

**INVESTIGATION OF B CELL SUBSETS AND
VACCINATION RESPONSES IN HIV-1
INFECTION AND CVID**

Melanie Sarah Hart

Imperial College London

Department of Medicine

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Declaration

I, Melanie Sarah Hart, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

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Abstract

The contribution of intrinsic defects in B and/or T cell function or impaired T-B cell interaction towards poor recall and neo-antigen vaccine responses in HIV-1 infection are not fully understood. Using CVID as a model for B cell maturation, we show patients with untreated HIV-1 infection have increased transitional and tissue like B cells and reduced IgM memory and class switched memory B cell proportions. Loss of IgM memory B cells is associated with progressive HIV-1. Antiretroviral therapy reduces transitional and tissue like B cell percentages but does not restore IgM memory or class switched memory proportions. Most HIV-1 patients on ART have reduced antibody levels post tetanus and pneumococcal vaccination. IgM memory B cell depletion associates with poor post vaccine IgM pneumococcal titres in HIV-1 suggesting loss of IgM memory B cells may be a risk factor for invasive pneumococcal disease. CVID patients with lung disease had lower memory B cells and a trend towards a loss of IgM memory B cells. IgM memory B cell percentages were protective against bronchiectasis in CVID patients displaying extremely low class switched B cell percentages.

Evaluation of proteins implicated in the pathogenesis of PID, autoimmunity and malignancy, showed increased expression of BAFF and APRIL in CVID and untreated HIV-1 and normalisation by ART. BAFF upregulation was associated with CD4 T cell decline without treatment. Expression of BAFF and APRIL ligands demonstrated decreased BAFF-R on class switched memory B cells in HIV-1 and increased TACI on tissue like and memory B cells. A loss of follicular helper T cells in untreated HIV-1 infection was reported, however this was not a selective depletion and numbers were normalised by ART.

In conclusion, we identify multiple novel defects in B cell composition in HIV-1 and suggest these may have implications for the design of effective vaccination strategies.

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Common Abbreviations

Ab	Antibody
Ag	Antigen
AIDS	Acquired Immune deficiency syndrome
APC	Antigen presenting cell
APRIL	A Proliferation-inducing Ligand
ART	Anti-Retroviral Treatment
BSA	Bovine Serum Albumin
BAFF	B cell Activating Factor of the TNF family
BAFF-R	BAFF Receptor
BCMA	B cell Maturation Antigen
BCR	B cell Receptor
CAML	Calcium-modulator and cyclophilin Ligand
CD	Cluster of Differentiation
CpG	C-Phosphate-G
CSB	Class switched memory B cell
CSR	Class switch Recombination
CV	Co-efficient of Variation
CVID	Common Variable Immune Deficiency
DC	Dendritic Cell
ELISA	Enzyme-linked Immunosorbent Assay
FCS	Foetal Calf Serum
FSC	Forward Scatter
FDC	Follicular Dendritic Cell
GC	Germinal Centre
HC	Healthy Control
HIV-1	Human Immunodeficiency virus-strain 1
HRP	Horse radish Peroxidase
ICOS	Inducible Co-stimulatory molecule
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IPD	Invasive Pneumococcal Disease

IQR	Inter quartile range
LPS	Lipopolysaccharide
LSS	Lymphocyte Subset analysis
MAb	Monoclonal Antibody
MBL	Mannose Binding Lectin
MFI	Median Fluorescence Intensity
MHC	Major Histocompatibility Complex
MZB	Marginal zone B cell/IgM memory B cell
NK	Natural Killer cell
OD	Optical Density
OPD	o-Phenylenediamine
PAMPs	Pathogen-Associated Molecular Patterns
PID	Primary immune deficiency
PMT	Photo Multiplier Tube
PRR	Pattern recognition receptor
PBS	Phosphate buffered Saline
PBST	Phosphate buffered Saline with Tween
RA	Rheumatoid Arthritis
RAG	Recombination Activation Gene
SHM	Somatic Hypermutation
SD	Standard Deviation
SLE	Systemic Lupus Erythrematosus
SSC	Side Scatter
TAC1	Transmembrane activator and calcium-modulator and cyclophilin ligand Interactor
TCR	T cell Receptor
Tg	Transgenic
Th	Helper T cell
TLR	Toll-Like Receptor
TSLP	Thymic stromal lymphopoetin
VDJ	V-D-J gene rearrangement
VL	Viral Load

1. Chapter I

1.1 General Introduction

‘I attained a triumph so complete that it is now rare to meet an American with marks of small pox on his face... Benefits are valuable according to their duration and extent, like the showers from heaven, but the benign remedy Vaccination saves millions of lives every century, like the blessing of the sun, universal and everlasting’.

— Benjamin Waterhouse (1754-1846)

The immune system is a powerful defence tool that protects the body against infectious agents and toxins using a diverse variety of effector cells and molecules. The immune system comprises the lymph organs such as the bone marrow, spleen, thymus, lymph nodes, the mucosal lymphoid tissues and the lymphatic vessels which drain free cells and extracellular fluid from tissues. Haematopoietic stem cell precursors in the bone marrow mature to become the white blood cells of the immune system, which then patrol the lymphatic system, peripheral blood and tissues. T and B lymphocytes and NK cells derive from lymphoid progenitor cells. Myeloid progenitor cells give rise to the other white blood cells: granulocytes, macrophages, mast cells and dendritic cells. Conversely, immune deficiency, both genetic and acquired, is a considerable problem in the twenty-first century and associated with high levels of morbidity and mortality.

1.2 Overview of immune response to infection

If a pathogen has been able to breach the physical barriers of the body such as epithelia and mucosa, the frontline of host defence is the innate immune system, which acts within minutes of microbial infection. If this innate mechanism is overcome, the adaptive immune system has evolved in vertebrates to fight infection in cooperation with the innate system. The adaptive immune response is slow, taking approximately five days to develop, primarily because antigen specific clonal selection and proliferation needs to occur.

1.3 The Innate Immune System

Innate immunity is the first line of defence in the immune response and is effective enough to prevent the body being overwhelmed by the majority of infectious agents (pathogens). The innate immune system senses microbes and tissue damage to clear localised infections rapidly without antigen specificity and initiates tissue repair. It comprises complement plasma proteins, cytokines and inflammatory cells. Recognition of pathogens is firstly by preformed non-specific effectors and specific effectors. The early induced innate response requires recognition of microbial-associated molecular patterns, which then cause inflammation, recruitment and activation of effector cells. One important example is the release of interferons upregulated by innate cell stimulation, which then activate NK cells to aid innate host defence. The innate immune system can also influence the level and type of adaptive immune response and also act to aid the adaptive immune response if necessary.

Inflammation is caused by cytokines, chemokines and other mediators acting on local blood vessels to cause heat, swelling, pain and endothelial permeability so that cells can migrate into tissues. Neutrophils in large numbers are chemo-attracted to the sites of inflammation, followed by monocytes which then mature into macrophages. Both neutrophils and macrophages have surface receptors for common bacterial molecules and complement, these scavenger cells can then engulf and destroy microorganisms.

Pattern recognition receptors (PRR) such as Toll-like receptors (TLRs) and collectins such as mannose-binding lectin (MBL) recognise broad classes of pathogens by the repeating patterns of structure on the surface of microorganisms, known as pathogen-associated molecular patterns (PAMPs). PRRs are non-clonal, are specifically inherited in the genome and can be categorised into secreted, transmembrane, or cytosolic forms. Each innate cell lineage expresses a defined set of PRRs allowing allocation of recognition responsibilities: some are phagocytic receptors, others are chemotactic and others induce production of effector molecules to influence both the innate and adaptive immune responses (Iwasaki and Medzhitov 2004).

The transmembrane PRRs include the TLR family and the C-type lectins. The evolutionarily conserved TLRs, first identified in mammals in 1994, have a key role

in immune defence against bacteria, fungi and viruses (Beutler 2009). Activation of transcription factors such as NF- κ B leads to the transcription of pro-inflammatory genes leading to inflammation and tissue repair.

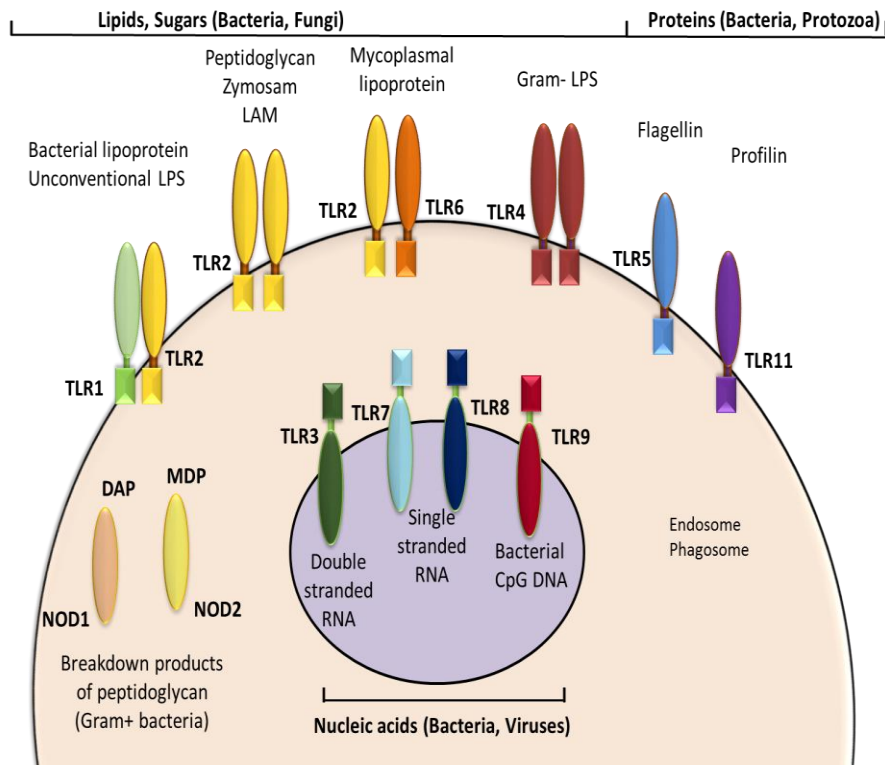


Figure 1: TLR families. Modified from (Rich 2008)

There are ten predominant TLRs described in man, cell surface expressed TLRs include TLR4 expressed by macrophages, which recognises LPS on bacteria by signalling through CD14 and TLR1/TLR2 which detect bacterial lipoproteins. Endosomal TLRs predominantly detect microbial nucleic acids: TLR3 stimulation by viral DsRNA induces production of interferon, whilst CpG dsDNA derived from bacteria is recognised by TLR9 (see Fig.1). Exogenous ligands are also able to trigger activation of adaptive immunity including immunoglobulin antibody responses (Iwasaki and Medzhitov 2010).

Mice deficient in two mutations encoding adaptor proteins (MyD88 and TRIF) do not have TLR signalling and are extremely immunocompromised, demonstrating the important non-redundant role of TLR in host immunity.

Secreted PRRs, which includes pentraxins, collectins and ficolins, bind to microbial cell surfaces, activate the classical and lectin complement pathways and opsonise pathogens for phagocytosis by neutrophils and macrophages. The cytosolic PRRs include the retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) and Melanoma differentiation factor 5 (MDA5), which detect viral pathogens. Recognition through RLRs leads to secretion of factors such as type I interferons (IFNs) to activate DCs. The nucleotide-binding domain and leucine rich repeat-containing receptors (NLRs) NOD1 and NOD2 are also cytosolic PRRs and detect breakdown products of intracellular bacteria.

1.4 Overview of the Adaptive Immune System

The adaptive or acquired immune system is only present in vertebrates. This system is based on antigen specific recognition of 'foreign' molecules that are mainly proteins or peptides to develop an appropriate inflammatory response. The adaptive immune response requires B and T cell activation. A hallmark of the adaptive immune response is immunological memory, which is absent in the innate immune response. Key players of the adaptive immune response are antibodies, T lymphocytes and B lymphocytes. The B and T lymphocytes have clonally rearranged receptors highly specific for antigen.

A key feature of acquired immunity is molecular distinction between self-components and non-self, such as pathogenic components, this role is performed mainly by T cells, so that foreign proteins are recognised as antigens whilst self-proteins are tolerated. T cells must generate specific antigen receptors called the T cell receptor (TCR). Subsequent encounter must ensure these T cells can bind self MHC molecules and non self antigenic peptides. T lymphocytes recognize antigens as a complex of short linear peptides bound to MHC molecules on the surface of APCs.

1.4.1 *T cell differentiation and T cell subsets*

T cells develop in the thymus and go through a process of positive and negative selection before entering the periphery. Thymocytes are initially double negative for CD4 and CD8, subsequently T cells express both CD4 and CD8 (double positive). Mature T cells become single positive for CD4 or CD8. A $\alpha\beta$ TCR is expressed by 85-100% of T lymphocytes in the peripheral blood, the remainder express a $\gamma\delta$ TCR. The two main subsets are labelled according to the TCR co-receptor molecules they singularly express: CD4 T cells and CD8 T cells. The CD4 T cells bind to a non-polymorphic region of MHC class II molecules in extracellular peptide recognition whereas CD8 T cells bind MHC class I containing intracellular peptides. T cells expressing CD4 are commonly referred to as T helper (Th) cells whilst CD8 T cells are known as cytotoxic effector T cells (CTLs). T helper (Th) cells can be subdivided into Th1 and Th2 cells. However, recent work has shown the CD4 T cell compartment can be subdivided into a whole number of subsets on the basis of cytokines secreted, effector functions and cell surface markers (see Fig. 2).

