this subset at baseline, and experienced the lowest mean expression with TLR stimulation. Expression of CD95 on mature naïve B-cells was significantly raised by 39.93% with R848 stimulation, from 21.82±25.15% at baseline, to 30.53±33.70% ($p<0.01$), while VIP inhibition resulted in a mean CD95 expression that was 86.21% below baseline, and 90.15% below that of the TLR-stimulated cells (both $p<0.05$; Fig. 3.20 C), which can be considered a near-complete abrogation. As with the majority of

markers on the HIV-^{ve} subset, there was no significant change in expression with TLR stimulation, and VIP inhibition, and expression in the HIV+^{ve} group was significantly higher in all conditions (Appendix A, Table 12).

In a manner consistent with expression patterns of the previous subsets, both CD305, CD300a expression on this subset was not significantly changed by either TLR stimulation, or VIP inhibition.

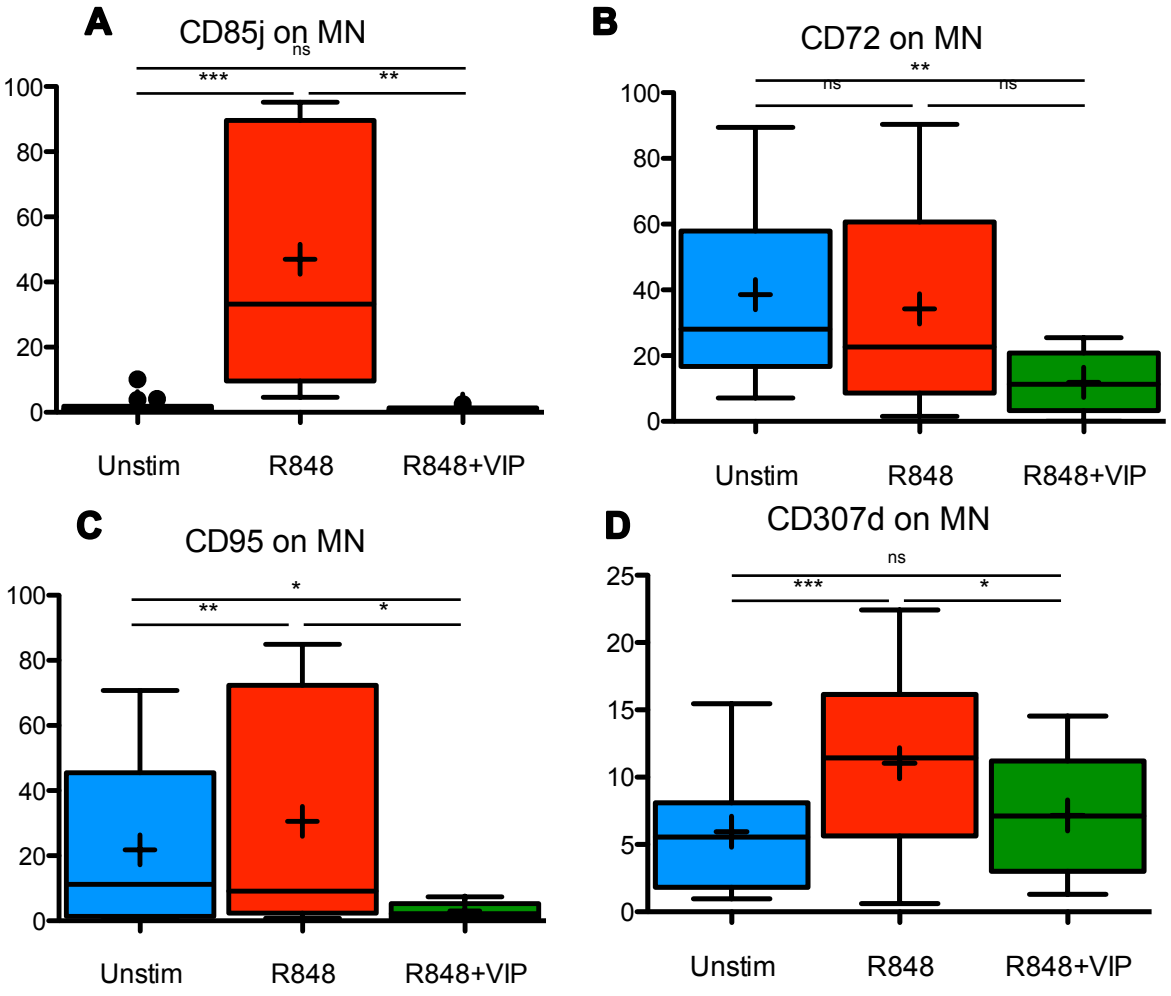


Fig. 3.20: Inhibitory (CD85j, CD72), and exhaustion marker (CD307d), and Fas (CD95) expression on mature naïve B-cells after R848 stimulation, and VIP inhibition*. A) CD85j expression was raised approximately 40-fold with R848 stimulation (p<0.001), and decreased back to baseline after VIP inhibition. B) CD72 expression on MN B-cells was not significantly changed by R848 stimulation, but decreased to levels 69% below that of the un-stimulated baseline (p<0.01). C) Stimulation with R848 resulted in a 40% increase in CD95 expression, while VIP inhibition resulted in <5% CD95 expression (p<0.05). D) CD307d expression on MN B-cells was increased approx. 2-fold with R848 stimulation, while VIP inhibition significantly down-regulated expression to levels not significantly different from that of the un-stimulated baseline.*

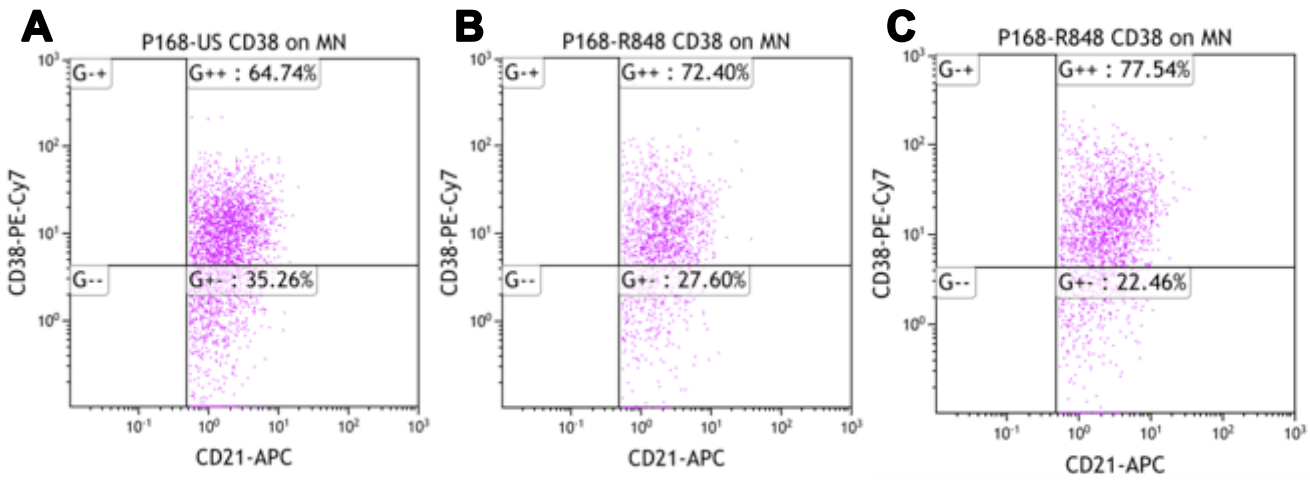


Fig. 3.21 CD38 expression at baseline, and with both R848 stimulation, and VIP inhibition *CD38 expression was greatest on this subset, and was not very responsive to VIP inhibition A) Baseline CD38 expression on MN B-cells on HIV+^{ve} patient P168 was 64.74%. B) R848 stimulation increased expression of CD38 to 72.40%, although this was not significant. C) VIP inhibition further increased CD38 expression on this subset to 77.54%, although the average expression of all the patients in this study had slightly decreased CD38 expression with VIP inhibition (60.94±21.49%).*

Chapter 4 Discussion

4.1 Whole Blood *Ex Vivo* phenotyping assay

HIV+^{ve} individuals have significantly higher baseline activation levels than HIV-^{ve} controls

Previous studies have shown a high incidence of B-cell abnormalities with HIV infection, namely hypergammaglobulinemia, polyclonal activation, and poor response to both neo- and recall antigen (Penesieroso, *et al*., 2013; Sciaranghella, *et al*., 2013; Shen & Tomaras, 2011). In this study, we assessed the B-cells of asymptomatic HIV+^{ve} individuals in order to ascertain their activation and inhibitory/exhaustive status both *ex vivo*, and after *in vitro* stimulation. The majority of studies have assessed B-cell function in end-stage AIDS, where all compartments of the immune system are dysfunctional, we therefore hypothesized that our study cohort, with mean CD4 counts of 472.3 cells/μL, would still have a relatively well-preserved immune system, allowing us to assess the impact of asymptomatic HIV infection on the B-cell compartment, with regard to cellular markers of activation, and inhibition/exhaustion. Several authors have also reported HIV-associated aberrant B-cell activation in asymptomatic HIV-infection (Shen & Tomaras, 2011; Moir, *et al*. 2008, 2010).