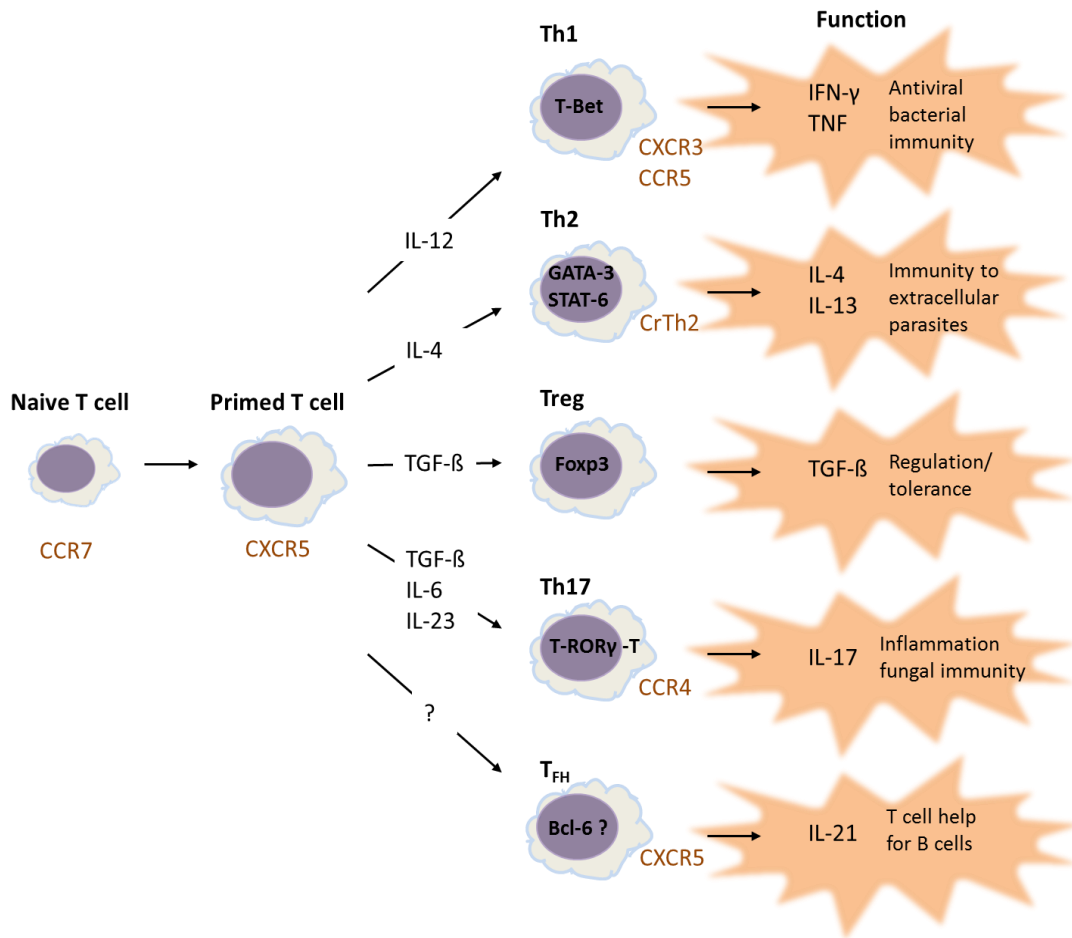


Figure 2: CD4 T cell differentiation model. Modified from (King, Tangye et al. 2008).

Figure 2 describes the development of five effector CD4 T cell subsets that have different, possibly distinct roles in the immune response, governed by the developmental expression of different transcription factors. These CD4 T cells express different chemokine receptors, secrete different cytokines and localise to different sites in the body. It is now believed that IL-21 producing T_{FH} T cells, rather than the previously attributed Th2 T cells are the predominant T cell subset involved in B cell help during the T cell dependent humoral response. Th1 CD4 T cells secrete IFN- γ and TNF, cytokines important for intracellular infection, whilst Th2 cells produce cytokines to aid defence against large extracellular parasites such as helminth infections e.g. IL-4 promotes Ig isotype switching to IgE, key to immune responses against parasitic infections. Foxp3 expressing Regulatory T cells (T_{reg}) are important in immune regulation and maintaining peripheral tolerance. Th17 cells are a recently discovered helper T cell subset that has a key role in the immune response against

fungi and mucosal bacterial infections. Th17 have some functional similarities to T_{FH} cells and also produce IL-21.

1.5 B Cell Immunology

1.5.1 B cells and antibodies in host defence

Identification of B cells only took place in the late 1960's and early 1970's with the advent of flow cytometry, studies of human immune deficiency and experimental animal models (LeBien and Tedder 2008). Each B cell produces antibody or immunoglobulin of a single specificity. Immunoglobulins have sequence variability in the light and heavy chain amino acids (Fab region), whilst antigen binding can be translated into effector functions based on the Fc region, the larger non variable portions of its heavy chains. Antibodies have three principle roles in immune defence: neutralisation, opsonisation and complement activation. In neutralisation, antibodies typically bind to molecules such as viral surface proteins, surface glycoproteins or bacterial toxins and block pathogenic interaction with host cells (attachment, penetration or blocking viral uncoating). This decreases viral replication. It is believed that neutralization *in vitro* is usually related to protection *in vivo*. During opsonisation, antibody or antibody activated complement binds to receptors on the cell membrane of a pathogen to label it for rapid degradation and removal by phagocytosis (opsonophagocytosis). Complement activation is another effector mechanism mediated by antibodies however human immunoglobulin isotypes vary considerably in their ability to activate complement. IgM, IgG1, and IgG3 isotypes are effective activators of the classical complement pathway.

1.5.2 Structure of the B cell Receptor

The B cell receptor (BCR) is a transmembrane bound immunoglobulin on the B cell surface that has specificity for antigen. Plasma cells secrete immunoglobulin of the same antigen specificity. Each antibody has a variable (V) and a constant region (C) which is less variable (see Fig.3). An antibody has a paired heavy and light chain arrangement. Five different classes of antibodies are described by variations in the constant region (IgG, IgA, IgM, IgE and IgD).

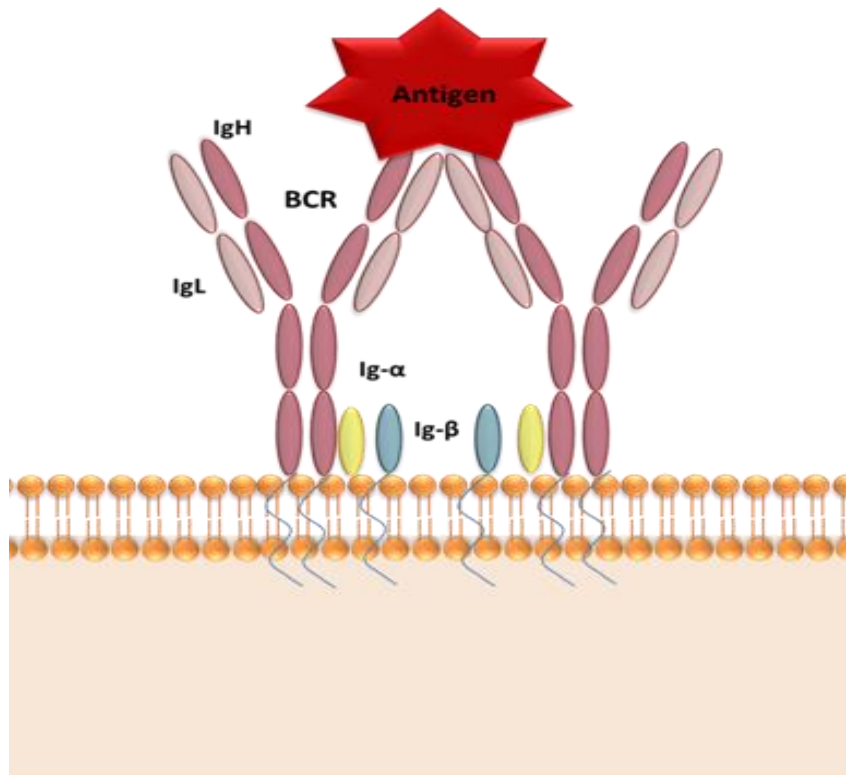


Figure 3: The B cell Receptor. Modified from (Rich 2008).

Ig α (CD79a) and Ig β (CD79b) also form part of the BCR complex (see Fig.3) and act to transduce binding signals inside the cell. B cell maturation cannot take place without an intact BCR complex, loss of function mutations cause B cell deficient agammaglobulinaemia (Vale and Schroeder 2010). Mutations in proteins involved in the BCR signal transduction: Bruton tyrosine kinase (BTK) or B cell linker protein (BLNK) also lead to B cell deficient agammaglobulinaemia (Vale and Schroeder 2010). A B cell co-receptor complex, composed of three cell surface proteins CD19/CD21/CD81, enhances antigen dependent signalling through the BCR by binding to its ligand. CD19 is required to modulate BCR transduction; human CD19 deficiency does not affect B cell numbers but causes panhypogammaglobulinaemia and CVID (van Zelm, Reisli et al. 2006). The role of CD81 remains to be determined. CD21 (complement receptor 2, CR2) is a receptor for the complement fragments C3d/C3dg (degraded C3d). Bacterial surface proteins bound to C3d cross-link the CD19/CD21/CD81 complex with the BCR and upregulate intracellular signalling to amplify B cell activation by activating additional signal pathways. Upregulated intracellular signal pathways may aid B cell differentiation and antibody production and induce the expression of co-stimulatory molecules on the B cell surface.

1.5.3 B cell Development

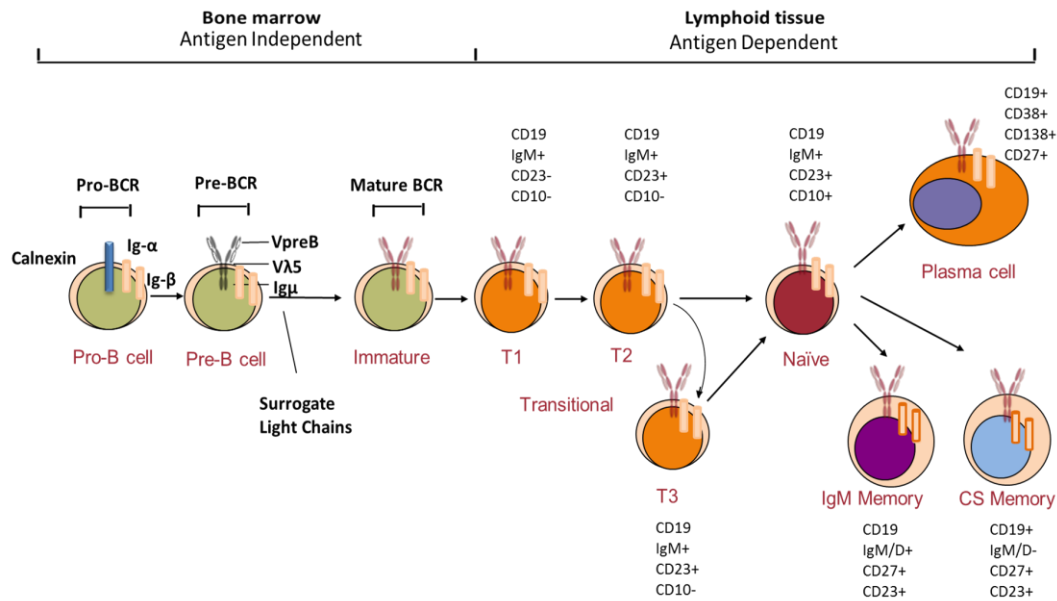


Figure 4: Stages of B cell development, focusing on antigen independent and antigen dependent stages. Adapted from (Cambier, Gauld et al. 2007).

In the bone marrow, the earliest B lineage cells (early pro-B cells) are derived from stem cell lymphoid progenitors interacting with IL-7 secreting stromal cells (see Fig. 4). Production of B cells declines with age, although these cells are produced by the bone marrow throughout life. A primary antigen specific receptor is generated by undergoing VDJ recombination as the cell matures. At this point in B cell development, the early pro B cell has little regenerative capacity and has started to rearrange the immunoglobulin heavy chain locus at the DH to JH joining region. At the late pro-B cell stage, VH to DJH joining occurs.

The large pre-B cell stage heralds the intracellular expression of an intact U heavy chain, some surface expression allows combination with surrogate light chain to form the pre-B cell receptor and complete heavy chain VDJ recombination. Division of the large pre-B cell creates a progeny of small pre-B cells, which re express RAG proteins and rearrange the light chain genes, first at the kappa locus, then if not successful, the lambda locus. Production of an IgM BCR at the cell surface signals the

cell as an immature B cell (Fig.4); expression of a mature functioning antigen receptor requires a selection process to ensure self- tolerance, at which time most B cells undergo apoptosis by negative selection, clonal deletion or undergo receptor editing.

Under the influence of B cell activating factor of the TNF family (BAFF), a proliferation inducing ligand (APRIL) (Schneider 2005), other chemokines such as thymic stromal lymphopoetin (TSLP) (Astrakhan, Omori et al. 2007), antigen naive IgM+IgD+CD27⁻ B cells exit the bone marrow, circulate peripheral blood as transitional T1 cells and home to the spleen or other secondary lymph organs, to further mature as T2 and T3 transitional B cells. BAFF and APRIL are primarily expressed by monocytes, DCs, B cells and macrophages and have three receptors (BCMA, TACI and BAFF-R). Further maturation into immunoglobulin secreting plasmablasts or memory CD27⁺ B cells requires complex interactions between survival factors such as BAFF, APRIL and antigen mediated BCR signals (see Chapter VI for further information on the BAFF /APRIL chemokine axis and receptors).