Our HIV+^{ve} patient cohort did however have increased levels of both CD38, and PD-1 expression on CD8 T-cells (46.25±22.94% vs. 28.33±17.95%; 31.13±19.79% vs. 18.29±12.64%, both $p<0.0001$), indicating that although their immune system could be described as “robust” based on CD4 count, there are abnormalities which suggests a significantly more activated state with corresponding levels of ‘immune exhaustion’. CD38 expression on CD8 T-cells is an indirect means of measuring immune activity against viral antigens, and as such has proven to be a useful measure of systemic immune activation (Sauce, *et al*., 2013). CD38 expression on CD8 T-cells (CD38/8) also correlated positively with HIV plasma viral load ($r=0.5147$, $p=0.041$), and negatively with CD4 count ($r=-0.4546$, $p=0.038$), thereby linking increased HIV plasma viral load with increased immune activation levels (CD38/8), and showing the link between immune activation and a decreased CD4 count

The aim of the *ex vivo* phenotyping was to determine whether there was a difference in the *ex vivo* expression of these markers between HIV+^{ve} individuals, and HIV-^{ve} controls. Statistics performed on the demographic data of the HIV+^{ve} and control samples showed no significant difference in age and sex distributions. *Ex vivo* activation, exhaustion, or inhibitory

marker expression of each of the various B-cell subsets was measured by multicolour flow cytometry, and evaluated on the entire B-cell population as well as on each of peripheral blood memory B-cell populations. The results demonstrated that B-cells from individuals with asymptomatic chronic HIV-1 infection have significantly higher and deregulated *ex vivo* activation, and inhibitory marker expression across all subsets. It was also found that there was a general increase in expression of inhibitory markers on activated memory (AMem) and tissue-like memory (TLMem) cells in these individuals. In addition, the putative marker of exhaustion, CD307d had the highest expression on AMem cells and to a lesser extent, on TLMem, and mature naïve (MN) B-cells. Lastly, we assessed *ex vivo* expression of the immunomodulatory receptor, VPAC2, the receptor for vasoactive intestinal peptide and found slightly decreased total expression in the HIV+^{ve} group.

4.1.1 Subset Distribution

Increased "exhausted" B-cells, and a decreased proportion of quiescent cells in chronic HIV infection

Previous studies have noted several perturbations within the B-cell compartment of HIV infected individuals (Moir & Fauci, 2008). Notably, there are disturbances in both the number of circulating memory B-cells and in the total number of circulating B-cells (Titanji, *et al.*, 2006). There have been differing opinions on whether the number of circulating B-cells is decreased or not with HIV infection, with Fogli, *et al.* (2012) reporting that there was no difference between the median B-cell counts of the HIV+^{ve} or HIV-^{ve} groups (129.5 vs. 143 cells/µl respectively). Moir, *et al.* (2008), however reported a significant reduction in median absolute B-cell count with untreated HIV infection (171 vs. 270 cells/µl, $p<0.0001$).

In concurrence with the study by Moir, the present study found a decreased absolute B-cell count (188.6±181.3 vs. 276.8±141.8 cells/µl, $p=0.0002$; Table 3.1). There was a significant reduction in the proportion of RMem B-cells (17.78±7.54% vs. 23.01±8.65%, $p=0.0481$), and a significant increase in the proportion of the putative "exhausted phenotype" TLMem B-cells in the HIV+^{ve} group (24.57±14.84% vs. 15.43±10.39%, $p=0.0314$ respectively). There was no significant difference in the distribution of any of the other peripheral blood B-cells, although the percentage of AMem B-cells correlated positively with PD-1 expression on CD8 T-cells, highlighting a possible link between T-cell exhaustion, and B-cell activation and exhaustion. Absolute CD4 count correlated positively with the percentage of resting memory (RMem) B-cells ($r=0.4533$, $p=0.039$), which means that either resting memory B-cells are being driven into the activated memory pool, or they are dying via apoptosis, thereby

correlating with loss of $CD4^+$ T-cells, which is driven by T-cell activation. This highlights a possible mechanistic link between CD4 T-cell loss and re-activation of RMem B-cells.

Interestingly, there was no significant difference in the number of $CD10^+$ transitional B-cells, which is a population that is essentially absent in healthy individuals, but expanded in lymphopenic diseases such as HIV infection (Ho, *et al*., 2006). This may be explained by the fact that the patients sampled for this study had relatively well-maintained CD4 counts (mean 427 cells/µl). Patients from the study by Ho, *et al.* (2006), with CD4 counts below 50 cells/µl had transitional B-cells that accounted for >60% of total B-cells. The decreased proportion of resting memory cells can be viewed as being a result of re-activation of this subset, which then become activated memory cells, and then due to chronic activation, become (functionally exhausted) TLMem cells (Penesieroso, *et al*., 2013). This is further explained by the fact that the percentage TLMem B-cells correlated negatively with the percentage of RMem B-cells ($r=-0.4844$, $p=0.26$), as well as the percentage of MN B-cells ($r=-0.7795$, $p<0.0001$).

As the proportions of MN, and RMem B-cells decrease, the proportion of TLMem B-cells increases, indicating the inherent link between the populations. Interestingly, the percentage of AMem B-cells correlated positively with the percentage TLMem B-cells ($r=0.6532$, $p=0.0013$), however, the percentage AMem also correlated negatively with the percentage MN ($r=-0.8457$, $p<0.0001$) which further strengthens the claim that MN B-cells become AMem B-cells upon activation, which in turn become RMem when the activation signals are down-regulated (Pensieroso, *et al*., 2013). The process of down-regulating activation signals does not occur with untreated HIV infection, and therefore RMem cells are re-activated, and MN cells become AMem, which in turn eventually become functionally-exhausted TLMem cells.

4.1.2 The State of the Total B-cell Population *ex vivo*

Variable changes in activation marker expression in the HIV+ total B-cell population is concomitant with increased inhibitory marker expression

Total CD25-expressing B-cells were decreased in the $HIV+^{ve}$ group, with 2.42±3.82% compared to 4.33±3.38% in the $HIV-^{ve}$ control ($p=0.0051$). The Il-2 receptor is up-regulated with increased IL-2 production, namely by co-stimulated T-cells (Sandoval-Montes & Santos-Argumedo, 2005). The down regulation of this activation marker is perhaps a result of the decreased number of $CD4^+$ T-cells in circulation, or possibly also as a means of downregulating response to IL-2, as is found in T-cells. van Grevenynghe, *et al*. (2011) previously described an IL-2 linked pathway of B-cell loss in (untreated) HIV infection,

although they defined memory B-cells as being $CD3^{+}CD19^{+}CD27^{+}$, and did not distinguish between AMem, and RMem B-cells, nor did they investigate the entire B-cell population. They showed a significant positive correlation between the proportion of memory B-cells and serum IL-2 levels ($r=0.5801$, $p<0.0001$), although there was no significant difference in CD25 expression on memory B-cells between $HIV+^{ve}$ subjects, and $HIV-^{ve}$ controls. However, the authors did note that interfering with the Foxo3a and TRAIL pathways (both of which are linked to IL-2 signaling) is a possible way of rescuing both B- and T-cell function during chronic HIV infection (van Grevenynghe, *et al.*, 2011).

While CD287 expression was not significantly different between the two groups, expression of CD284 was significantly raised on the $HIV+^{ve}$ group's total B-cell population (40.86±30.50% vs. 24.56±17.51%, $p=0.048$), which may be indicative of increased levels of systemic LPS, a direct consequence of HIV-associated enteropathy. Moir &Fauci (2009), however reported that B-cells do not express LPS receptors, although this may be referring to CD14, which is the so-called "classical" LPS receptor that acts as a co-receptor to TLR4. CD14 is only found on myeloid antigen-presenting cells such as monocytes and dendritic cells, and not B-cells (Murphy, *et al.*, 2008: pp.13). The data here may indicate an additional pathway of chronic B-cell stimulation, not previously described.

Total B-cells expressing CD72 was also increased in $HIV+^{ve}$ group (60.98±19.50% vs. 38.49±23.68%, $p=0.0038$). CD72 is expressed on all B-cell subsets except for plasma cells and both positively and negatively influences BCR signaling (Wu & Bondada, 2009). Adaci *et al.* (2000) found that CD72 constitutively down-regulates signaling via the BCR, and thereby establishes a threshold of signaling for B-cell activation. Several authors have reported elevated CD72 expression with HIV infection, however exact figures have not been published (Kardava, *et al.*, 2011; Silva, *et al.*, 2011; Moir & Fauci, 2014). Inhibitory receptors, also known as immunomodulatory receptors, are critical mediators of immune function, which, upon ligation, down-regulate activation signals in order to attenuate an immune response (Silva, *et al.*, 2011). In healthy individuals, cell surface receptors bearing cytoplasmic ITIMs, negatively regulate activation signals, however in diseases such as HIV, the persistent expression of inhibitory receptors can lead to decreased functionality, and even anergy and cellular exhaustion (Kardava, *et al.*, 2011).

4.1.3 VPAC2 expression on the total B-cell population

Vasoactive intestinal peptide is a neurohormone that is part of the glucagon/secretin family, whose receptors VPAC1 and VPAC2 are coupled to adenylyl cyclase, which when engaged down-regulates cellular production of pro-inflammatory cytokines (such as IL-2) and

activation markers (Pozo, *et al*., 2000). VPAC1&2 receptor expression has been well described on T-cells, however there has been very little published regarding B-cell VPAC1&2 expression (Gaena & Delgado, 2002). Previous studies have determined that murine B-cells (at rest, and LPS-stimulated) do not express either VPAC1 or VPAC2 (Lara-Marquez, *et* al., 2001). We determined, by use of FMO techniques that B-cells express low levels of VPAC2 *ex vivo*, with significantly down-regulated expression in the HIV+^{ve} group (1.07±0.77% vs. 2.26±2.22%, p=0.0296), and that VPAC1 expression was not detected (data not shown). We were unable to measure VPAC2 expression on the various B-cell subsets due to limitations in fluorochrome availability, and it is possible that even though there are low levels of the total B-cell population, there may be increased levels of expression on one of the memory B-cell subsets. Therefore it is difficult to infer whether this de-regulation of VPAC2 expression is a reflection of HIV-associated immune exhaustion, or if it is due to conflicting factors (such as under-, or over-represented populations making up the total B-cell compartment).