1.5.4 B Cell Subsets

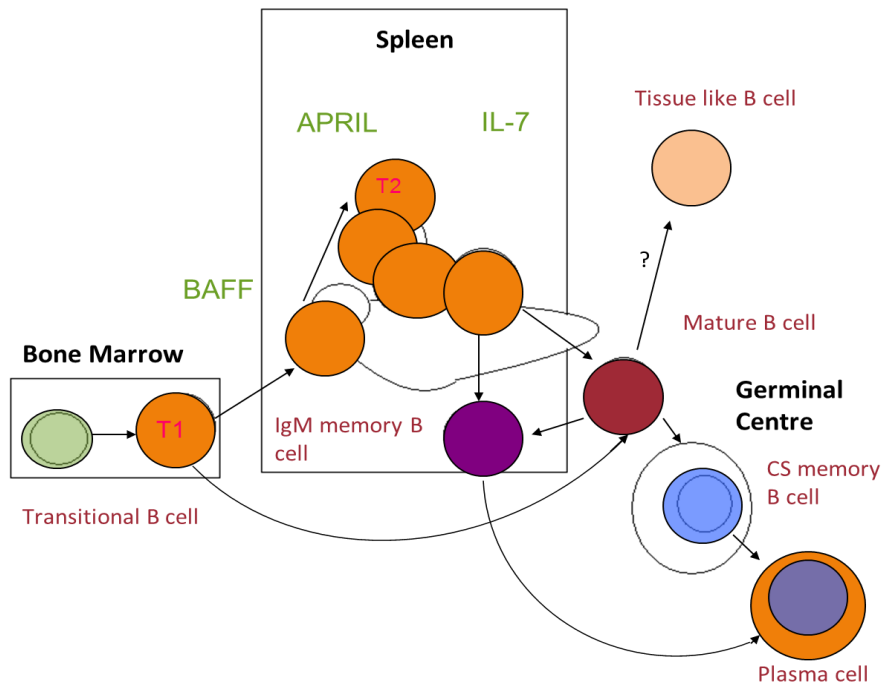


Figure 5: Origin of memory B cells. Adapted from (Carsetti, Rosado et al. 2004).

B cell subsets are currently divided into transitional B cells, naïve mature B cells, tissue like B cells, IgM memory, class switched memory and plasma cells based on cell surface markers and function (see Fig. 5). Transitional B cells are described as $CD19^+IgM^{++}CD38^{++} CD24^+CD27^-CD10^+$ and represent B cells at an immature stage of B cell development, that are found at increased frequencies in cord blood, are increased in the peripheral blood of infants and decline with age in the healthy host, representing <1-10% of adult peripheral B cells [14]. Forty per cent of transitional B cells are self-reactive and so approximately half of all transitional B cells are removed from the B cell repertoire before maturation into mature naïve B cells can occur. Transitional B cells are poorly characterised in man, since most of our current understanding is derived from murine data. In mice, transitional B cells have been split into three maturational subsets (T1, T2, and T3), however, in man, subsets are not as well defined. See Chapter III discussion for a description of recent advances in transitional B cell biology. Transitional B cells exit the bone marrow and migrate to the spleen to mature into mature naïve or memory B cells. Mature naïve B cells, so called because they are naïve to antigen, phenotypically express $CD19^+IgD^+CD27^-$

and represent the majority of peripheral blood B cells (50-70%). Somatically mutated memory B cells express CD27 (Klein, Rajewsky et al. 1998), persist after antigen challenge, expand quickly in response to secondary antigen responses and are able to differentiate into terminally differentiated plasma cells. CD27⁺ memory B cells represent a large proportion (40%), of the B cell pool in the blood and lymph nodes of healthy individuals (Weller, Braun et al. 2004). CD27⁺ memory B cells comprise two subsets: IgM memory B cells are CD19⁺CD21⁺CD27⁺IgD⁺IgM⁺ and are important in T cell independent immune responses early in infection, whilst class switched memory B cells are CD19⁺CD21⁺CD27⁺IgD⁻IgM⁻ and are important for adaptive T cell dependent humoral responses. Plasma cells (plasmablasts) are thought to be differentiated from both CD27⁺ memory pools (IgM memory and class switched memory B cells), secrete large amounts of specific immunoglobulin and are phenotypically CD19⁺CD27⁺⁺CD38⁺⁺. Plasma cells are extremely long-lived and are replenished from the pool of memory B cells only when required (DiLillo, Hamaguchi et al. 2008). As shown in Fig.5, B cell maturation in man is unclear: two developmental pathways have been proposed: the first suggests transitional B cells mature into splenic marginal zone B cells (MZB cells, also known as IgM memory B cells) which secrete IgM or instead mature into class switched B cells memory B cells in the germinal centre (also known as germinal centre B cells). The alternative model proposes that transitional cells mature into germinal centre derived B cells and then a proportion differentiate into a IgM memory B cell population (Liu, Oldfield et al. 1988; Vinuesa, Sze et al. 2003). The ontogeny of the recently discovered tissue like B cell subset is unclear. These cells have a CD19⁺CD21⁻CD27⁻CD10⁻FCLR4⁺CD11c⁺ phenotype and are believed to be enriched in tissues such as the tonsils (Ehrhardt, Hsu et al. 2005).

1.5.5 IgM memory B cells

IgM memory B cells have a CD19⁺CD27⁺IgM⁺IgD⁺CD21⁺ phenotype and circulate in the peripheral blood and the marginal zone of the spleen (Weller, Braun et al. 2004). IgM memory B cells produce high titre specific IgM antibodies to neutralise viruses e.g. H1N1 (Throsby, van den Brink et al. 2008) and influenza (Baumgarth, Herman et al. 2000) and act with complement during opsonophagocytosis of encapsulated bacteria such as *Streptococcus pneumoniae*. The loss of the IgM memory B cell subset may be responsible for the increased risk of invasive

pneumococcal disease in primary immune deficiency (PID) (Carsetti, Rosado et al. 2005), the elderly, young infants and those with hyposplenism (Carsetti, Pantosti et al. 2006). These individuals cannot mount effective T-independent immune responses against encapsulated bacterial pathogens that can suddenly cause a systemic infection (*Haemophilus influenzae B*, *Streptococcus pneumoniae*) and cannot be immunised with unconjugated polysaccharide vaccines (Timens, Boes et al. 1989). In young infants, it has been recently shown that a IgM memory B cell subset is present (at reduced percentages to adults) but seemingly immature since the IgM memory B cells did not seem to have any signs of antigen driven expansion or activation using H-CDR3 spectra typing (Weller, Mamani-Matsuda et al. 2008), suggesting immaturity of IgM memory B cells may be a key factor in predisposition to invasive pneumococcal disease in infants. Whilst the ontogeny of IgM memory B cells is unclear, IgM memory B cells have been shown to arise from CD24⁺CD38⁺ transitional B cells in response to CpG antigens (Capolunghi, Cascioli et al. 2008) and are thought to be already prediversified whilst antigen naïve (Weller, Braun et al. 2004). In this study, one pre mutated IgM clone was shown to be expanded in response to polysaccharide vaccination, IgM and IgG plasma clones derived from this clonotype were still found in the spleen and blood four weeks later (Weller, Braun et al. 2004). Unlike class switched memory B cells which show a oligoclonal CD3 spectra typing profile, IgM memory B cells have a polyclonal profile, consistent with a non-T cell antigen driven response (Weller, Mamani-Matsuda et al. 2008).

Controversy surrounds whether IgM memory B cells are *bona fide* memory B cells. Accepted hallmarks of memory are somatic hypermutation within Ig V region genes, antigen binding ability and CD27 expression (Tangye and Tarlinton 2009). Isotype switching is also an established marker of memory B cells, a process which does not take place during IgM memory B cell development (Coffman and Cohn 1977). Some authors believe IgM memory B cells are simply B cells that have aborted class switch recombination in the germinal centre, however studies have proven that IgM memory B cells have undergone SHM and that SHM takes place before isotype switching. Some conclude that IgM memory B cells are not *bona fide* memory B cells because SHM may have occurred during antigen naïve development of the pre-immune repertoire and IgM memory B cells are involved in T cell independent immune responses outside a GC (Weill, Weller et al. 2009). This is controversial, since GC

formation may not always be a prerequisite for memory, or GC formation exclusively for T cell dependent reactions involving class switched memory B cells. For instance, small GCs are found in XLP patients deficient in class switched memory B cells but IgM memory B cell populations are still present (Ma, Pittaluga et al. 2006). Some authors prefer these cells are called marginal zone B cells (MZB), due to the fact that these IgM memory B cells also express IgD, albeit at low levels (Tangye and Good 2007; Lanzavecchia and Sallusto 2009). Additionally, others believe that IgM memory B cells are a separate B cell lineage entirely as described in rodents and should be referred to as MZ B cells to avoid confusion (Weill, Weller et al. 2009). Further work is needed to de mystify the molecular processes of B cell memory and B cell maturation before this debate can be closed. For ease of description, this thesis refers to IgM memory B cells.

1.5.6 Class switched memory B cells

Class switched memory B cells are classical CD27⁺ memory B cells so-called because isotype-switching of the BCR has taken place, therefore these cells express either IgG, IgA or IgE, depending on the cytokine milieu in the germinal centre. Early studies of the B cell compartment determined that most memory B cells in the primary immune response express IgG (Coffman and Cohn 1977; Liu, Oldfield et al. 1988). Class switched B cells are part of the adaptive immune system and have a key role in T cell dependent humoral immune responses to infection or vaccination with protein antigens. These highly somatically hypermutated memory B cells form a long-lived memory B cell pool to provide durable humoral memory in adults. Immunisation causes the memory B cell pool to proliferate and produce predominantly antigen specific plasma cells in approximately seven days, accompanied by an increase in serum antibody levels at ten days or so. Re-infection or a booster vaccination leads to an enhanced immune response typified by rapid differentiation of antigen specific memory B cells into antibody-producing B cells. Serological immune memory is a long-lived response, applying to both plasma cells and class switched memory B cells (Lanzavecchia and Sallusto 2009), studies have shown that protein vaccine antibodies have a half-life of 15 to 20 years, whilst responses to attenuated viral vaccines are even longer.

Class switched memory B cells are dependent on T cell help for maturation and function. A lack of class switched memory B cells is seen in many primary immune deficiency diseases associated with considerable mortality and morbidity, indicating the importance of these memory B cells to a functioning immune system. Studies involving humoral immune deficiencies (XLP, Hyper-IgM syndrome, ICOS deficiency) in which cognate CD4 T cell help does not occur, have defective/absent/immature germinal centre formation and lack of class switched memory B cells. IgM memory B cells, which do not require T cell help may be present (Tangye and Good 2007). The main reservoir of class switched B cells is in lymphoid tissues such as bone marrow or spleen.

1.5.7 Response to infection by B cells and plasma cell differentiation

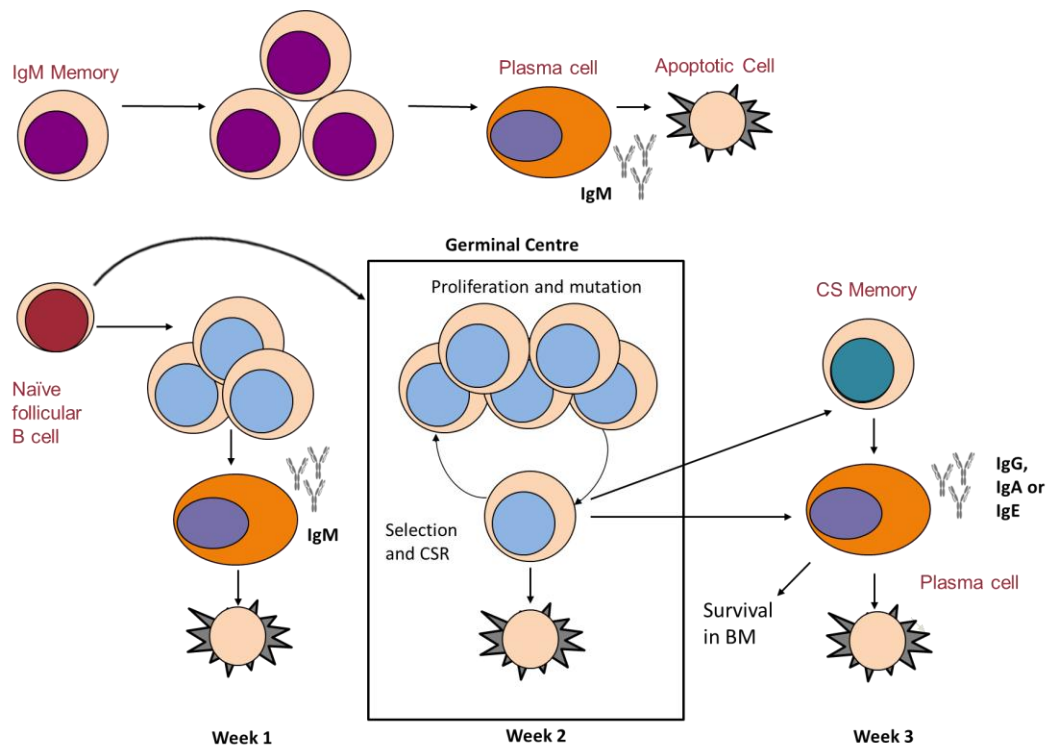


Figure 6: Plasma Cell Formation. Modified from (Shapiro-Shelef and Calame 2005)

Humoral immune responses take place in the secondary lymph organs such as the lymph nodes or spleen and can be T cell dependent or T cell independent depending on the nature of the antigen. Innate T cell independent humoral immunity acts at three to four days post antigen stimulation to rapidly produce low affinity short lived IgM+

antibody responses to capsular carbohydrates. The T cell dependent adaptive response is only effective against protein antigens such as tetanus toxoid. During a T cell dependent B cell response, optimal expansion, differentiation, and class switching of naïve B cells requires BCR triggering, T cell help and a third signal from TLR agonists or by cytokines secreted by activated dendritic cells. The T cell dependent pathway starts acting at five to six days post antigen stimulation, a germinal centre reaction and CSR follows, eventually producing long lived high affinity antibody responses by class switched memory B cells. Some long-lived plasma cells will reside in the bone marrow (see Fig. 6).