4.1.4 The State of Activated Memory B-Cells *ex vivo*

A higher proportion of AMem B-cells are terminally-differentiated in chronic HIV, but with slightly decreased co-stimulatory capacity

The HIV+^{ve} AMem subset had markedly increased CD38 expression (38.63±11.82% vs. 24.21±8.96%, p=0.0097), and slightly decreased CD86 expression (7.09±6.24% vs. 9.46±5.23%, p=0.048). CD38 is a well-described marker of both T-cell and B-cell activation, and in B-cells it is associated with the CD21/CD19 complex, and has been implicated in BCR-signaling (Sandoval-Montes & Santos-Argumedo, 2005). CD38 has also been identified as a marker of terminal differentiation in B-cells, which has been strongly associated with HIV viraemia (Moir & Fauci, 2009). CD86, also known as B7.2 is a marker of antigen presentation, and of early activation through T-cell co-stimulation (Pieper, Grimbacher, & Eibel, 2013). CD86 is up regulated when CD40 and MHC-peptide complexes on B-cells interact with CD40L and the TCR-CD4 complex respectively on T-cells. CD80 and CD86 interact with CD28 on T-cells, resulting in the production and release of pro-inflammatory cytokines such as Il-6 and TNF (Moir & Fauci. 2009).

One would expect an increase in CD86 expression in the HIV+^{ve} group, similar to that of CD38 due to the increased levels of T-cell activation; however CD86 was only up regulated upon antigen stimulation. It is known that the CD38 pathway is intrinsically different to that of the CD86 pathway: CD38 is up-regulated upon mature naïve B-cell activation and differentiation (Sandoval-Montes & Santos-Argumedo, 2005). Whereas, in support of the current study findings, Malaspina, *et al*. (2010) reported impaired co-stimulatory capacity in

the B-cells of HIV-viraemic individuals, manifest as a decreased expression of the CD80 & CD86 receptor pair.

Variable inhibitory receptor expression on AMem B-cells in the HIV+^{ve} group, but significant exhaustion marker CD307d expression

In the HIV+^{ve} activated memory subset, CD72 was found to be elevated (33.33±20.08%, vs. 20.74±17.66%, p=0.0396), while CD300a and CD305 had decreased expression in the HIV+^{ve} group (60.91±17.88% vs. 70.74±11.26%, p=0.048; & 19.67±10.17% vs. 29.89±13.64%, p=0.0213 respectively). Silva, *et al*. (2011) reported that CD300a expression is mostly absent on mature naïve B-cells, and variably-expressed on memory cell and plasmablast/cell subsets. The same authors also stated that CD305 expression is lowest on the memory subsets, and highest on mature naïve B-cells, and that CD307d is almost absent on mature naïve B-cells, and mainly expressed on the tissue-like memory subset (Silva, *et al.*, 2011). In the context of HIV-infection however, it was reported that markers like CD85j (which is mostly absent in healthy individuals), CD300a, and CD305 are aberrantly expressed with untreated infection and concomitant with the expansion of the exhausted tissue-like memory phenotype (Moir, *et al*., 2008).

The data in the present study supports that of Silva, *et al*. (2011), where CD300a and CD305 expression was down-regulated with HIV-infection. CD305 recognises collagen as its primary ligand, suggesting a potential role in regulation of tissue damage and inflammation (Meyaard, 2008). Furthermore, CD300a has recently been identified as a receptor for phosphatidylserine and phosphatidylethanolamine, which are both expressed on cell surface with cell death (Simhadri, *et al.*, 2012). Indeed, the fact that both CD305 and CD300a are down-regulated, while CD72 remains up-regulated, seems to be rather counter-intuitive, due to the increased inflammation and immune activation seen in HIV infection (Klatt, *et* al., 2013). This is perhaps indicative of the state of AMem B-cells in untreated HIV infection, where there is dysfunctional BCR signaling (due to up-regulation of markers which have opposing effects thereon), and where at least a quarter of all AMem B-cells express the exhaustive marker CD307d.

In healthy individuals, insignificant levels of inflammation (lower CD38/8) were found alongside higher levels of CD305 and CD300a, both of which could play a role in the clearance of damaged and apoptotic tissue. Decreased levels of CD305 and CD300a in HIV infection may therefore contribute towards increased levels of inflammation (as indicated by increased CD38/8), a fact that may be further influenced by increased expression of both

inhibitory, and exhaustion markers. These cells may consequently be entering a state of exhaustion, and have BCR function that is inhibited, thereby also inhibiting effector functions such as responding to tissue damage, and interacting with T-cells via co-simulation.

4.1.5 The State of Tissue-Like Memory B-cells *ex vivo*

Putative exhaustion marker CD307d expression is primarily associated with AMem, not TLMem B-cell in chronic HIV infection

CD307d is technically an inhibitory receptor, bearing intracellular ITIMs, which was initially discovered in a population of $CD21^{low}CD27^{low}$ human tonsillar cells, and subsequently in peripheral blood in $CD21^{low}CD27^{low}$ "tissue-like" memory B-cells (Moir, *et al*., 2008). These tissue-like memory B-cells have been shown to express a homing and inhibitory receptor profile similar to that of the antigen-specific exhausted T-cell phenotype, and the same authors have demonstrated that this population of B-cells proliferates poorly in response to B-cell stimuli, and have a predominantly HIV-specific response (when comparing challenge with influenza haemagglutinin and HIV gp120) (Moir, *et al., 2008*). Contrary to literature findings, we demonstrated that FcRL4 (CD307d) was present mainly on activated memory cells and to a lesser extent on tissue-like memory cells and mature naïve B-cells of both $HIV+^{ve}$ and $HIV-^{ve}$ individuals; and not primarily on $HIV+^{ve}$ tissue-like memory cells. CD307d expression was significantly up-regulated in activated memory cells (25.75% vs. 15.27%, p=0.0015), tissue-like memory cells (12.93±10.32% vs. 7.50±5.10%, p=0.045), and mature naïve B-cells (7.49% vs. 2.58%, p=0.014). Until recently, the ligand for CD307d was unknown, until Wilson, *et al.* (2012) implicated IgA as the primary ligand for FcRL4, thereby proving its status as a genuine Fc receptor.

TLR activation pathways are up-regulated with HIV infection

TLRs are involved in recognition of highly-conserved pathogen-associated molecular patterns (PAMPs), of which TLR7 (CD287) recognises internalised viral single-stranded RNA within endosomes, while TLR4 (CD284) recognises bacterial LPS. CD287 expression was at 1.12±1.32% in the $HIV-^{ve}$ group, while there was an approximate 8-fold increase in the $HIV+^{ve}$ group (p=0.0128); while CD284 expression was relatively similar between the two groups (medians approx. 27%). Persistent viraemia and bacterial translocation are both hallmark features of untreated chronic HIV-infection, thereby possibly explaining the 8-fold increase in CD287 expression in the $HIV+^{ve}$ group, but not the unchanged CD284 expression (Klatt, *et* al., 2013; Moir & Fauci, 2014). It is possible that exposure to LPS causes a downregulation of CD284 expression, however, there may also be an as yet unknown bacterial mechanism of B-cell exhaustion that is separate to the TLR pathway.

Tissue-like memory B-cell have down-regulated BCR function, and increased expression of a leukocyte immunoglobulin-like receptor (LILRs)

LILRs consist of several immunomodulatory receptors whose function is to regulate the effector functions of professional APCs, most importantly immune activation in a variety of disease contexts, such as HIV infection (Lichterfeld & Yu, 2012). The same authors reported that the function of CD85j is to down-regulate immune responses via phosphorylation of intracellular ITIM domains (Lichterfeld & Yu, 2012). Expression of CD85j on B-cells (which is effectively absent from healthy individuals) is linked to BCR signaling, and a decreased memory B-cell response (i.e. proliferation and antibody secretion) during HIV (Sauce, Elbim, & Appay, 2013). CD85j expression was increased almost 3-fold on the TLMem subset with HIV infection (19.38±23.18% vs. 7.09±10.39% p=0.0463). CD72 expression was also increased in the HIV+^{ve} TLMem subsets (70.88±16.54% vs. 28.83±25.68%, p<0.0001). Both these receptors have been shown to down-regulate BCR signaling, and their expression on this subset is indicative of decreased functional capacity, and therefore immune exhaustion. Moir & Fauci (2010) reported an increase in CD95 expression with HIV infection, and while one would expect this population to be pre-disposed to apoptosis, CD95 expression was not significantly different between the two groups. It did, however approach significance (27.49±16.27% vs. 19.37±17.70%; p=0.0783), and a larger sample size may yield significant results.

4.1.6 The State of Resting Memory, & Mature Naïve B-cells *ex vivo*

Patterns of marker expression indicate apparent increased antigenic stimulation, both directly (via TLR pathways), and indirectly (via IL-6 signaling) in resting memory B-cells

As previously mentioned, the relative proportion of resting memory cells was significantly decreased in the HIV+^{ve} group. In addition, there was a significant increase in activation marker expression within this subset, with increased CD126, CD287, and CD284 expression compared to the HIV-^{ve} control (39.01±25.59% vs. 14.89±9.48%, p=0.0008; 10.60±13.10% vs. 3.44±2.61%, p=0.0199; & 51.43±30.12% vs. 24.84±10.07%, p=0.0057). IL-6, the ligand for CD126, is up-regulated with B-cell activation and plays an important role in maturation of activated B-cells (Baenziger, *et al.*, 2009). Constant antigenic stimulation, which is characteristic of HIV-infection, results in increased B-cell activation and proliferation, which is due, in part to increased serum IL-6 concentration (Leeansyah, *et al.*, 2013). This may be a likely mechanism for the increased CD126 expression in this subset, due to the fact that resting B-cells should not have high CD126 expression. CD126 expression was also markedly increased on the HIV+^{ve} mature naïve B-cell subset, which accounted for the majority of B-cells within both HIV+^{ve} and HIV-^{ve} individuals (43.56±17.33% vs.

49.03±18.20% of total B-cells). 21.56±32.25% of HIV+^{ve} mature naïve B-cells expressed CD126 (compared to 5.91±7.83% in the HIV-^{ve} control p=0.024), which is a direct consequence of chronic B-cell stimulation-related IL-6 release. Indeed, in healthy individuals, mature naïve B-cells express very low levels of activation markers due to the fact that they are cognate Ag naïve, and mount weak responses to innate stimuli. These cells can also be in the early stages of activation, which requires both BCR-stimulation and T-cell help (Ruprecht & Lanzavecchia, 2006). These mature naïve B-cells, in a sense, have a predisposition towards activation at a very early stage, a state that may very well be a major contributing factor towards HIV-associated B-cell dysfunction.

In agreement with Lanzavecchia & Sallusto (2007), mature naïve B-cells in our HIV^{+ve} cohort lacked TLR4 expression. The same authors stated that antigen naïve B-cells only express TLR7 when primed by APCs such as DCs (Lanzavecchia & Sallusto, 2007). This is in concert with our finding of consistent up-regulation of CD287 across all B-cell subsets (except for the mature naïve subset, on which there are extremely low levels of expression) is highly indicative of persistent viraemia. Similarly, CD284, the LPS-recognizing TLR, had up-regulated expression on resting memory cells, and not mature naïve B-cells, indicating prior exposure to LPS.

5.2 Responses of isolated B-cells to R848, LPS, and VIP

B-cells enriched by negative selection were cultured for 18 hours with either LPS or R848 alone, or in combination with VIP (hereafter referred to as “VIP inhibition”). B-cells from HIV+^{ve} individuals had a higher activation status after culture along with an increased inhibitory and exhaustive phenotype compared to that of the HIV-^{ve} control group. We further showed that HIV+^{ve} B-cells are more sensitive to TLR stimulation than HIV-^{ve} controls and that in certain instances, VIP inhibition results in alteration of receptor expression to levels similar to that of the HIV-^{ve} control. In other words, compared to the stimulated conditions, co-incubation with VIP resulted in significant down-regulation of activation, inhibition, and exhaustion marker expression. We also demonstrated that B-cells express a functional LPS receptor in the form of TLR4, in contrast to previous reports which suggest that B-cells do not express LPS receptors (Moir & Fauci, 2009)

Innate stimuli such as LPS and R848 (an agonist for TLR7, which is the innate sensor of viral RNA) were used in this assay to represent two important contributing arms of chronic stimulation by HIV, namely indirectly, via increased systemic LPS and directly, via viraemia respectively. B-cells in the current study displayed varying expression of markers of activation (increased CD126 in RMem cells and decreased CD86 and CD25 expression on AMem cells). In fact, it is well known that the HIV+^{ve} immune system has a higher baseline

activation status than that of healthy individuals, and as such have a predisposition towards cellular activation (Appay & Sauce, 2009). The fact that TLR-mediated activation of antigen-naïve B-cells occurs predominantly after CD40 and BCR activation is indeed supported by our data, in that our TLR ligands effectively induced activation and inhibitory marker expression (Allman & Pillai, 2008).

5.2.1 Subset Distribution

TLR stimulation has little effect on B-cell subset distribution in vitro

The TLR stimulation data support the *ex vivo* findings of a decrease in the relative percentage of resting memory cells in the HIV+^{ve} group across the stimulation conditions compared to the HIV-^{ve} control. The different stimulation conditions had little effect on overall subset distribution, although there was a trend towards a decrease in the HIV-associated subsets (tissue-like memory, and transitional B-cells) with VIP inhibition, as well as an increase in the proportion of resting memory and mature naïve B-cells, however this was statistically insignificant.

5.2.2 The Total B-cell Population after TLR Stimulation and VIP Inhibition

R848 provided the best stimulation for total B-cells and resulted in up-regulated activation marker expression

CD38 and CD86 are markers of activation, where CD38 is a marker of terminal B-cell differentiation, and CD86 a marker of co-stimulatory ability (Moir & Fauci, 2009). CD86 expression was significantly lower than baseline after VIP inhibition ($p<0.01$), and was also not significantly different from the HIV-^{ve} group in the same conditions. VIP therefore was able to down-regulate CD86 expression on the total B-cell population to levels that are similar to that of the HIV-^{ve} group. In the presence of VIP the total B-cell population would therefore be at a lower state of activation, and be less likely to prime T-cells via co-stimulation, thereby contributing towards lowering T-cell activation in HIV infection. CD38 expression on the total B-cell population appears to be largely expressed on mature naïve B-cells, as it is supposedly lost on mature lymphocytes (Deaglio, *et al*., 2003). Furthermore, Deaglio, *et al.* (2003) reported that CD38 appears to have maturation stage-, and microenvironment-specific function, where it is involved in lymphopoeisis blocking in the bone marrow, and down-regulating apoptosis in germinal centre B-cells. Perhaps, the fact that CD38 is up-regulated with VIP inhibition indicates that its function on mature lymphocytes concerns apoptosis regulation, and up-regulated expression confers a protective effect on the B-cell population. Sandoval-Montes and Santos-Argumedo (2005) demonstrated that CD38 is also associated with the CD21/CD19 complex and plays a role in

BCR signaling. Up-regulated CD38 expression in this instance may confer more tightly-regulated BCR signaling and function.

TLR4 (CD284) and TLR7 (CD287) expression is down-regulated with VIP inhibition in total B-cell populations, as well as in all measured subsets

Interestingly, CD287 expression on the total B-cell population was unresponsive to R848 stimulation, whereas CD284 expression was responsive. Inhibition with VIP resulted in a highly significant down-regulation of receptor expression to levels that were both below baseline, and insignificantly different from that of the HIV-^{ve} population. The same was true for CD284 and CD287 expression of AMem, RMem, TLMem, and MN B-cells in the HIV+^{ve} group, with the VIP inhibition scenarios having the lowest expression within the HIV+^{ve} group alone. Interestingly, CD284 also has low baseline expression on mature naïve B-cells in the HIV-^{ve} group compared to that of the HIV+^{ve} group, indicating that HIV+^{ve} individuals have high systemic LPS, and subsequently, higher levels of activation marker expression.

Stimulation for 8 hours with TLR agonists has been shown to up-regulate mRNA levels of genes critical to B-cell proliferation and differentiation (Hanten, *et al.*, 2008). Lester, *et al.* (2008) have also shown increased TLR4 and TLR7 expression on PBMCs isolated from HIV+^{ve} women from Kenya, both of which correlated with HIV viraemia, and also that TLR expression levels returned to levels comparable to HIV-uninfected individuals with the initiation of ART. This study however, also measured TLR-mRNA levels, and not direct expression on cells, and therefore also did not investigate TLR expression on each cell subset. Furthermore, Baenziger, *et al*. (2009) found that chronic TLR7 stimulation in mice induces similar immune activation and lymphoid system disruption to that of HIV infection. Their findings indicate that TLR7 plays a major role in HIV-mediated pathology, and our data suggests that administration of VIP in a dose of 1 x10^{-8}M can significantly reduce TLR7 expression across all B-cell subsets to levels not dissimilar to that of HIV uninfected individuals. To our knowledge, this is the first study to investigate TLR expression after stimulation, via multicolour flow cytometry, as well as to investigate the effect of VIP inhibition on the expression of TLR7 (CD287) and TLR4 (CD284).

Although the LPS-recognizing TLR, CD284, had down-regulated expression in presence of VIP, there were still approx. 48% of total B-cells expressing this receptor. These levels, however were not significantly different from that of the HIV-^{ve} group, and little is known as to the expression patterns of TLR4 on B-cells in HIV infection (and healthy individuals), so it is difficult to draw a clear conclusion from these results. It may be likely that CD284 expression

is in fact downregulated in response to high levels of systemic LPS. A recent study by Rauch, *et al.* (2012) described CD284 expression on B-cells in the context of sepsis, and they reported constitutive expression of this LPS receptor.

VPAC 2 expression, although somewhat higher on the B-cells of HIV-^{ve} individuals, is not effected by TLR stimulation

VPAC2 expression on the total B-cell population tended towards having higher expression in the HIV-^{ve} group, although this was not statistically significant. Due to limitations in antibody availability, we were unable to determine VPAC2 expression on the different B-cell subsets, although pilot data (not shown) did indicate that VPAC2 was expressed only on $CD19^{+}CD21^{-}$ B-cells, thereby excluding resting memory and mature naïve B-cells. It is most likely that VPAC 2 expression is up-regulated upon activation, and therefore would be present on plasmablasts, activated memory, and perhaps tissue-like memory cells. According to our data, the other cell types responded to VIP inhibition, so further studies into the dynamics of VPAC2 expression on the B-cell subsets is warranted. Indeed, VPAC2 levels on $CD4^{+}$ T-cells correlated positively with Fas ligand (FasL) expression (Ipp, *et al*., 2014). It is possible that the fact that there is no de-regulation of VPAC2 expression with HIV infection, indicates that it could be a potential therapeutic target.

Molecular studies have elucidated that lymphocytes may indeed be producers of VIP, along with peptidergic nerve fibres in the lymphoid tissue, whose downstream effects include inhibition of pro-inflammatory cytokines such as IL-6, and IL-12; and later stimulation of anti-inflammatory IL-10 production (Pozo, *et al*., 2000).