1.5.8 Somatic hypermutation and Class Switch Recombination

Somatic hypermutation (SHM) creates affinity maturation of the antibody repertoire following exposure to antigen or repeated immunisation. SHM is often dependent on T cell help. Proliferating B cells in the germinal centre dark zone introduce single nucleotide changes into the rearranged variable genes of the Ig molecules. SHM is dependent on activation-induced cytidine deaminase (AID) and involves the transcription of the BCR locus. At least two separate mechanisms are involved: one involves mutation hot spots within the RGYW (purine/G/pyrimidine/A) motif and the second involves error-prone DNA synthesis that may lead to a nucleotide mismatch between the original template and the mutated DNA strand. If this rearrangement creates a receptor with higher affinity for antigen presented by an FDC, this B cell is preferentially activated to differentiate into a memory B cell or plasma cell. Many other B cell progeny with lower antigen affinity are thought to die by apoptosis.

Class switch recombination (CSR) is the mechanism which allows the B cell to express alternative BCRs to lead to the specific production of effector IgG, IgA or IgE antibodies. Once B cells have left the bone marrow and entered the periphery, the maturing B cell expresses an IgD BCR in addition to IgM. IgM and IgD antibodies use the same VHDJH-exon and promoter and co-expression is due to differential termination of transcription and splicing of the C μ and C δ primary transcripts. To express alternative Ig BCRs, activated B cells class switch from expression of their C μ VHDJH-exon to expression of the same VHDJH-exon with one of the downstream CH genes (C α 1,2, C γ 1,2,3,4, or C ϵ) to express IgG, IgA or IgE. CSR can be induced by signals derived from T cells (e.g. CD40L binding to CD40 on the B cell) or T-cell-

independent signals (e.g. LPS). The choice of CH gene transcribed by a B cell is dependent on the cytokine milieu. Secretion of IFN- γ targets CSR to IgG2 in humans. Signals from cytokines and B-cell activation-inducing ligands activate TATA-less promoters located in front of the switch regions to start transcription, which stops after the specific CH gene sequence has been transcribed.

1.5.9 Effector B cells

Each of the antibody classes (IgG, IgA, IgM, IgE) contributes a different variety of immune functions to the immune response. IgM is the principle immunoglobulin class formed during the first encounter with antigen known as the primary immune response. It is initially expressed as an antigen receptor on the surface of B lymphocytes as a monomeric structure comprised of two light (κ or γ) and two heavy (μ) chains. IgM is secreted in a pentameric form: five of the monomeric subunits are held together by a joining (J) chain. IgM is a multivalent antigen binder that fixes complement efficiently as an important component of the early immune response. The synthesis of IgM is not dependent upon the activity of T lymphocytes (T independent) and so this antibody can be thought to be part of the innate response, the so called 'natural antibody' of low affinity and specificity that can aid clearance of microorganisms. IgM is thought to be abundant in the intravascular compartment.

In man, IgG is the most abundant immunoglobulin in serum at concentrations of 5-15g/L. IgG has four subclasses: IgG1, IgG2, IgG3 and IgG4. IgG is the principal antibody type of an adaptive (memory) immune response. IgG has a crucial role in complement fixation (except IgG4). Phagocytes and NK cells express Fc γ receptors to detect IgG bound to microbial antigens. The structure of an IgG molecule comprises two light (κ or γ) and two heavy (μ) chains joined by disulphide bridges. Unlike other immunoglobulin classes, IgG is able to cross the placenta allowing infants to be born with a full repertoire of antigen diversified maternal IgG antibodies. This provides an infant with an important level of antibody protection during the first few months, when antigen-driven immune responses have not developed.

IgA is found at low levels in human serum (0.5-3g/L) but is the most abundant antibody type in the bodily secretions, being found at high concentrations in tears, saliva, and the secretions of the respiratory, genito-urinary and gastrointestinal

systems (resistant to enzymatic digestion). It is actively secreted as a dimer across mucous membranes by attachment of a secretory component. IgA is also abundant in breast milk to provide passive immunity to the neonatal gastrointestinal system. IgA is thought to help prevent a breach of the mucous membrane surface by microbes or toxins. IgA does not fix complement by the antibody-dependent pathway and therefore does not aid phagocytosis.

IgE is involved in Type 1 hypersensitivity reactions and is thought to have evolved to play a protective role in host defence against parasitic infections, especially to helminths. However, IgE is best recognised as a key component of the aberrant allergic immune response seen in susceptible individuals to common aerosol antigens e.g. grass pollen and food borne antigens like peanut, causing asthma, rhinitis and other allergic manifestations including systemic anaphylaxis, which can be fatal. Cross-linking of IgE and antigen by high-affinity receptors for IgE (Fcε) present on mast cells and basophils leads to degranulation, releasing potent biological mediators such as histamine. A biological role for secreted IgD is still unclear but IgD is known to be important as an antigen membrane receptor on mature B cells such as IgM memory B cells or naive B cells. Molecular mechanisms promoting isotype switching from IgM to IgM/IgD are T cell independent.

1.5.10 T cell dependent immune response

The humoral response to protein antigens is dependent on cognate B-T cell germinal centre interactions in the follicular compartments of secondary lymphoid tissues. CD4 T cells are necessary for the formation of the germinal centre (GC) reaction (Liu and Banchereau 1996). Activation of T cells by APCs induces CXCR5 upregulation (Fig.7). Germinal centre (GC) founder B cells bind and process antigen presented by FDCs. T cell help is elicited by CD40 on GC founder B cells binding to its ligand CD40L on the surface of CD4⁺ follicular helper T cells (T_{FH} cell). T_{FH} cells secrete IL-21 and IL-10 to give B cells 'help' to differentiate into antibody secreting cells through IL-21R. This aids the creation of germinal centres where memory B cells with a IgD-IgM-CD27⁺ phenotype (class switched memory B cells) develop and produce high affinity, class switched immunoglobulins of the IgG and IgA isotype as part of the adaptive immune response (Fig.7). The creation of high affinity antibodies during affinity maturation is dependent on T_{FH} cells selecting GC B cells expressing

antibody to bind with high affinity to antigen. Self-reactive B cells/ low affinity B cells are unable elicit T_{FH} help and are removed from the periphery by undergoing apoptosis. T_{FH} cells were initially defined by CXCR5, whose expression gave these T cells the ability to home to B cell follicles (chemo-attracted by its ligand CXCL13).

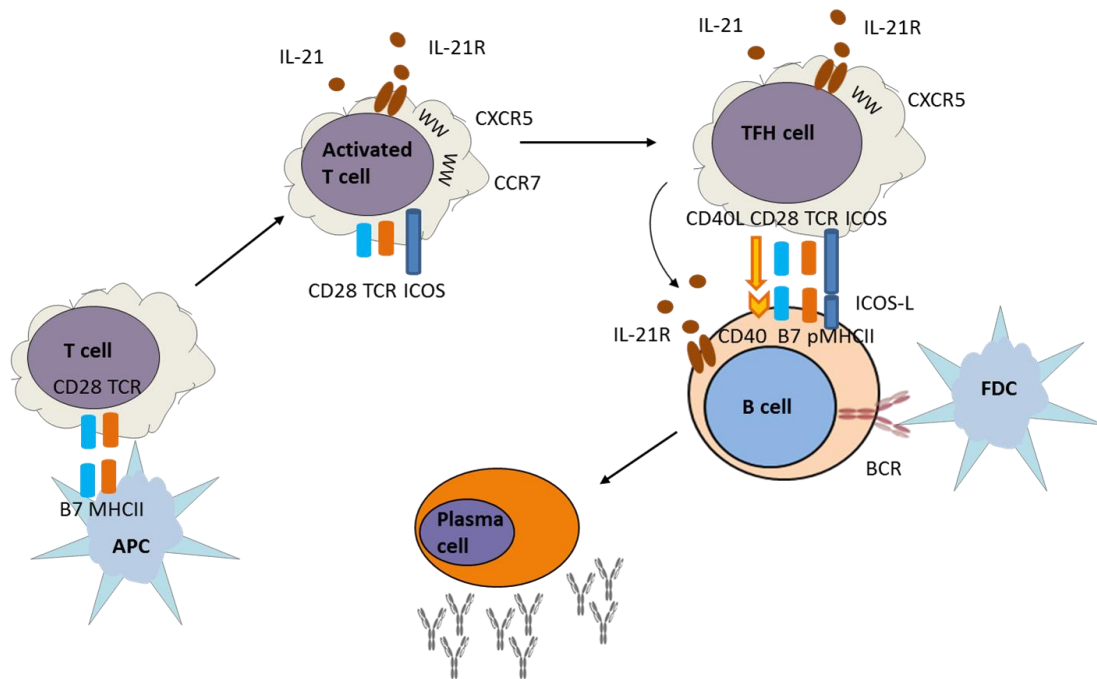


Figure 7: Generation of T cell dependent immune responses and activation of T_{FH} cells. Modified from (King, Tangye et al. 2008).

T_{FH} have a $CD4^+CXCR5^+CD45RO^+$ phenotype and express high levels of the inducible co-stimulatory molecule (ICOS) and signalling lymphocytic activation molecule (SLAM) associated protein (SAP) (see Fig. 7). Functionally, T_{FH} are able to induce GC B cells to secrete antibody (Breitfeld, Ohl et al. 2000; Schaerli, Willimann et al. 2000) through co-stimulatory molecules CD28, OX40 and ICOS (Yu, Batten et al. 2009). T_{FH} cells also express PD-1, CD95, BCL-6 and IL-21R and decreased levels of CCR7 and CD62L expression. T_{FH} cells have recently been shown to be key T cells in the production of IL-2, IL-4 (Reinhardt, Liang et al. 2009), IL-10 and IL-21 (for further details on T_{FH} cells, see Chapter VII).

1.5.11 T cell independent immune response

T cell independent antigen responses by B cells are activated by carbohydrate antigens of bacterial capsular polysaccharides and by Toll-like-receptor (TLR) ligands derived from micro-organisms. The T-independent immune response is principally mediated by marginal zone like B cells of the splenic marginal zone compartment (IgM memory B cells) (Kruetzmann, Rosado et al. 2003; Carsetti, Rosado et al. 2004). Physiologically, the splenic marginal zone compartment is well placed to filter blood borne antigens. It is the first lymphoid organ where B cells can come into contact with blood borne pathogens and has low blood flow, allowing prolonged contact (Harms, Hardonk et al. 1996). The T cell dependent germinal centre is seemingly entirely redundant in the T-independent immune response, as demonstrated *in vivo* by primary immune deficient patients suffering from X-linked hyper IgM syndrome (XHM) (Weller, Braun et al. 2004). XHM patients lack mature lymph node germinal centres, yet still retain mature IgM⁺ memory B cells with high affinity immunoglobulin gene hypermutations.

1.6 HIV-1 infection

1.6.1 HIV-1 Epidemic

The acquired immune deficiency syndrome (AIDS) was first reported in 1981 (Anon MMWR 1981). Five young homosexual men were described as suffering from abnormal CD4:CD8 T cell ratios and amongst other unusual infections, *Pneumocystis carinii* pneumonia, a rare opportunistic pathogen that only causes disease in an immunocompromised host (Anon MMWR 1981). A disease causing gradual loss of CD4+ T cells that was transmitted by sex or blood was suspected but no other similar syndrome had ever been reported (McElrath and Haynes 2010). Two groups independently identified the presence of a retrovirus in the blood of AIDS patients, which was later confirmed to be the same virus, termed Human Immune Deficiency Virus (HIV) and the cause of AIDS (Gallo, Salahuddin et al. 1984; Levy, Hoffman et al. 1984; Montagnier, Chermann et al. 1984). Barré-Sinoussi *et al* (1983) reported cells morphologically resembling a retrovirus and reverse transcriptase activity in lymph node cells taken from a patient with pre-AIDS syndrome. Popovic *et al* (1984) characterized a retrovirus from patients with AIDS patients and showed the presence of antibodies to the virus in AIDS patients. Characterisation of the aetiological agent raised hopes that a vaccine or cure would be developed quickly (Gallo, Salahuddin et al. 1984; Levy, Hoffman et al. 1984; Montagnier, Chermann et al. 1984). Unfortunately, since then, HIV infection has become a global pandemic, with 25 million fatalities already attributed to this disease. The most recent 2009 UNAIDS estimates suggest over 33 million people are living with HIV, with two-thirds of infected cases arising from Sub-Saharan Africa (UNAIDS 2007).