Markers of inhibition were down-regulated by VIP inhibition

Expression of CD72 was significantly lowered by LPS, and not R848 stimulation, while CD85j, and CD305 expression was not significantly changed by either. VIP inhibition resulted in significant further down-regulation of CD72 and CD85j; however this was still significantly higher than that of the HIV-^{ve} group. Based on this data, CD72 expression seems to be reversible to a certain extent, although additional studies may be required to elucidate the significance of this. CD72 seems to be involved in setting the threshold for BCR-mediated activation, so down-regulation of this marker may prove useful in counteracting activation-mediated cellular exhaustion (Adaci *et al.*, 2000). This could indicate that B-cells, even in asymptomatic HIV infection, have progressed to the stage where markers of exhaustion are prominent on the total B-cell population, and that some of these markers remain elevated even with VIP inhibition.

The apoptotic receptor CD95 (Fas) was down-regulated by VIP

CD95 expression on the total B-cell population responded strongly to R848 stimulation, and was significantly down-regulated with VIP inhibition. The high baseline levels of CD95 on this population was indicative of an increased propensity towards apoptosis (43% of cells expressed CD95). The subsequent increase in CD95 expression with TLR stimulation further strengthens this hypothesis. Therefore, VIP has shown efficacy in down-regulating CD95 expression on the total B-cell population (to levels similar to that of healthy individuals), and has the potential to play an important role in ameliorating the effects of chronic HIV infection.

5.2.3 The State of Tissue-Like Memory B-cells

The exhausted B-cell population displayed evidence of viral, and bacterial stimulation, and VIP was unable to down-regulated activation marker expression

CD287 expression was absent on TLMem B-cells in the HIV-^{ve} group, and VIP inhibition elicited the same response in the HIV+^{ve} group. More than 60% of TLMem B-cells expressed CD284 at baseline, while VIP inhibition down-regulated expression to levels comparable with the HIV-^{ve} control. The high expression levels of these two markers at the un-stimulated baseline, as well as the up-regulation of CD287 with stimulation indicates that this population has had prior antigenic stimulation via LPS and viral RNA, and it primed to respond rapidly to viral RNA. This supports the findings that viraemeia contributes towards B-cell exhaustion (Kardava, *et al.*, 2011; Moir & Fauci, 2009).

The exhausted B-cell population displayed evidence of inhibited BCR signaling as well as increased apoptotic potential, and appeared unable to fully recover with VIP inhibition

Inhibitory marker expression was up-regulated at baseline on TLMem B-cells (where the HIV-^{ve} group had <1% expression of inhibitory receptors). CD72, CD300a, CD307d, and CD85j expression was slightly down-regulated upon VIP inhibition; however levels of these markers never reached levels comparable to that of the HIV-^{ve} group. Similarly, CD95 expression on this subset remained significantly raised (compared to the HIV-^{ve} control) even after VIP inhibition. The TLMem B-cell population was therefore unable to fully recover from its inhibitory and pro-apoptotic state with VIP inhibition. The tissue-like memory population can indeed be regarded as being functionally “exhausted”, in that their inhibitory state persisted, even after our intervention. These cells are therefore beyond the point where they could be rescued from their fate, and an intervention should rather be targeted at B-cells before they become exhausted.

CD307d expression was somewhat insensitive to TLR stimulation in the HIV+^{ve} group

The putative marker of B-cell exhaustion, CD307d was equally sensitive to both LPR and R848 stimulation, although, there was only a significant reduction in expression with LPS & VIP inhibition, where the HIV+^{ve} mean expression was comparable to that of the HIV-^{ve} group. This was observed in mature naïve, tissue-like memory, and activated memory B-cells, while although there was a trend towards down-regulated expression with VIP inhibition, this was not statistically significant. Total B-cell CD307d expression was, however, down-regulated with both dual-stimulation events, although not in any of the three subsets individually.

FcRL4 may act as a molecular switch in memory B-cells, which down-regulates adaptive immune signaling, and up-regulates the innate response in reaction to chronic antigenic stimulation, as seen in untreated HIV infection (Sohn, *et al*., 2011). This explains the similar CD307d response in both LPS, and R848 stimulation, although it leads one to wonder why VIP only down-regulates this marker in LPS-stimulated cells, and not in R848-stimulated cells. It is possible that like LPS, VIP binds to a receptor on the cell surface, while ligands for TLR7 are intracellular (via endosomal transportation), and therefore R848 stimulated cells are activated in a manner that is intrinsically different to that of the LPS stimulated cells.

5.2.4 The State of Mature Naïve, and Activated and Resting Memory B-cells

Activated memory B-cells responded to VIP inhibition and down-regulated almost all activation and inhibitory markers

AMem B-cells at baseline displayed evidence of prior antigenic stimulation, as well as increased cellular activation as measured by CD86, and CD126 expression. The exhaustive status of these cells was also high, due to increased expression of multiple inhibitory markers. AMem B-cells in the HIV+^{ve} group were able to down-regulate TLR4 and TLR7 expression, as well as CD86, CD72, CD305, and CD95, after VIP inhibition. Therefore the phenotype of these cells returned to one that is comparable with the HIV-^{ve} group. CD126, and CD307d, however remained elevated, even after VIP inhibition. This is perhaps indicative of the fact that CD126 expression is required for activation of resting memory B-cells (Haas, *et al*., 2011); while cellular exhaustion (as measured by CD307d expression) remained higher (although only marginally so, where p=0,034). Perhaps a higher dose of VIP is needed to down-regulate expression of CD307d, or indeed an additional signal may be required to down-regulate its expression. What is quite interesting is that there was a

near complete down-regulation of CD95 expression post VIP inhibition ($p<0.05$), indicating that the apoptotic potential of these cells was essentially similar to that of the HIV-^{ve} group.

Resting memory B-cells responded the most efficiently to VIP inhibition, with near complete down-regulation of activation, and inhibitory markers.

RMem B-cells at baseline also displayed evidence of prior antigenic stimulation, as well as increased cellular activation as measured by CD25, CD86, and CD126 expression. The exhaustive, and pro-apoptotic status of these cells was also high, due to the expression of CD307d, CD305 and CD95. All markers were down-regulated upon VIP inhibition, and interestingly, CD25 expression (the IL-2 receptor) was down-regulated to levels below that of the HIV-^{ve} control. RMem B-cells could therefore prove to be an effective therapeutic target in early HIV infection, as these are the cells that become re-activated upon stimulation (hence their depletion).

Mature Naïve B-cells responded to VIP inhibition, although several of activation, and inhibitory markers remained up-regulated

It is interesting to note that this subset was able to down-regulate most activation and inhibitory markers upon VIP inhibition (despite having a pre-disposition towards activation, as indicated by their response to TLR ligands). MN B-cells in the HIV-^{ve} group did not respond to TLR stimulation, and indeed, should not, due to the fact that there was no prior BCR engagement (mature naïve B-cells are by definition, naïve to BCR engagement). VIP inhibition was unable to down-regulate CD126 expression, indicative of the prior IL-6 stimulation *in vivo*. Similarly, CD307d, and CD95 expression remained up-regulated post VIP inhibition, which is perhaps indicative of a predisposition towards cellular exhaustion, and early apoptosis signaling in HIV infection. CD85j, an Ig-like receptor, had insignificant baseline expression, but was markedly up-regulated with R848 stimulation, and returned to baseline levels with VIP inhibition. This also shows the underlying predisposition towards activation in HIV infection, due to the lack of a similar response in the HIV-^{ve} group.

5.3 Limitations of the Study,

A major limiting factor if this study was the small sample size, which can be attributed to the high cost associated with multicolour flow cytometry. This study has proven which novel markers are expressed significantly, so future studies may be able to decrease costs by reducing the number of antibodies required. A further limitation is that we only investigated surface marker expression, and not intracellular mRNA levels, which would give a more accurate depiction of gene upregulation. TB co-infection is a major problem in the HIV+^{ve} population, so investigating the effects of both TB-HIV co-infection, and mono infection would be of significance in future studies.

5.4 Concluding Remarks

This study has demonstrated that, *ex* vivo, B-cells from HIV+^{ve} individuals have significantly higher expression of both activation and inhibitory markers, as well as low levels of the immunomodulatory receptor, VPAC2. There was a profound loss in both the absolute number of circulating B-cells, as well as the number of resting memory cells in HIV infection and concomitantly, an expansion of a tissue-like memory phenotype, which has been shown to display a similar phenotype to that of the exhausted T-cell. Thus even in this group of clinically well HIV+^{ve} participants, with relatively well maintained CD4 counts; significant B cell abnormalities can be detected. Earlier administration of antiretroviral therapy possibly in conjunction with an immune-modulatory agent such as VIP, may limit the dysfunction that develops in the B cell compartment.

We have also shown that isolated B-cells are extremely responsive to TLR stimulation *in vitro*, with high expression of receptors that recognize LPS, and viral RNA. We proved that LPS can effectively activate B-cells *in vitro*, despite contrary reports in literature, although R848-stimulation was more effective than that of LPS. Mature naïve B-cells from HIV+^{ve} individuals had a predisposition towards activation such that they were able to up-regulate activation markers after stimulation while those in the HIV-^{ve} group did not. Furthermore, we provided evidence for the potential role of vasoactive intestinal peptide as an immunomodulatory agent in a concentration of 1 x10^{-8} M. Inhibition with VIP with either of the TLR-ligands (LPS or R848) significantly down-regulated both activation marker (CD126, CD86), and inhibitory marker (CD85j, CD300a) expression on various B-cell subsets. VIP was also able to down-regulate CD95 expression on all subsets of HIV+^{ve} B-cells (except for activated memory cells). Our data has shown that VIP appears to almost completely inhibit pathway-specific activation pathways (through CD86 expression, and not CD38), while it

was able to nearly-completely downregulate CD95 expression, and therefore Fas-mediated apoptosis signaling.