Studies of archived paraffin biopsy sections and molecular dating techniques have determined that HIV-1 originates from an African non-human primate retrovirus (SIVcpz) (Worobey, Gemmel et al. 2008; Korber, Muldoon et al. 2000). It is believed the pandemic group M virus was transmitted by a single chimpanzee to human event in Southern Cameroon (Keele, Van Heuverswyn et al. 2006), probably from hunting for meat (Worobey, Gemmel et al. 2008) and dated to have taken place between 1930 and 1950 (Keele, Van Heuverswyn et al. 2006; Ho and Bieniasz 2008; Worobey, Gemmel et al. 2008). SIVcpz is a Simian Immune Deficiency Virus (SIV), which is naturally non-pathogenic in the natural host (SIVsmm, in sooty mangabeys, SIVcpz in

chimpanzees, SIVagm in African green monkeys). However, SIV is pathogenic to new hosts, animal or human and repeated cross transmission events have led to pathogenic HIV-1, HIV-2 infection in humans and SIVmac infection in Asian macaques (Ho and Bieniasz 2008). Research evaluating immunological differences between pathogenic and non-pathogenic primate lentiviral infection provide useful insights into pathogenesis of HIV and may play an important role in eliminating HIV infection.

1.6.2 Virology

HIV is a lenti or 'slow' retrovirus, that causes a gradual decline in CD4 counts (Rowland-Jones 1999) until symptomatic opportunistic fatal infection (AIDS) occurs in an average time of ten years without therapy. HIV has two genetic subgroups: HIV-1 and HIV-2. HIV-2 is less transmissible and is mainly confined to Western Africa (Duvall, Jaye et al. 2006). Both subgroups of HIV can be transmitted by blood products, needles shared by intravenous drug users, vertical transmission from mother to infant but are predominantly transmitted sexually. Unprotected sexual contact allows cell-free virus in infected bodily fluids such as semen or cell-associated virus in infected mucosal surfaces to cause infection. Despite the documented inefficacy of this mode of transmission, heterosexual infection is now the most common mode of transmission globally. In this introduction, information regarding the natural history of disease and initial infection will focus on sexual transmission of HIV-1 infection.

1.6.3 Life cycle of HIV-1

Upon infection of a target T cell or macrophage, the HIV-1 external glycoprotein (gp120) and envelope protein (gp41) form spikes on the host cell surface. HIV-1 attaches to the target host cell membrane via CD4 and binds to one of its co receptors: CCR5 or CXCR4. CCR5 trophic virus is found early in the infection, whilst in 50% of individuals, CXCR4 trophic virus is seen in advanced disease. Fusion occurs and the virus enters the target cell by pore formation (Fig.8). The viral core is then disassembled to allow viral reverse transcriptase to reverse transcribe the single stranded viral genome for integration into host DNA. HIV reverse transcriptase is highly error prone and has no proofreading activity. Coupled with the high rate of replication of the HIV virus (10^9 to 10^{10} virions per day), the extremely high mutation

rate quickly leads to development of quasi-species and potential drug resistance. HIV integrase mediates the insertion of the viral DNA into the cell chromosomal DNA.

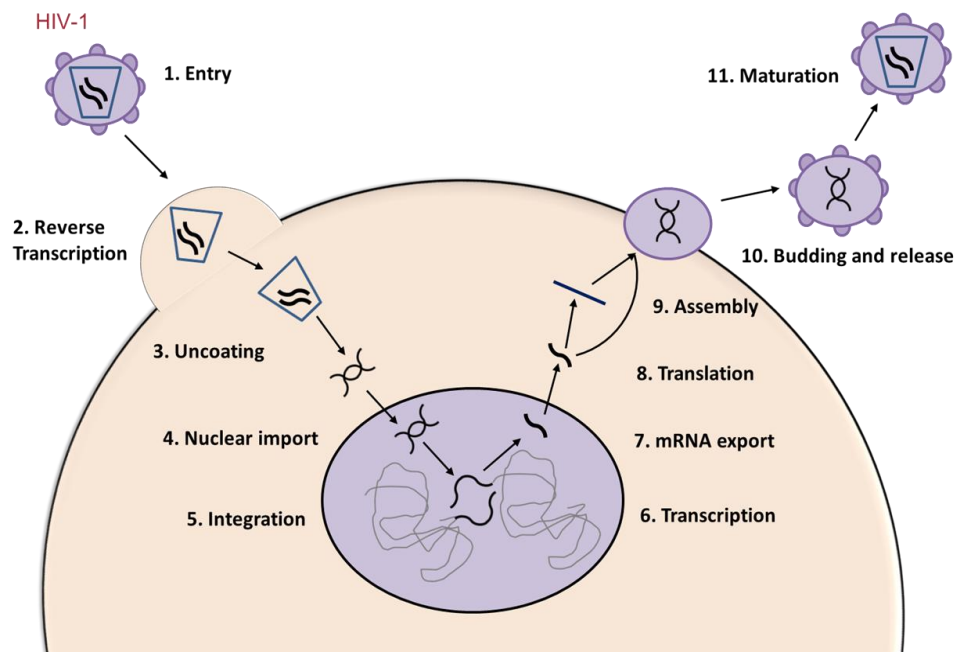


Figure 8: HIV life cycle. Adapted from (Ho and Bieniasz 2008)

The integrated viral genome utilizes the cell's transcription and translation mechanism to encode for the small regulatory proteins Tat and Rev, which amplify and facilitate transcription and translation of HIV genome. The envelope proteins are transported to the cell membrane, while the gag structural proteins form the capsid, incorporating two RNA molecules in the process. In the final stage, the viral particle buds from the host cells, taking a fragment of the cell membrane with the envelope proteins.

1.6.4 Natural History of HIV-1 infection

The initial site of infection during sexual transmission is extra lymphoid: the mucosa of the reproductive tract, the gastrointestinal tract or the skin. HIV-1 preferentially infects CCR5+CD4 T cells, therefore targeting key HIV specific cells (Douek 2002). Monocytes and dendritic cells and Langerhans cells in the foreskin and other sites can also be infected and also possibly contribute to HIV transmission, based on evidence that circumcision offers some protection against transmission (Hladik and McElrath 2008). The virus rapidly migrates via draining lymph nodes to the gut-associated

lymphoid tissue (GALT). Viral replication in the draining lymph nodes also helps the spread of the HIV virus into the bloodstream.

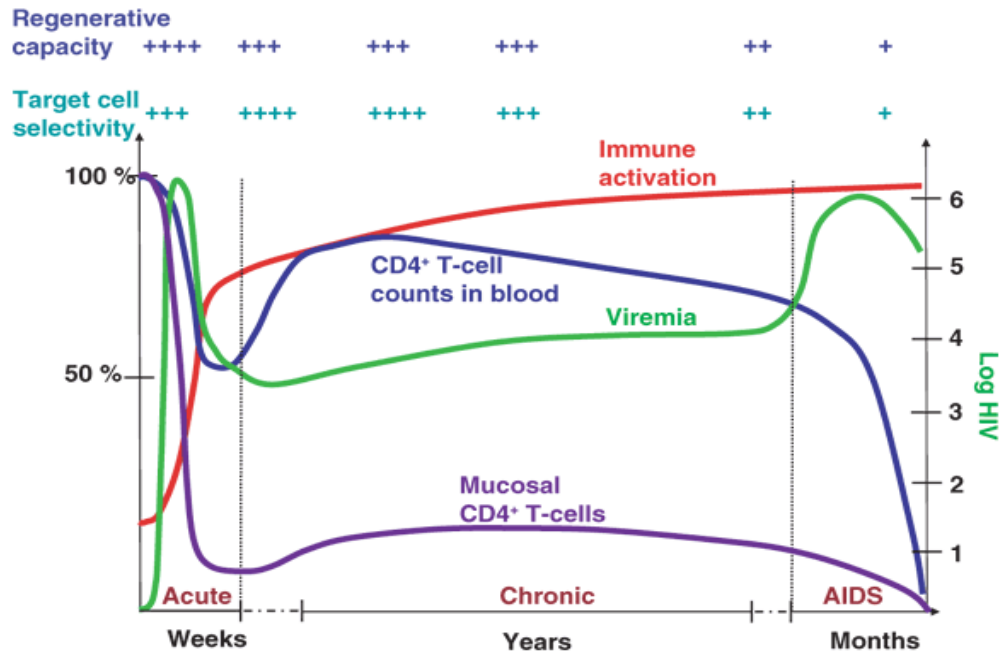


Figure 9: Natural history of HIV infection (Grossman, Meier-Schellersheim et al. 2006)

The virus systemically disseminates to the GI tract, the spleen and other lymphoid organs, where persistent lymphoid tissue viral reservoirs are established. Within days of infection, there is almost an absolute loss of gastro-intestinal mucosal lamina propria CD4 T cells, necessary for effective adaptive immune responses in the tissues (Simon, Ho et al. 2006) (see Fig. 9). Gut associated lymphoid tissue is particularly susceptible to HIV mediated cell death and loss of CCR5+CD4 cells is strongly associated with bacterial translocation and the development of systemic immune activation (Brenchley, Price et al. 2006).

In the absence of an effective immune response, massive viraemia takes place during acute infection. Viraemia rises to a peak at two to four weeks post infection associated with a sharp fallen peripheral CD4 T cell counts (Fig. 9). Loss of CD4 T cells may be caused by bystander apoptosis or direct killing of target cells. At this time, 50 to 90% of infected individuals suffer from acute HIV syndrome or acute seroconversion

illness, an acute febrile flu-like illness and may present to the healthcare system at this point, although few are diagnosed.

During chronic infection, the acute viraemia is partially controlled by HIV specific effector CD8 T cells which reduce and contain the viral load to a stable set point level. CD4 T cell counts recover to become relatively stable for a period of five or more years. Some patients do not progress to AIDS for many more years and control viral load levels without treatment, these so called 'elite controllers' make up less than 1% of the HIV infected subjects.

Immune activation is a dominant feature of the chronic phase of infection and is possibly a mechanism for CD8 immune exhaustion, poor regenerative capacity of T cells and a terminal decline of CD4 T cell numbers into fulminant AIDS without anti-retroviral treatment (ART). AIDS is defined by CD4 T cell counts depleted to <200 cells/ μ l and the presence of AIDS defined opportunistic infections such as *Pneumocystis carinii* pneumonia (PCP) caused by *P. jiroveci*, which may overwhelm the weakened immune system. The exact mechanism of CD4 T cell loss in HIV-1 infection is not known: activation induced cell death (AICD), increased apoptosis, increased cell turnover and HIV-1 direct viral killing may all contribute.

1.6.5 Humoral Immunity in HIV-1 infection

The role of B cells in the natural immune response to HIV-1 infection is controversial. Neutralising antibodies against the virus may appear to aid HIV viraemic control. Clinically, preservation of humoral immunity must be considered in the management of HIV infected patients to prevent invasive disease by encapsulated bacteria, to respond effectively to vaccinations against vaccine preventable diseases such as typhoid or invasive pneumococcal disease and to respond to potential HIV vaccines designed to prevent disease or minimise HIV-1 related immune progression.

1.6.6 B cell defects in HIV-1 infection

Some of the earliest laboratory observations suggested HIV-1 is able to disturb B cell homeostasis in addition to the loss of CD4 T cells (Lane, Masur et al. 1983). Immunoglobulin levels are raised in HIV-1 infection yet HIV-1 does not infect B cells directly, which led observers to aberrantly believe that the humoral immune system was not detrimentally affected by HIV. However, additional studies reported polyclonal activation, increased B cell activation markers (Malaspina, Moir et al. 2003), decreased *in vitro* responses to B cell mitogens such as Pokeweed Mitogen (PWM) (Lane, Masur et al. 1983) and poor *in vivo* response to T cell dependent (Moir, Ogwaro et al. 2003) and T cell independent antigens, even before the CD4 T cell decline had taken place in HIV-1 infected individuals (Miedema, Petit et al. 1988). B cell apoptosis and B cell turnover were found to be increased (Moir, Malaspina et al. 2004). Later, HIV-1 infection was shown to be associated with alterations in B cell subsets in blood and loss of peripheral CD27⁺ memory B cells (Moir, Malaspina et al. 2004; Titanji, Chiodi et al. 2005).

1.6.7 Appearance of undefined CD21^{low} B cells in the peripheral blood of HIV infected individuals

The appearance of an unusual B cell population expressing decreased or absent levels of CD21 (CD21^{low}) in the peripheral blood of viraemic HIV infected individuals has been recognised since 1992 (Benedetto, Di Caro et al. 1992; Scott, Landay et al. 1993). CD21^{low} B cells were shown to have reduced ability to produce immunoglobulins after PWM stimulation (Benedetto, Di Caro et al. 1992) and poor proliferation to B cell stimulation (Moir, Malaspina et al. 2004), yet little else about the definition of these cells was known. Again, this population has been poorly

defined functionally and phenotypically and as such did not fit into known subsets of B cell differentiation. A lack of multiparametric flow cytometry and variable gating or different B cell phenotypic markers used by different research groups to characterise B cell subsets has caused confusion to such an extent that it has been unclear whether some early papers were describing the more recently characterised transitional B cell subset (Sims, Ettinger et al. 2005) which also has decreased CD21 expression and is also expanded in HIV, CVID and SLE (Malaspina, Moir et al. 2006). Recent work has shown that the CD21^{low} B cell fraction is heterogeneous and contains several different cell populations aberrantly expanded in conditions with disturbed B cell homeostasis. CD21^{low} cells have differing functions and variable low expression of CD21: immature transitional B cells (CD21^{int}CD10⁺CD38⁺⁺IgM⁺CD24⁺), activated memory B cells (predominantly characterised in HIV infection: CD21^{int}CD27⁺CD10⁻) and most recently, tissue like B cells (CD21^{low/-}CD27⁻CD38⁻FCLR4⁺/CD11c⁺) have been characterised to date.

1.6.8 Impact of ART on HIV B cell dysregulation

The introduction of ART has made it possible to study which B cell defects remain despite viraemic control and which are reversible defects associated with on-going high levels of viral replication. Those that are reversed by ART include hypergammaglobulinaemia, spontaneous secretion of Ig by B cells in culture, markers of B cell activation and expansions of CD21^{low} B cells (transitional B cells and tissue like B cells). High levels of B cell turnover and apoptosis seen in untreated individuals is also restored to normal levels in ART treated individuals.

The aim of effective ART is effective immune reconstitution and control of viraemia to undetectable levels. If responses to T cell dependent antigens are entirely T cell dependent, there is an expectation that reconstitution of CD4 T cell counts after commencing ART therapy should restore T cell dependent humoral immune responses. However, T and B cell immune reconstitution is often incomplete in ART treated HIV-1 infected individuals and this is a huge obstacle to vaccine development/immune function in pre-existing HIV-1 infection. Functionally, ART treated individuals respond poorly to vaccination. Additionally, there is some evidence that B cell memory is not restored by treatment: total CD27⁺ B cell numbers are reduced in untreated HIV-1 infection (De Mito, Morch et al. 2001; Nagase,

Agematsu et al. 2001; Chong, Ikematsu et al. 2004) and possibly in treated HIV infection (Chong, Ikematsu et al. 2004). However, few studies have examined which of the CD27+ memory B cell subsets (IgM memory B cells or class switched memory B cells) are decreased in untreated and treated HIV-1 infection.

1.6.9 Neutralising antibodies and B cell memory

Natural immunity to HIV does not seem to be protective in man, however, it has been experimentally documented that a few B cells have the ability to produce neutralising antibodies necessary for an effective immune response against HIV (Mascola and Montefiori 2010). Neutralising antibody production commences after seroconversion. In man, in a small minority of HIV-1 infected patients, heterogeneous polyclonal broadly neutralising antibodies are found directed against HIV-1 envelope glycoprotein (Env) epitopes (Mascola and Montefiori 2010). HIV virus fuses with target cells using a trimeric complex involving gp120 and gp41. Neutralising antibodies seem to function by preventing the trimer binding to the CD4 co-receptor (neutralising antibody b12) or by preventing fusion with the target cell (neutralising antibodies 4E10 or 2F5) (Parren and Burton 2001).

Experiments using passive transfer of some of these neutralising antibodies (directed against HIV gp120) have been shown to subsequently protect against HIV infection in a simian model (Baba, Liska et al. 2000) and to delay HIV-1 rebound after stopping ART (Trkola, Kuster et al. 2005). There is also recent evidence *in vivo* to demonstrate that B cell function and neutralising antibodies control long-term viraemia *in vivo* in HIV-1 infection: B cell depletion using Rituximab for a pre-existing lymphoplasmacytoid lymphoma in an HIV-1 infected individual resulted in a decline in autologous neutralizing antibody (NAb) responses and a large rise in HIV-1 plasma viral load (VL) (Huang, Bonsall et al.). In addition, HIV-1 sequences diversified faster than usual and NAb-resistant mutants were selected for growth (Huang, Bonsall et al.).

The existence of some neutralising antibodies is promising and encouraged by the International Aids Vaccine Initiative's Neutralising Antibody Consortium (NAC), who hoped more basic research into Env and monoclonal antibody function would provide better rationale for successful vaccine design (Burton, Desrosiers et al. 2004).

Formulated vaccines so far have not been protective nor produced protective antibody responses. Vaccine design must consider that B cells have other important functions such as antibody-dependent cellular cytotoxicity (ADCC) or complement fixation which may be required to assist viral clearance. Therefore, the humoral immune system is thought to be important to HIV-1 control and to the design of a successful HIV vaccine.

1.6.10 Clinical features of HIV infection: risk of invasive bacterial disease

Patients with HIV-1 infection are at a higher risk of invasive bacterial disease from encapsulated bacteria such as *Streptococcus pneumoniae* and *Salmonella typhi*. *S.typhi* causes the disease known as typhoid fever, is caught from infected food or water and is more commonly found in Asia, Latin America and Africa. The gram negative bacilli are transmitted through the faecal-oral route. Symptoms start several days after infection. Gastrointestinal symptoms commonly occur, multi organ involvement or intestinal haemorrhage are severe complications. Infections can be life threatening if not treated promptly with antibiotics. Patients with HIV infection have an increased risk of *S.typhi* bacteraemia, a longer duration of disease or relapsing disease (Gotuzzo, Frisancho et al. 1991; Khan, Coovadia et al. 1997; Wolday and Erge 1998; Geretti, Brook et al. 2008).

Patients with AIDS are at risk of death from typhoid fever associated colitis or diarrhoea (Geretti, Brook et al. 2008). Non typhoid *Salmonella* (NTS), for example, *Salmonella enterica Typhimurium*, also cause gastroenteritis, which is self-limiting in healthy individuals but a common opportunistic pathogen causing bacteraemia in HIV-1 infection before the era of ART (Prevention 1992; MacLennan, Gilchrist et al. 2010). In developing countries, with poor access to ART, invasive NTS bacterial disease is still causing considerable morbidity and mortality (Chimalizeni, Kawaza et al. 2010).

S.pneumoniae are encapsulated commensal gram positive bacteria that can cause infections of the upper and lower respiratory tract. There are >90 strains of *S. pneumoniae*, however most cases of disease are generally caused by relatively few serotypes. Invasive pneumococcal disease (IPD) is characterised by severe and life threatening complications such as pneumonia, meningitis, pericarditis, septicaemia

and osteomyelitis, that cause disease in a susceptible host. IPD is diagnosed by the isolation of *S. pneumoniae* from a normally sterile site such as the blood, joints and cerebrospinal fluid. Over one million children under five years old die from pneumonia or IPD each year (Bryce, Boschi-Pinto et al.). Factors that predispose to invasive pneumococcal disease are: extremes of age (under five years or over 65 years of age), a non-functioning spleen (splenectomised patients, asplenicism or functional hyposplenism from coeliac disease, sickle cell disease or liver cirrhosis) and immune deficiency, both primary forms and secondary such as HIV infection.

Co-infection with *S. pneumoniae* is a major cause of vaccine-preventable morbidity and mortality amongst HIV-1 infected patients (Redd, Rutherford et al. 1990). Consistent with the general population, *S. pneumoniae* is the most likely cause of bacterial pneumonia and bacterial community acquired pneumonia in HIV-1 infection, however, the risk of invasive pneumococcal disease (IPD) is estimated at 60 times (Jordano, Falco et al. 2004) to 300 times higher than the general population, depending on the stage of HIV disease (Breiman, Keller et al. 2000). Low peripheral CD4 T cell counts seen in advanced disease (<200 cells/ μ l), are associated with the greatest incidence of pneumococcal and bacterial pneumonias (Gebo, Moore et al. 1996; Dworkin, Ward et al. 2001; Lopez-Palomo, Martin-Zamorano et al. 2004) and two pneumonias within a year is recognised by the CDC as a sign of AIDS. Despite the advent of ART being attributed to a decline in IPD (Mocroft, Vella et al. 1998; Heffernan, Barrett et al. 2005), IPD is still a prominent cause of morbidity and mortality in ART treated HIV infection worldwide (Gebo, Moore et al. 1996; Dworkin, Ward et al. 2001; Lopez-Palomo, Martin-Zamorano et al. 2004). The likelihood of nasopharyngeal colonisation by *S. pneumoniae* is also increased in HIV-1 infection even in pneumococcal vaccinated HIV-1 patients on ART and seems to be additionally linked to cigarette smoking (Madeddu, Laura Fiori et al. 2010).

1.6.11 General Principles of Vaccination

Vaccination is one of the greatest triumphs of modern medicine and has led to the eradication of diseases such as smallpox. Vaccine development is on-going for many infectious diseases: recent vaccine successes include two vaccines for Human Papilloma virus (HPV), a virus now associated with cervical cancer development (Gillison, Chaturvedi et al. 2008). However, many vaccines have been developed

empirically by using live weakened or related strains of the disease-causing organism and in a few cases e.g. mumps, the protective immune mechanisms are not understood at all. In addition, natural infections with some pathogens, such as influenza, do not confer immunity. In these cases, alternative mechanisms for improving response to vaccination are sought, such as the use of immunogens e.g. the use of highly immunogenic single recombinant peptides and/or the use of adjuvants to amplify immune responses. Alongside enormous viral diversity of HIV-1 and early establishment of latent infection, lack of basic knowledge and lack of natural immunity are key factors contributing to the lack of a protective vaccine for HIV infection, despite considerable research efforts.

In a healthy individual, the vaccine response is influenced by various factors: dose, route of immunisation, the persistency of the antigen and perhaps memory B cell subset percentages. It is known that in many cases, protection conferred by vaccines involves the humoral immune response and the production of specific antibodies. Thus prevention of infection by vaccination correlates with the induction of specific antibodies (Plotkin 2010). These antibodies aid rapid phagocytosis of the invading organism (opsonophagocytosis) or neutralise bacterial toxins. Vaccine-induced antibody responses are often long-lived, requiring little or no additional boosters to sustain protection from pathogens (Amanna, Carlson et al. 2007). Rare exceptions include the BCG and VZV vaccines, where vaccine protection is mainly cell mediated (Plotkin 2010). The humoral antibody response is complex; perhaps the best vaccines are those that elicit broad ranges of immune responses from both the innate and adaptive immune system that leads to a vigorous humoral antibody response. Indeed, systems biology techniques that measure gene upregulation during successful vaccination indicate many innate genes are upregulated early on after vaccination in addition to adaptive immune system genes (Pulendran, Li et al. 2010).

In the clinical laboratory, measurement of specific antibodies and reference ranges including minimum protective levels are considered correlates of protection for a wide range of organisms including tetanus, *Haemophilus influenzae*, *S.pneumoniae* and hepatitis B. However, some bacteria have evolved mechanisms to evade the immune system including the presence of a capsule therefore not all antibodies are

considered protective. It has been known for many years that *S.pneumoniae* antibodies against cell-wall polysaccharide are not protective, so these are adsorbed before measurement of IgG or IgM total antibodies against Pneumovax II in the routine clinical laboratory. In addition, recent evidence shows that antibodies against NTS include protective outer-membrane protein antibodies and inhibitory antibodies against LPS. These inhibitory antibodies cause defective killing of NTS and contribute to bacteraemia in HIV-1 infected patients, despite higher overall levels of serum antibodies (MacLennan, Gilchrist et al. 2010).

1.6.12 Vaccination in HIV-1 infection

Patients with HIV-1 infection are generally at a higher risk of disease and at risk of more severe disease than the healthy population, therefore, where possible, vaccination is pre-indicated for vaccine-preventable diseases. More boosters may be required due to possible suboptimal level and/or duration of responses to vaccination. The BHIVA guidelines for Immunisation of HIV-infected Adults (2008) (Geretti, Brook et al. 2008), recommend Hepatitis B, pneumococcal PPV23 (Pneumovax II), influenza (parental only) and the combined tetanus-diphtheria-pertussis vaccine are given to all HIV infected individuals. Other vaccines are recommended for a subset of patients at greater risk of disease e.g. Hepatitis A, *H.influenzae* and Meningococcal C (MenC) and for those planning to travel (e.g. rabies, tick-borne encephalitis vaccines). Live vaccinations such as the smallpox vaccine or BCG used to protect against tuberculosis (TB), are generally contraindicated due to risk of disease in an immunocompromised host, however the live measles, mumps and rubella (MMR), yellow fever and varicella vaccines are deemed suitable for HIV-1 infected patients with a CD4 count >200 cells/ μ l. See Chapter IV Introduction, for a detailed description of pneumococcal and tetanus immunisation in HIV-1 infected individuals.

1.6.13 Treatment of HIV-1 infection

Since the advent of highly active antiretroviral treatment (ART) in 1996, the natural history of HIV and AIDS has been revolutionised (Mocroft, Vella et al. 1998), leading to the reclassification of HIV as a 'chronic illness'. However, due to drug toxicity, side effects such as lipodystrophy (fat redistribution syndrome) and the risk of drug resistance, it is recommended that treatment generally commenced only in established chronic HIV infection, when the CD4 T cell count has dropped to under 350 cells/ μ l

or if the patient shows signs of an AIDS defining illness (BHIVA 2008) (Gazzard, Bernard et al. 2008) (Table 1). The CD4 T cell count and plasma viral load are used as a marker of immune recovery and monitoring of efficacy of treatment, however, there is gathering evidence that other lymphocyte subsets detrimentally affected by HIV-1 infection may not reconstitute fully using ART.