Inhibition with VIP resulted in CD287 expression indistinguishable from that of an HIV-^{ve} individual, thereby proving its ability to dampen the potential harm of persistent viraemia, by down-regulating the innate viral sensors on these B-cells. Silva, *et al*. (2011) reported that CD305 expression is lowest on the memory subsets, and highest on mature naïve B-cell *ex vivo*, which supports our earlier findings, however this was practically reversed with culture and stimulation of cells, where mature naïve B-cells consistently express the lowest levels of the collagen receptor, CD305. Perhaps it is useful to down-regulate CD305 expression on the antigen-specific memory subsets to limit the already present propensity towards inhibition and exhaustion. The fact that there was no response in mature naïve cells is perhaps indicative of their lack of BCR engagement.

The marker of exhaustion, CD307d was significantly higher on the tissue-like memory and activated memory subsets of the HIV+^{ve} group, even after VIP inhibition. It appears that in most cases, the HIV+^{ve} TLM subset is past the proverbial “point-of-no-return” for VIP to effectively down-regulate activation and inhibition markers. Further studies may shed light onto this, and in fact one could expect to see a decrease in the numbers of TLM cells, with VIP, as is seen with ART. Mature Naïve B-cells should be the primary target of a subset-specific intervention, due to the fact that these cells have had the least exposure to stimulation of any peripheral blood subset.

Currently in South Africa, the guidelines for commencing ART are at a CD4 count of 350cells/µl. This study has shown clearly that even at CD4 counts above this level; significant B cell abnormalities and dysfunction have already occurred *in vivo*. It would therefore be important for earlier interventions to take place to determine which patients have a more ‘activated and/ or exhausted’ phenotype; targeting them for earlier therapies. This study also highlighted the potential for a novel immune-based intervention strategy with the use of the agent VIP. Future studies may benefit from investigating the effects of different types of stimulation (i.e. cognate Ag, *de novo* Ag, mitogen stimulation). Furthermore, while propensity towards apoptosis signaling was only measured via Fas cell surface expression, future studies may further benefit from investigating the various apoptosis pathways (via caspase activation, bcl signaling, and even NK-mediated cell killing) and the endpoints of apoptosis, such as Annexin V expression.

References

Allman D, & Pillai S (2008). Peripheral B-cell subsets. *Current Opinion in Immunology*; 20:149-157

Appay V & Sauce D (2009). Immune activation and inflammation in HIV-1 infection: causes and consequences. Journal of Pathology; 214:231-241

Agarwal K, Aggarwal M, Aggarwal VK, Pujani M, & Nain M (2010). Increased hematogones in an infant with bicytopenia and leucocytosis: a case report. *Cases Journal;*3:75-78

Badley AD, Pilon AA, Landay A, & Lynch DH (2000). Mechanisms of HIV-associated lymphocyte apoptosis. *Blood*; 96(9):2951-2964

Baenziger S, Heikenwalder M, Johansen P, Schlaepfer E, Hofer U, Miller RC, Diermand S, Honda K, Kundig TM, Aguzzi A, & Speck RF (2009). Triggering TLR7 in mice induces immune activation and lymphoid system disruption, resembling HIV-mediated pathology. *Blood*; 113(2):377-388

BD Biosciences. A setup system for compensation: BD compbeads plus BD FACSDiva software. Viewed 13 November 2013. Available at:

http://www.bdbiosciences.com/documents/BD_FACSDiva_setup_system.pdf

Boasso A & Shearer GM (2008). Chronic innate immune activation as a cause of HIV-1 immunopathogenesis. *Clinical Immunology*; 126:235-242

Boliar S, Murphy MK, Tran C, Carnathan DG, Armstron WS, Silvestri G, & Derdeyn CA (2012). B-Lymphocyte dysfunction in chronic HIV-1 infection does not prevent cross-clade neutralization breadth. *Journal of Virology*; 86(15):8031-8040

Bokaei PB, Mab X, Sakacd D, & Branch DR (2007). HIV-1 integration is inhibited by stimulation of the VPAC2 neuroendocrine receptor. *Virology*; 362(1):38–49

Branch D (2011). Neuropeptide Receptors: Novel Targets for HIV/AIDS Therapeutics. *Pharmaceuticals*; 4:485-493

Brenchly JM & Douek DC (2008) [1]. The mucosal barrier and immune activation in HIV pathogenesis. *Current Opin HIV AIDS*; 3(3):356-361

Brenchly JM & Douek DC (2008) [2]. HIV infection and the gastrointestinal immune system. *Nature: Mucosal Immunology*; 1:23-30

Cadogan M, & Dalgleish AG (2008). HIV immunopathogeneis and strategies for intervention. *The Lancet Infectious Diseases*; 8:675-684

Capello D, Scandurra M, Poretti G, Rancoita PMV, Mian M, Gloghini A, Deambrogi C, Martini M, Rossi D, Greiner TC, Chan WC, Ponzoni M, Moreno SM, Piris MA, Canzonieri V, Spina M, Tirelli U, Inghirami G, Rinaldi A, Zucca E, Favera RD, Cavalli F, Larocca LM, Kwee I, Carbone A, Gaidano G, & Bertoni F (2009). Genome wide DNA-profiling of HIV-related B-cell lymphomas. *British Journal of Haematology*;148:245–255

Cotugno N, Douagi I, Rossi P, & Palma P (2012). Suboptimal immune reconstitution in vertically HIV infected chidren: A view on how HIV replication and timing of HAART initiation can impact in T and B-cell compartment. *Clinical and developmental immunology*. Doi:10.1155/2012/805151

Dauby N, De Wit S, Delforge M, Necsoi VC, &Clumeck N (2011). Characteristics of non-AIDS-defining malignancies in the HAART era: a clinic-epidemiological study. *Journal of the International AIDS Society*; 14(16).Doi 10.1186/1758-2652-14-16

Das A, Xu H, Wang X, Yau CL, Veazey RS, & Pahar B (2011). Double-positive CD21+CD27+ B cells are highly proliferating memory cells and their distribution differs in mucosal and peripheral tissues.*PLOS One*;6(1):e16524. doi: 10.1371/journal.pone.0016524.

De Milito A (2004). B lymphocyte dysfunctions in HIV infection. *Current HIV Research*; 2:11-21

De Milito A, Nilsson A, Titanji K, Thorstensson R, Reizenstein E, Narita M, Grutzmeier S, Sönnerborg A, & Chiodi F (2004). Mechanisms of hypergammaglobulinemia and impaired antigen-specific humoral immunity in HIV-1 infection. *Blood*; 103(6):2180-2186

Deaglio S, Capobianco A, Bergui L, Durig J, Morabito F, Duhrsen U, & Malavasi F (2003). CD38 is a signaling molecule in B-cell chronic lymphocytic leukemia cells. *Blood*; 102(6):2146-2155

Delgado M, & Ganea AD (2000). VIP and PACAP Inhibit Activation Induced Apoptosis in T Lymphocytes. *Annals of the New York Academy of Sciences*; 921:55-67

Desai S & Landay A (2010).Early immune senescence in HIV disease. *Current HIV/AIDS Reports*: 7;4-10

Eisenberg R, & Looney RJ (2005). The therapeutic potential of anti-CD20, what do B-cells do? *Clinical Immunology*; 117:207-213

Erdei A, Isaák A, Török K, Sándor N, Kremlitzka M, Prechl J, & Bajtay Z (2009). Expression and role of CR1 and CR2 on B and T lymphocytes under physiological and autoimmune conditions. *Molecular Immunology*; 46(14):2767-73.

Ettinger R, Sims GP, Fairhurst A, Robbins R, da Silva YS, Spolski R, Leonard WJ, & Lipsky PE (2005). IL-21 induces differentiation of human naïve and memory B-cells into antibody-secreting plasma cells. *Journal of Immunology*; 175(1):7869-7879

Evans D (2013). Ten years on ART – Where to now?. *South African Medical Journal*; 103(4):229-31. doi: 10.7196/samj.6835

Fairfax KA, Kallies A, Nutt SL, & Tarlinton DM (2008). Plasma cell development: from B-cell subsets to long-term survival niches. *Seminars in Immunology*; 20(1):49-58

Fauci AS. Immunopathogenic mechanisms of HIV infection (1996) 654-655. In Fauci AS, moderator: Immunopathogenic mechanisms of HIV infection. *Ann Intern Med*; 124(7):654-663

Fogli M, Torti C, Malacarne F, Fiorentini S, Albani M, Izzo M, Giagulli C, Maggi F, Carossi G, & Caruso A (2012). Emergence of exhausted B-cells in asymptomatic HIV-1-infected patients naïve for HAART is related to reduce immune surveillance. *Clin Dev Imunol*; DOI 10.1155/2012/829584

Filippatos GS, Gangopadhyay N, Lalude O, Parameswaran N, Said SI, Spielman W, & Uhal BD (2001). Regulation of apoptosis by vasoactive peptides. *American Journal of Phusiology – Lung Cellular and Molecular Physiology*; 281:749-761

Gaena D, & Delgado M (2002). Vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase-activating polypeptide as modulators of both innate and adaptive immunity. *Critical Reviews in Oral Biology & Medicine*; 13(3):229-237

Gonzalez SF, Degn SE, Pitcher LA, Woodfurr M, Heesters BA, & Carrol MC (2011). Trafficking of B-cell antigen in lymph nodes. *Annual Review of Immunology;* 29:215-233

GraphPad Software. GraphPad Prism User Guide. Graphs: Symbols, bars, lines, and legends. Viewed 27 November 2014, available at: http://graphpad.com/guides/prism/6/user-guide/index.htm?box-and-whiskers.htm

Haas A, Zimmerman K, & Oxenius A (2011). Antigen-dependent and –independent mechanisms of T and B cell hyperactivation during chronic HIV-1 infection, *Journal of Virology*; 85(23):12012-12113

Haase AT (2010). Targeting early infection to prevent HIV-1 mucosal transmission. *Nature*; 464:217-223

Hardy RR, & Hayakawa K (2001). B-cell development pathways. *Annual Review of Immunology*; 19:595-621

Hazenberg MD, Otto SA, van Benthem BHB, Roos MTL, Coutinho RA, Lange JMA, Hamann D, Prins M & Miedema F (2003). Persistent immune activation in HIV-1 infection is associated with progression to AIDS. *AIDS*; 17:1881-1888

He X, Jia H, Jing Z, & Liu D (2013). Recognition of pathogen-associated nucleic acids by endosomal nucleic acid-sensing toll-like receptors. *Acta Biochim Biophys Sin (Shanghai)*; 45(4):241-58. doi: 10.1093/abbs/gms122

Ho J, Moir S, Malaspina A, Howell ML, Wang W, DiPoto AC, O'Shea MA, Roby GA, Kwan R, Mican JA, Chun T, & Fauci AS (2006). Two overrepresented B cell populations in HIV-infected individuals undergo apoptosis by different mechanisms. *PNAS;* 103(51):19436-19441

Huang J1, Zhu C, Zhang P, Zhu Q, Liu Y, Zhu Z, Wang M, Li W, Yang G, Dong N, Liu J, Chen L, Zhang Y, Yang R, Deng L, Fan J, Wang X, Liu J, Ma B, Fu Q, & Wu K (2013). S100+ cells: a new neuro-immune cross-talkers in lymph organs. *Scientific Reports*; 3:1114. doi: 10.1038/srep01114.