Table 1: When to treat. Modified from BHIVA Guidelines 2008 (Gazzard, Bernard et al. 2008)

Presentation	Guidelines
Primary HIV Infection	Treatment in clinical trial Or Neurological involvement Or CD4 <200 cells/ μ l >3/12 months Or AIDS-defining illness
Chronic HIV Infection	
CD4 <200 cells/ μ l	Treat
CD4 201-350 cells/ μ l	Treat as soon as patient is ready
CD4 >350 cells/ μ l	Consider enrolment into 'when to start' trial
AIDS diagnosis	Treat (except for TB when CD4 >350 cells/ μ l)

The classes of ART drugs are so-named by their mode of action: nucleoside reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs) and protease inhibitors (PIs). These are used in appropriate combinations to suppress viral load and HIV progression and increase peripheral CD4 counts, however, no current drug formulation can eradicate HIV infection. An NRTI and Efavirenz is now recommended for the first line treatment for HIV by the British HIV Association (BHIVA) (Gazzard, Bernard et al. 2008). Newer types of ART drugs have been formulated, primarily to treat highly drug resistant patients with few treatment options. Integrase inhibitors such as Raltegravir (MK-0518) and Elvitegravir (GS-9137) have been developed which have activity against strains of HIV resistant against the commonly used protease inhibitors and reverse transcriptase inhibitors. The US Food and Drugs Administration (FDA) have approved Raltegravir for use in treatment experienced patients. Enfuvirtide is a 36 amino acid peptide derived from the extracellular domain of gp41, an HIV envelope protein. Approved in 2003, Enfuvirtide is a fusion inhibitor, by binding to the transmembrane domain it

prevents HIV from fusing to the host cell surface. Again, its use is limited to patients that have failed ART and it does have drawbacks, it has to be administered subcutaneously and is expensive. CCR5 receptor agonists have also been developed, such as Maraviroc and prevent the HIV virus from binding to the cell via CCR5.

1.7 Primary Immune Deficiency

Primary immune deficiency (PID) describes intrinsic disorders of the immune system of genetic origin, whilst HIV-1 infection is an example of secondary (or acquired) immune deficiency. PID includes severe-combined immune deficiency (SCID), combined immune deficiency (CID) and primary antibody deficiency. Antibody deficiencies are responsible for 50 to 70% of all cases of primary immune deficiency (Wood 2010). All patients with PID who have a genetic B cell defect (excluding selective IgA deficiency) are at a high risk of recurrent, invasive or severe pneumococcal disease (Picard, Puel et al. 2003). Patients with intrinsic T cell defects such as CD40L deficiency are associated with impaired antibody responses and also suffer from invasive pneumococcal disease.

1.7.1 CVID

In 1953, Janeway and colleagues reported the first known case of Common Variable Immune Deficiency (CVID), involving a 39 year-old patient with recurrent sinopulmonary infections, *H. influenzae* meningitis and bronchiectasis (Park, Li et al. 2008), yet much is still to be learnt about this syndrome. CVID is a heterogeneous primary immune syndrome simply characterised by recurrent sinopulmonary infections and antibody deficiency in the absence of other known causes. The condition is relatively common, affecting at least 1:50,000 of the UK population and has a bimodal age distribution, peaking in childhood and early adulthood. What triggers CVID in these patients is not understood. Sex distribution is equal. A frequent consideration regarding CVID disease is the lengthy delay in diagnosis that many patients face (median 4.7years), even despite displaying symptoms associated with CVID. Misconceptions that primary immune deficiencies are quite rare, that patients are extremely ill or only seen in childhood, may be responsible (Park, Li et al. 2008). Diagnostic delay is associated with a higher incidence of recurrent pneumococcal disease and a greater need for replacement Ig therapy. Ten to 25% of patients with CVID display familial inheritance of the disease, yet the remaining percentage seem to be sporadically occurring cases. CVID is characterised in the laboratory by low serum immunoglobulin levels (hypogammaglobulinaemia) of at least two isotypes, predominantly IgG and IgA, plus an impaired functional antibody response demonstrated by a failed vaccination to two or more common recall protein antigens

such as tetanus toxoid, *H.influenzae* and a lack of response to polysaccharide *Pneumococcal spp.* (ESID diagnostic criteria) (Conley, Notarangelo et al. 1999). Measurement of isohaemagglutinins can also be used to determine functional antibody production. The level of the antibody response to vaccination is important, since some physicians may decide not to immediately treat the patient with replacement Ig on the results of these tests (Buckley 2006). Due to cases of transient hypogammaglobulinaemia of infancy, which by definition spontaneously resolves, diagnosis of CVID should not be applied to infants under the age of two years (ESID) or even four years old (Cunningham-Rundles 2010). Since other causes of primary or secondary antibody deficiency must be excluded, differential diagnosis of CVID must include secondary hypogammaglobulinaemia (e.g. protein losing enteropathy (PLE), haematological malignancies, burns, certain drugs) (see Table 2).

Table 2: Differential diagnosis of hypogammaglobulinaemia. Modified from (Warnatz and Schlesier 2008)

Conditions associated with hypogammaglobulinaemia	
Drug Induced	Genetic disorders
Antimalarial agents, Captopril, Carbamazepine, Glucocorticoids, Fenclofenac, Gold salts, Penicillamine, Phenytoin, Sulfasalazine	Ataxia Telangiectasia Autosomal or X linked forms of SCID Class Switch recombination defects (CD40L, CD40, AID, UNG) Transcobalamin II deficiency and hypogammaglobulinaemia Agammaglobulinaemia (btk, I chain, a.o.m.) X-linked lymphoproliferative disorder (XLP)
Chromosomal anomalies	Systemic disorders of Immunodeficiency caused by hypercatabolism of Ig
(Chr. 18q- Syndrome, Monosomy 22, Trisomy 8 or 21) Myotonic dystrophy (DM1 and DM2)	Immunodeficiency caused by excessive loss of immunoglobulins (nephrosis, severe burns, lymphangiectasia, severe diarrhoea)
Malignancy	Infectious diseases
Chronic Lymphocytic Leukaemia Immunodeficiency with Thymoma Non-Hodgkin's lymphoma	Congenital infection with HIV or rubella Congenital infection with CMV or Toxoplasma gondii Epstein-Barr virus

Primary hypogammaglobulinaemia with recurrent infections can also be caused by selective IgA deficiency, selective IgG subclass deficiency, X-linked agammaglobulinaemia (XLA), X-linked lymphoproliferative disease (XLP), autoimmune recessive agammaglobulinaemia or one of the Hyper IgM syndromes. Patients with IgG subclass deficiency and IgA deficiency are often asymptomatic. Protein analysis or genetic testing and careful clinical history of patients can be used to help rule out these diseases. Although not an exhaustive list, protein/genetic tests include the measurement of BTK for XLA, SH2DIA for XLP, BLNK for AR agammaglobulinaemia and CD40L for Hyper-IgM syndromes respectively.

1.7.2 Clinical Features of CVID

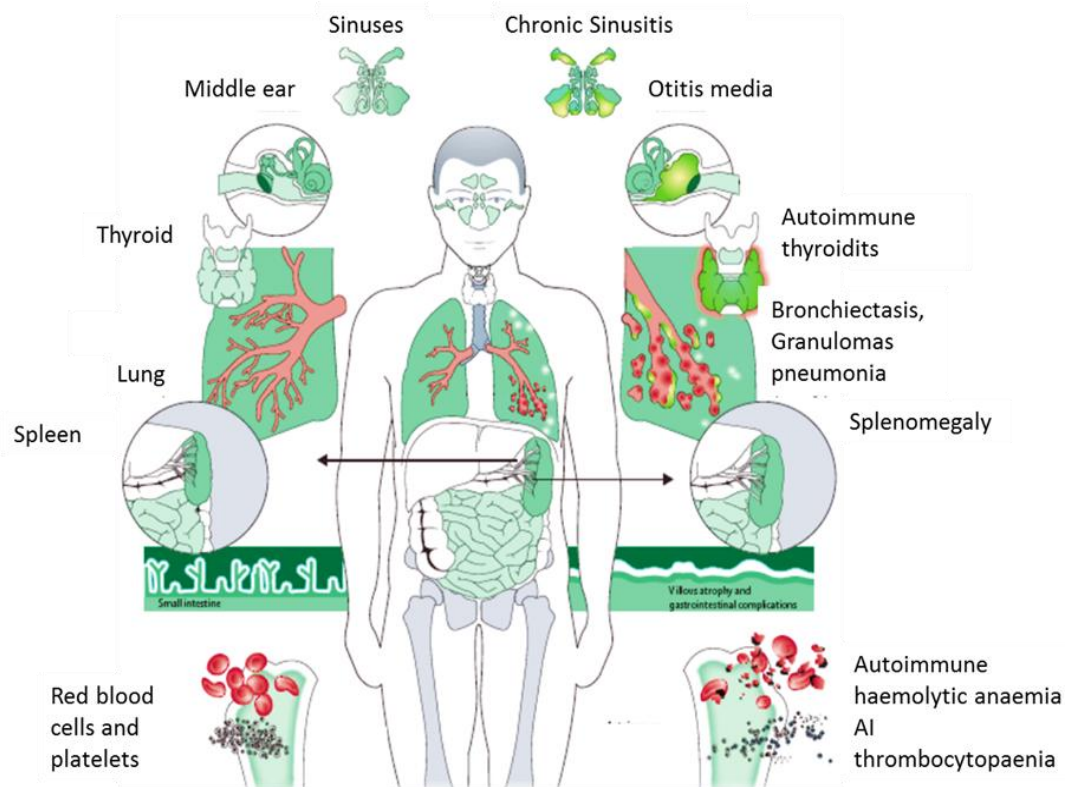


Figure 10: Clinical features of CVID. Modified from (Park, Li et al. 2008)

Approximately 90% of CVID patients present with a history of frequent infections (Cunningham-Rundles 2010). Infections of the respiratory tract contribute the greatest

burden of infectious disease and pneumonia caused by encapsulated bacteria such as *pneumococcus spp*, *H.influenzae* or mycoplasma is the most prevalent presentation in CVID (Cunningham-Rundles and Bodian 1999; Quinti, Soresina et al. 2007). Recurrent chronic sinusitis, bronchitis, otitis media are common manifestations of bacterial infection (see Fig. 10). Chronic lung damage is a common outcome of recurrent respiratory infections and may continue after treatment, leading to bronchiectasis (irreversible bronchial wall thickening and dilatation). For some patients with severe lung disease, heart/lung transplantation or continuous oxygen therapy may be necessary.

CVID is a multi-organ disease in many patients: gastrointestinal infections such as *Giardia enteritis* can occur, the spleen can be enlarged (splenomegaly), thyroid function can be affected, lung granulomas and nodules may be present, the joints can become inflamed and red blood cells and platelet function can be abnormal. Severity of clinical manifestations can vary widely, reflecting the heterogeneity of CVID.

The risk of cancer is increased in CVID, since up to 15% of patients suffer from malignancy. Gastric cancer and non-Hodgkin's lymphoma are the most common malignancies. In addition, lymphoid hyperplasia with splenomegaly is seen in 20% of CVID patients. Around a quarter of patients with CVID have manifestations of autoimmune disease, which is likely to be a consequence of B cell dysregulation. Autoimmune thrombocytopenic purpura (ITP) and Autoimmune haemolytic anaemia (AIHA) or both (Evan's Syndrome) are the most reported autoimmune complications, affecting up to eight per cent of CVID patients. Intravenous immunoglobulin therapy may be protective against AIHA or ITP. Other reported autoimmune conditions include autoimmune neutropenia, thyroiditis, pernicious anaemia, primary biliary cirrhosis and rheumatoid arthritis.

Granulomatous disease affects up to 10% of CVID patients and apparently resembles sarcoidosis, unfortunately this can lead to erroneous diagnoses and can delay diagnosis of CVID (Cunningham-Rundles 2010). The granulomas, defined as spherical masses of immune cells are well formed and non-caseating. Giant cells or non-necrotizing epitheloid cells may be present. An intense lymphoid infiltration may surround lung granulomas, associated with a poor outcome (granulomatous

lymphocytic interstitial lung disease). The lungs are the most affected organ, then lymph nodes and spleen however sites such as the brain, liver, skin, gastrointestinal tract, kidney and bone marrow may be affected. There is some evidence that presence of Human Herpes Virus 8 is associated with granulomatous disease and lymphoproliferative disorders (Morimoto and Routes 2005; Wheat, Cool et al. 2005). Another study reported decreased levels of regulatory CD4+FOXP3+CD25+T cells (T_{regs}) in CVID patients with granulomatous disease or autoimmune cytopenia (Horn, Manguiat et al. 2009; Arumugakani, Wood et al.). Ig replacement therapy is not thought to be helpful for granulomatous disease.

The T cell compartment is likely to be defective in a high proportion of patients with CVID. Reduced expression of CD40L on activated T cells has been reported in CVID and both frequency and absolute T cell numbers can be reduced. Naïve CD4+ T cells have been shown to be reduced in some patients with CVID and may reflect the severity of disease (Giovannetti, Pierdominici et al. 2007). Recently, a new subset of CVID has been reported, with a severe T cell defect, termed Late-Onset Combined Immune Deficiency (LOCID) (Malphettes, GÃrard et al. 2009). LOCID diagnosis is confined to patients with non-bacterial opportunistic infections such as cryptosporidia, CMV retinitis and aspergillosis and who have a marked loss of the CD4 compartment, especially CD4+CD45RA+ CCR7+ T cells. Patients with LOCID had a significantly higher prevalence of splenomegaly, GI tract disease and granuloma than classical CVID patients. This requires further detailed evaluation since poor T cell function has been reported to be associated with death at an earlier age (Cunningham-Rundles and Bodian 1999).

1.7.3 Genetic Defects associated with CVID

To date, mutational defects in just four genes account for 10 to 15% of CVID cases. Mutations in the tumour necrosis factor super family (TNFRSF) account for two of the four genes identified so far: TNFRSF13B (TACI) and TNFRSF13C (BAFF-R). (See Chapter VI for details of the roles of TACI and BAFF-R in the B cell chemokine axis).