Ipp H, Nkambule BB, Reid TD, de Swardt D, Bekker LG, & Glashoff RH (2014). CD4+ T cells in HIV infection show increased levels of expression of a receptor for vasoactive intestinal peptide, VPAC2. *Immunologic Research*; 60(1):11-5. doi: 10.1007/s12026-014-8487-2.

Jain V, & Deeks SG (2010). When to start antiretroviral therapy. *Curr HIV/AIDS Rep*; 7:60–68.DOI 10.1007/s11904-010-0044-6

Joint United Nations Programme on HIV/AIDS (UNAIDS) (2012): AIDS Epidemic Update 2012. Viewed 15 January 2013, Available at:

http://www.unaids.org/en/media/unaids/contentassets/documents/epidemiology/2012/gr2012/20121120_UNAIDS_Global_Report_2012_with_annexes_en.pdf

Joint United Nations Programme on HIV/AIDS (UNAIDS) (2014): Global AIDS response progress reporting 2014. Viewed 29 November 2014, available at:

http://www.unaids.org/sites/default/files/media_asset/GARPR_2014_guidelines_en_0.pdf

Kardava L, Moir S, Wang W, Ho J, Buckner CM, Posada JG, O'Shea MA, Roby G, Chen J, Sohn HW, Chun T, Pierce SK, &Fauci AF (2011). Attenuation of HIV-associated human B-cell exhaustion by siRNA downregulation of inhibitory receptors. *Journal of Clinical Investigation*; 121(7):2614-2624

Kiazyk SA & Fowke KR (2008). Loss of CD127 expression links immune activation and CD4+ T cell loss in HIV infection. *Trends in Microbiology*; 16(12):567-573

Klatt NR, Funderburg NT, & Brenchley JM (2010). Microbial translocation, immune activation, and HIV disease. *Trends in Microbiology*; 21(1):6-13

Klatt NR, Chomont N, Douek DC, & Deeks SG (2013). Immune activation and HIV persistence: implications for curative approaches to HIV infection. *Immunological Reviews*; 254(1):326-342

Klein U, tu Y, Stolovitzky GA, Keller JL, Haddad J, Milijkovic, Cattoretti G, Califano A, & Dalla-Favera R (2003). Transcriptional analysis of the B cell germinal center reaction*PNAS*; 100(5):2639-2644

Küppers R (2008). Memory B-cells of a special kind. *Immunology and Cell Biology*; 86:635-636

Kyewski B, & Klein L (2006). A central role for central tolerance. *Annu Rev Immunol*; 24:571-606

Lamoreaux L, Reoderer M, & Koup R (2006). Intracellular cytokine optimization and standard operating procedure. *Nature Protocols*; 1(3):1570-1516

Lanzavecchia A & Sallusto F (2007). Toll-like receptors and innate immunity in B-cell activation and antibody responses. *Curr Opin Immunol*; 19(3):268-74.

Lester RT, Yao X, Ball TB, McKinnon LR, Kaul R, Wachihi, Jaoko W, Plumer FA, & Rosenthal KL (2008). Toll-like receptor expression and responsiveness are increased in viraemic HIV-1 infection. *AIDS*; 22(6):685-694

Leeansyaha E, Malonea DFG, Anthonyb DA, & Sandberga JK (2013). Soluble biomarkers of HIV transmission, disease progression and comorbidities. *Current Opinion in HIV & AIDS*; 8:117-124

Levesque MC, Moody MA, Hwang K, Marshall D, Whitesides JF, Amos JD, Gurley TC, Allgood S, Haynes BB, Vandergrift NA, Plonk S, Parker DC, Cohen MS, Tomaras GD, Goepfert PA, Shaw GM, Schmitz JE, Eron JJ, Shaheen NJ, Hicks CB, Liao H, Markowitz M,

Kelsoe G, Margolis DM & Haynes BF (2009). Polyclonal B cell differentiation and loss of gastrointestinal tract germinal centers in the earliest stages of HIV-1 infection. PLoS Med 6(7): e1000107. doi:10.1371/journal.pmed.1000107

Li Y, O'Dell S, Walker LM, Wu X, Guenaga J, Feng Y, Schmidt SD, McKee K, Louder MK, Ledgerwood JE, Graham BS, Haynes BF, Burton DR, Wyatt RT, & Mascola JR (2011). Mechanism of neutralization by the broadly neutralizing HIV-1 monoclonal antibody VRC01. *Journal of Virology;* 85(17):8954-67. doi: 10.1128/JVI.00754-11

Lichtenstein KA (2009). Antiretroviral treatment interruptions and risk of non-opportunistic diseases. *Current HIV/AIDS Reports*; 6:77-82

Lichterfeld M, & Yu XG (2012). The emerging role of leukocyte immunoglobulin-like receptors (LILRs) in HIV-1 infection. *J Leukoc Biol;* 91(1): 27-33.

Llinàs L, Lázaro A, de Salort J, Matesanz-Isabel J, Sintes J, & Engel P (2011). Expression profiles of novel cell surface molecules on B-cell subsets and plasma cells as analysed by flow cytometry. *Immunology Letters*; 134:113-121

Macker HT, & Trotter J. (2006). Flow Cytometry Controls, Instrument Setup, and the Determination of Positivity. *Cytometry Part A* ; 69A:1037–1042

Malaspina A, Moir S, Kottilil S, Hallahan CW, Ehler LA, Liu S, Planta MA, Chun T &Fauci AS (2003). Deleterious effect of HIV-1 plasma viraemia on B-cell costimulatory function. *Journal of Immunology*; 170:5965-5972

Mascola JR & Montefiori DC (2010). The role of antibodies in HIV vaccines. *Annu Rev Immunol*; 28:413-44. doi: 10.1146/annurev-immunol-030409-101256.

Mauri C &Bosma A (2012). Immune regulatory function of B-cells. *Ann. Rev. Immunol;* 30:221-241

McMichael AJ, Borrow P, Tomaras GD, Goonetilleke N, & Haynes BF (2010). The immune response during acute HIV-1 infection: clues for vaccine development. *Nature Reviews Immunology*; 10(1):11-23

McGuire AT, Glenn JA, Lippy A, & Stamatatos L (2014). Diverse recombinant HIV-1 Envs fail to activate B cells expressing the germline B cell receptors of the broadly neutralizing anti-HIV-1 antibodies PG9 and 447-52D. *Journal of Virology*; 88(5):2645-57. doi: 10.1128/JVI.03228-13

Melchers F, ten Boekel E, Seidl T, Kong XC, Yamagami T, Onishi K, Shimizu T, Rolink AG, & Andersson J (2000). Repertoire selection by pre-B-cell receptors and B-cell receptors, and genetic control of B-cell development from immature to mature B cells. *Immunological Reviews*; 175:33-46

Melchers F (2005). The pre-B-cell receptor: selector of fitting immunoglobulin heavy chains for the B-cell repertoire. *Nature Reviews. Immunology;* 5(7):578-84.

Meyaard L (2008). The inhibitory collagen receptor LAIR-1 (CD305). *Journal of Leukocyte Biology*; 83:799-803

Moir S & Fauci AS (2008). Pathogenic mechanisms of B-lymphocyte dysfunction in HIV disease. *J Allergy Clin Immunol*; 122(1):12-19

Moir S & Fauci AS (2009). B-cells in HIV infection and Disease. *Nat Rev Immunol*;9(4): 235–245

Moir S & Fauci AS (2013). Insights into B cells and HIV-specific B-cell responses in HIV-infected individuals. *Immunological Reviews*; 254: 207–224

Moir S & Fauci AS (2014). B-cell exhaustion in HIV infection: the role of immune activation. *Current Opinion in HIV and AIDS*; 9(5):472-7

Moir S, Malaspina A, Ho J, Wang W, DiPoto AC, O'Shea MA, Roby G, Mican JM, Kottilil S, Chun T, Proschan MA, &Fauci AS (2008). Normalization of B-cell counts and subpopulations after antiretroviral therapy in chronic HIV disease. *The Journal of Infectious Diseases*; 197:572–579

Moir S, Ho J, Malaspina A, Wang W, DiPoto AC, O'Shea MA, Roby G, Kottilil S, Arthos J, Proschan MA, Chun T & Fauci AS (2008) Evidence for HIV-associated B-cell exhaustion in a dysfunctional memory B-cell compartment in HIV-infected viraemic individuals. *Journal of Experimental Medicine*; 205(8):1797-1805

Moir S, Buckner CM, Ho J, Wang W, Chen J, Waldner AJ, Posada JG, Kardava L, O'Shea MA, Kottilil S, Chun T, Proschan MA, & Fauci AS (2010). B cells n early and chronic HIV infection: evidence for preservation of immune functions associated with early initiation of antiretroviral therapy. *Blood*; 116(25):5571-5579

Murphy K, Travers P, & Walport M (2008). *Janeway's Immunobiology*, 7th Ed. New York: Garland Science

Ostrowski SR (2010). Immune activation in chronic HIV infection. *Danish Medical Bulletin*; 57:B4122

Parham P (2009). *The Immune System*, 3rd Ed. New York: Garland Science

Pensieroso S, Cagigi A, Palma P, Nilsson A, Capponi C, Freda E, Bernardi S, Thorstensson R, Choldi F, & Rossi P (2009). Timing of HAART defines the integrity of memory B-cells and the longevity of humoral responses in HIV-1 vertically-infected children. *PNAS*; 106(19):7939-7944

Phan TG, Paus D, Chan TD, Turner ML, Nutt SL, Basten A, & Brink R (2006). High affinity germinal center B-cells are actively selected into the plasma cell compartment. *The Journal of Experimental Medicine*; 203(11):2419-2424

Pieper K, Grimbacher B, & Eibel H (2013). B-cell biology and development. *J Allergy Clin Immunol*; 131(4):959-71. doi: 10.1016/j.jaci.2013.01.046.

Pinto D, Montani E, Bolli M, Garavaglia G, Sallusto F, Lanzavecchia A, & Jarrossay D (2013). A functional BCR in human IgA and IgM plasma cells. *Blood*; 121(2):4110-4114

Pozo D, Delgado M, Martinez C, Guerrero JM, Leceta J, Gomariz RP, & Calvo JR (2000). Immunobiology of vasoactive intestinal peptide (VIP). *Immunology Today*; 21(1):7-11

Pozo D, & Delgado M (2004). The many faces of VIP in neuroimmunology: a cytokine rather than a neuropeptide? *The FASEB Journal;* 18:1325-1334

Pulendran B, & Ahmed R (2011). Immunological mechanisms of vaccination. *Nature Immunology*; 12(6):509-517

Rauch PJ, Chudnovskiy A, Robbins CS, Weber GF, Etzrodt M, Hilgendorf I, Tiglao E, FigueiredoJ , Iwamoto Y, Theurl I, Gorbatov R, Waring MT, Chicoine AT, Mouded M, Pittet MJ, Nahrendorf M, Weissleder R, & Swirski FK (2012). Innate Response Activator B Cells Protect Against Microbial Sepsis. *Science*; 335(6068): 597–601

Rawlings DJ, Schwartz MA, Jackson SW, & Meyer-Bahlburg A (2012). Integration of B cell responses through Toll-like receptors and antigen receptors. *Nature Reviews Immunology*; 12(4):282-94

Ruffin N, Lantto R, Pensieroso S, Sammicheli S, Hejdeman B, Rethi B, & Chiodi F (2012). Immune activation and increased IL-21R expression are associated with the loss of memory B cells during HIV-1 infection. *Journal of Internal medicine*; DOI: 10.1111/j.1365-2796.2012.02550.x

Ruprecht CR, & Lanzavecchia A (2006). Toll-like receptor stimulation as a third signal required for activation of human naïve B-cells. *European Journal of Immunology;* 36:810-816

Sanz E, Muñoz-A N, Monserrata J, Van-Den-Ryma A, Escollc P, Ranzc I, Álvarez-Mon M & de-la-Hera A (2010). Ordering human CD34+CD10-CD19+ pre/pro-B-cell and CD19- common lymphoid progenitor stages in two pro-B-cell development pathways. *PNAS*; 107(13):5925-5930

Sandoval-Montes C, & Santos-Argumedo L (2005). CD38 is expressed selectively during the activation of a subset of mature T cells with reduced proliferation but improved potential to produce cytokines. *Journal of Leukocyte Biology*; 77(4):513-521

Sauce D, Elbim C, & Appay V (2013). Monitoring cellular immune markers in HIV infection: from activation to exhaustion. *Current Opinion in HIV & AIDS*; 8:125–131

Shen, X & Tomaras, GD (2011). Alterations of the B-cell response by HIV-1 replication. *Curr HIV/AIDS Rep;* 8:23-30

Siegrist C, & Aspinall R (2009). B-cell responses to vaccination at the extremes of age. *Nature Reviews Immunology*; 9(3):185-194

Siewe B, Keshavarzian A, French A, Demarais P, & Landay A (2013). A role for TLR signaling during B cell activation in antiretroviral-treated HIV individuals. *AIDS Res Hum Retroviruses*; 29(10):1353-60. doi: 10.1089/AID.2013.0115

Slilciano JA, & Siliciano RF (2010). Biomarkers of HIV infection. *Current Opinions in HIV & AIDS*; 5:491-497

Silva R, Moir S, Kardava L, Debell K, Simhadri VR, Ferrando-Martinez S, Leal M, Peña J, Coligan JE, & Borrego F (2011). CD300a is expressed on human B-cells, modulates BCR-mediated signaling and its expression is down-regulated in HIV infection. *Blood*; 117(22):5870-5880

Simhadri VR, Andersen JF, Calvo E, Choi S, Coligan JE, & Borrego F (2012). Human CD300a binds to phosphatidylethanolamine and phosphatidylserine and modulates the phagocytosis of dead cells. *Blood*; 119(12):2799-2809

Sims GP, Ettinger R, Shirota Y, Yarboro CH, Illei GG, & Lipsky PE (2005). Identification and characterization of circulating human transitional B-cells. *Blood*; 105(11):4390-4398

Sohn HW, Krueger PD, Davis RS, & Pierce SK (2011). FcRL4 acts as an adaptive to innate molecular switch dampening BCR signaling and enhancing TLR signaling. *Blood*; 118(24):6332-6341

Sportès C, Babb RR, Krumlauf MC, *et al.* (2010). Phase I Study of Recombinant Human Interleukin-7 Administration in Subjects with Refractory Malignancy. *Clin Cancer Res*; 16:727-735

Steinbrink K, Mahnke K, Grabbe S, Enk AH, & Jonuleit H (2009). Myeloid dendritic cell: From sentinel of immunity to key player of peripheral tolerance? *Human Immunology*; 70(5):289-93

Steinman RM & Banchereau JB (2007). Taking dendritic cells into medicine. *Nature*; 449:419-426

Tan YV, Abad C, Wang Y, Lopez R, & Waschek J (2014). VPAC2 (vasoactive intestinal peptide receptor type 2) receptor deficient mice develop exacerbated experimental autoimmune encephalomyelitis with increased Th1/Th17 and reduced Th2/Treg responses. *Brain Behaviour and Immunity;* doi: 10.1016/j.bbi.2014.09.020

Tangye SG, & Tarlinton DM (2009). Memory B-cells: Effectors of long-live immune responses. *European Journal of Immunology*; 39:2065-2075

Tarlinton DM (2006). B-cell memory: are subsets necessary? *Nature Reviews Immunology*; 6(10):785-790

Thapa DR, Bhatia K, Bream JH, D'souza G, Rinaldo CR, Wolinsky S, Detels R, & Martinez-Maza O (2012). B-cell activation induced microRNA-21 is elevated in circulating B cells preceding the diagnosis of AIDS-related non-Hodgkin lymphomas. *AIDS*; 26(9):1177-1180

Thayer JF & Sternberg EM (2010). Neural aspects of immunoodulation: Focus on the vagus nerve. *Brain, Behaviour & Immunity*; 24(8):1223-1228

Titanj, K, De Milito A, Cagigi A, Thorstensson R, Grützmeier S, Atlas A, Hejdeman B, Kroon FP, Lopalco L, Nilsson A & Chiodi F (2006). Loss of memory B cells impairs maintenance of long-term serologic memory during HIV-1 infection. *Blood*; 108:1580-1587

van Grevenynghe J, Cubas RA, Noto A, DaFonseca S, He Z, Peretz Y, Filali-Mouhim A, Dupuy FP, Procopio FA, Chomont N, Balderas RS, Said EA, Boulassel M, Tremblay CL, Routy J, Sékaly R, & Haddad EK (2011). Loss of memory B cells during chronic HIV infection is driven by Foxo3a- and TRAIL-mediated apoptosis. *J Clin Invest*;121(10):3877-88.

Vinuesa CG & Chang PP (2013). Innate B cell helpers reveal novel types of antibody responses. *Nat Immunol*; 14(2):119-26. doi: 10.1038/ni.2511

Wang Y & Carter RH (2005). CD19 regulates B-cell maturation, proliferation, and positive selection in the FDC zone of murine splenic germinal centres. *Immunity*; 22:749-761

Wiik P, Opstad PK & Boyum A (1985). Binding of vasoactive intestinal peptide by human blood monocytes: demonstration of specific binding sites. *Regulatory Peptides*; 12:145-153

Wilson TJ, Fuchs A, & Colonna M (2012). Cutting edge: Human FcRL4 and FcRL5 are receptors for IgA and IgG. *The Journal of Immunology*; 188(10):4741-4745

Wu H & Bondada S (2009). CD72, a coreceptor with both positive and negative effects on B lymphocyte development and function. *J Clin Immunol*; 29:12-21

Zhou T, Georgiev I, Wu X, Yang ZY, Dai K, Finzi A, Kwon YD, Scheid JF, Shi W, Xu L, Yang Y, Zhu J, Nussenzweig MC, Sodroski J, Shapiro L, Nabel GJ, Mascola JR, & Kwong PD (2010). Structural basis for broad and potent neutralization of HIV-1 by antibody VRC01. *Science*; 329(5993):811-7. doi: 10.1126/science.1192819