Monogenic TACI mutations are the most common genetic defect reported so far and reflect eight to ten per cent of CVID disease (Castigli, Wilson et al. 2005; Salzer,

Chapel et al. 2005), with heterozygous mutations in two coding variants of the TACI gene C104R and A181E, accounting for most described TACI mutations. Mutations in TNFRSF13B, the gene encoding TACI protein, first reported in 2005, were associated clinically with lymphoproliferation, splenomegaly, tonsillar hyperplasia, IgA deficiency and increased incidence of autoimmune thyroiditis (Castigli, Wilson et al. 2005; Salzer, Chapel et al. 2005). However, the TACI mutation genotype-phenotype correlation is controversial since later work on TNFRSF13B has shown mutations in this gene can be present in the healthy population and most family members of patients with TACI mutations are asymptomatic (Castigli, Wilson et al. 2007; Pan-Hammarstrom, Salzer et al. 2007). In addition, TACI mutations are not associated with selective IgA deficiency as previously reported (Castigli, Wilson et al. 2007; Pan-Hammarstrom, Salzer et al. 2007). Recent data has further highlighted that many mutations of uncertain significance exist in the TACI gene (Lee, Ozcan et al. 2008).

Mutations in BAFF-R have so far only been identified in two related individuals. These BAFF-R deficient patients displayed absent protein BAFF-R expression on B cells, increased transitional cell numbers and reduced memory B cell counts (Warnatz, Salzer et al. 2009). ICOS deficiency has also been reported in patients with CVID and associated with absent expression of ICOS on activated T cells, extremely reduced numbers of class switched B cells, nodular lymphoid neoplasia and autoimmunity (Grimbacher, Hutloff et al. 2003). CD19 deficiency has been reported in four patients, in which homozygous gene mutations were associated with low/absent expression of CD19 on normal numbers of B cells (B cell counts measured using CD20 B cell expression). In these patients, memory B cells were found to be reduced in number (van Zelm, Reisli et al. 2006).

1.7.4 Treatment of CVID

The mainstay of treatment for CVID to reduce morbidity and mortality is prophylactic antibiotics and lifelong adherence to replacement immunoglobulin therapy (IVIG). Whilst replacement immunoglobulin therapy is effective and reduces sinopulmonary bacterial infections, lung disease etc., it is a human blood product that requires regular infusion and carries risks of infection, notably an outbreak of Hepatitis C occurred from contaminated IVIG in 1994 (Healey, Sabharwal et al. 1996). Some

hypogammaglobulinaemic CVID patients manage to avoid the need for replacement IVIG therapy, responses to functional antibody level testing may predict which patients require treatment (Buckley 2006).

1.7.5 Classification schemes for CVID

The varied nature of CVID disease has led to attempts to subcategorise patients for treatment and prognosis, firstly by an *in vitro* immunoglobulin production method (Bryant *et al.*, 1990) (Bryant, Calver *et al.* 1990) and then by phenotyping peripheral memory B cell subsets based on CD27 expression (Warnatz, Denz *et al.* 2002; Piqueras, Lavenu-Bombled *et al.* 2003). The Freiburg classification scheme, published in 2002, categorised switched memory B cell numbers as a percentage of peripheral blood lymphocytes and further divided subgroups by CD21 expression (Warnatz, Denz *et al.* 2002). Group 2 patients had normal class switched memory B cells, whilst Group 1 had reduced class switched memory B cells (<0.4%) and was further subdivided into Group 1a- (>20% of CD21low B cells) or Group 1b (normal levels of CD21low B cells).

The Paris scheme, published the subsequent year, (Piqueras *et al.*, 2003) adapted the previous classification system to include IgM memory B cells and simplified the analysis of B cells by classifying patients according to percentages of total CD27+ memory, class switched memory and IgM memory B cells (Piqueras, Lavenu-Bombled *et al.* 2003). Phenotypic utility and good agreement with the Freiburg scheme was achieved by using three subgroups based on percentage of CD19+ B cells. The MBO group was defined as <11% total CD19+CD27+ memory and had the greatest defect in B cell differentiation. The MB1 group classified patients with reduced (<8%) class switched memory B cells and normal numbers of IgM memory B cells (>8%). The MB2 group defined those patients with higher numbers of memory B cells that did not fit into MBO or MB1.

In 2008, an updated B cell memory classification system was proposed. The 'EURO Class' system aimed to merge the Paris and Freiburg classification schemes into one scheme (Wehr, Kivioja *et al.* 2008) (see Fig. 11). The EURO Class classification scheme is overly complex, serving to highlight that CVID is a heterogeneous syndrome (Wehr, Kivioja *et al.* 2008).

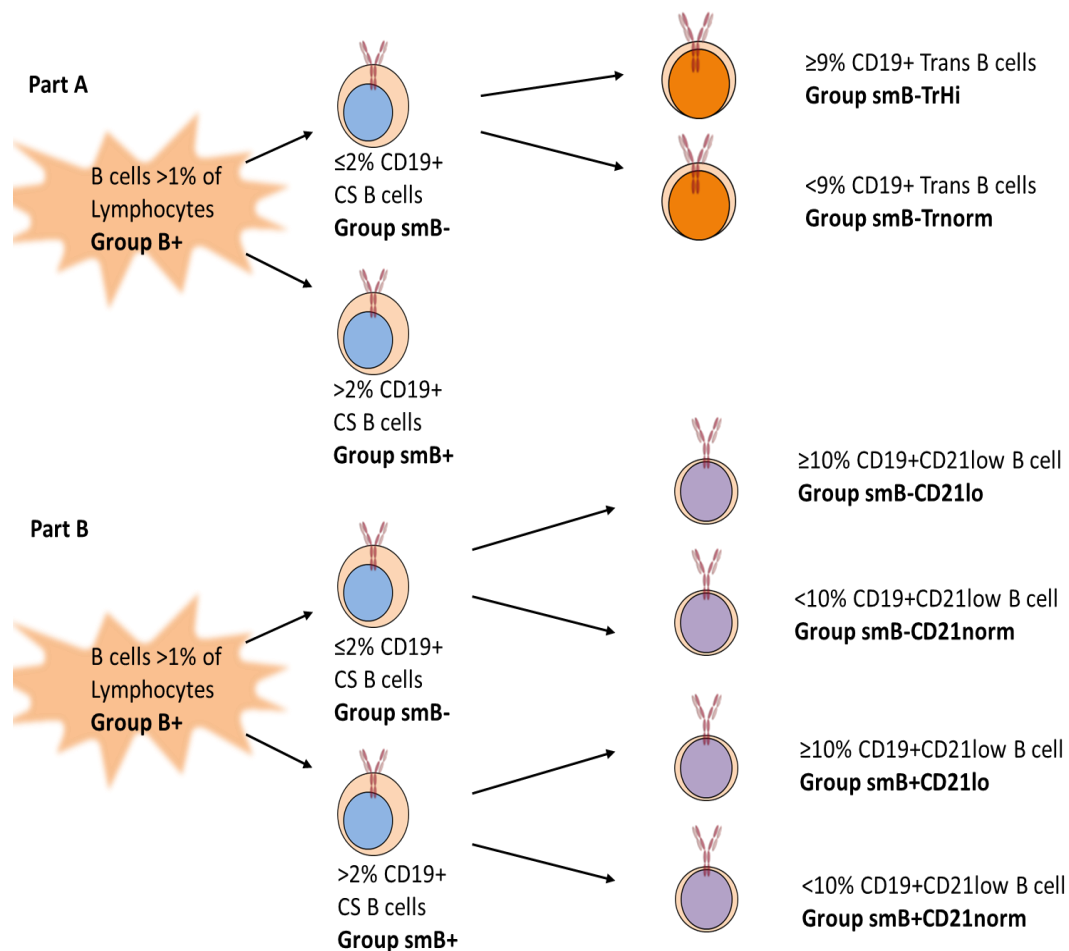


Figure 11: EURO Class Classification scheme. Modified from (Wehr, Kivioja et al. 2008).

Patients with $\leq 1\%$ of B cells are classified into Group B- and not classified further. In Part A, patients are classified into whether they have greater or less than 2% switched memory B cells and further subdivided on the basis of transitional B cell numbers ($<$ or $\geq 9\%$) (Fig.11). In Part B, patients are again classified on the number of class switched memory B cells and then subdivided on the number of CD21low B cells ($<$ or $\geq 10\%$). Some useful clinical correlations have arisen: ICOS deficiency presents with less than 2% switched memory B cells for example. High levels of transitional B cells or CD21low B cells are associated with lymphadenopathy and splenomegaly respectively. However, the scheme does not include IgM memory B cells, an important omission which has been noted by eminent researchers in this field (Wehr, Kivioja et al. 2008). IgM memory B cell measurement is useful, CVID patients with reduced IgM memory B cells have a higher incidence of bronchiectasis (Carsetti,

Rosado et al. 2005). At the present time, perhaps because the EURO Class scheme is relatively new, there are few papers correlating EURO Class classifications with clinical phenotypes or disease prognosis.

1.8 Aims and Hypothesis

The hypothesis of this study is that susceptibility to invasive pneumococcal infection in acquired immune deficiency such as HIV-1 infection is associated with loss of peripheral B cell subsets which gives rise to functional hyposplenism and humoral immune deficiency. Loss of these subsets could be due to maturational arrest at the transitional stage or apoptosis of the IgM memory and switched memory B cells.

The aims of this study are to investigate:

- 1) The distribution of transitional and memory B cell subsets, pneumococcal and tetanus antibody levels in patients with HIV-1 infection and to compare these with other patient groups at increased risk of invasive pneumococcal disease (CVID and splenectomised patients).
- 2) To assess functional serological immunisation responses to *S. pneumoniae* (T cell independent) and tetanus toxoid (T cell dependent) in HIV patients on ART and healthy controls.
- 3) To establish whether there is an association between peripheral B cell subsets and functional antibody levels post vaccination.
- 4) To investigate likely underlying mechanisms in HIV patients and disease controls. Analysis of expression of B cell chemokine receptors, B cell exhaustion, role of follicular helper T cells and plasma B cell chemokine levels will be determined.

2. Chapter II: Materials and Methods

2.1 Patient cohorts and trial design

2.1.1 *Functional Immunity Study Cohort*

HIV-1 infected patients included in Chapter III and IV were recruited from the Kobler Clinic, St Stephen's Centre, Chelsea and Westminster Hospital by Dr. Alan Steel and Dr. Peter Kelleher. These comprised 29 HIV-1 infected untreated patients and 55 HIV-1 patients on ART that had been recruited into a randomised trial to assess the efficacy of ART on pneumococcal and tetanus toxoid vaccination. Inclusion criteria for this trial included an undetectable viral load for \geq two consecutive months. Exclusion criteria included a pneumococcal or tetanus toxoid vaccine within the last five years. HIV-1 infected patients on ART were matched for nadir CD4 counts and immunised with Pneumovax II (T cell independent carbohydrate antigen), tetanus toxoid vaccine (T cell dependent protein antigen) or a placebo vaccine. The antibody response to vaccination was assessed at screening, baseline, 4 weeks, 12 weeks and 24 weeks post immunisation. 17 healthy laboratory workers from the Immunology laboratory and 66 samples from a sera bank (serology only) were also tested to act as control subjects.

2.1.2 *B cell chemokine axis and tissue like B cell studies*

For the tissue like B cell, follicular helper T cell and chemokine studies (Chapters V and VI), HIV-1 infected patients were again recruited from the Kobler Clinic, St. Stephen's Centre, Chelsea and Westminster Hospital. Drs. Peter Kelleher and Emma Page recruited and consented the HIV-1 infected patients for this work. Fourteen untreated patients with HIV-1 infection, 14 HIV-1 infected patients on ART and 11 healthy controls were included. HIV infected patients on treatment had an undetectable viral load for >6months.

2.1.3 *CVID cohorts*

For the measurement of transitional B cells, memory B cell subsets and T cell activation (Chapter III), 28 patients diagnosed with Common Variable Immune

Deficiency (CVID) and eight individuals with a clinical history of splenectomy were recruited from the Lind Ward Respiratory Clinic at the Royal Brompton Hospital by Dr. Peter Kelleher. In Chapter III, 36 patients with CVID were studied to assess whether memory B cell subsets were associated with respiratory complications. 28 patients were from the described cohort above and an additional eight patients were included, also diagnosed by ESID criteria and from the Lind Ward Respiratory Clinic. Bronchiectasis was defined clinically on chest CT scan according to standardised clinical criteria. Spirometry (FEV1, RV, KCO and MEF) and vitalographs were performed in all patients as part of clinical care and analysed by Dr. Peter Kelleher. For Chapters V and VI, 24 patients with CVID were recruited by Dr. Peter Kelleher from the Lind Ward Respiratory Clinic (as before) to further examine defects in the B cell chemokine axis, tissue like B cells and follicular helper T cell immunity. Patients with primary immune deficiency defined as CVID fulfilled the ESID diagnostic criteria: i.e. had low Ig levels of at least two isotypes, presented at greater than two years of age, displayed a failure to respond to vaccination and other causes of hypogammaglobulinaemia were excluded (Conley, Notarangelo et al. 1999). Most of the CVID cohorts were on replacement immunoglobulin therapy.

2.1.4 Ethics

Ethics approval was approved by the Riverside Ethics Committee (RREC1108 and PREC04/Q0406/119) and a consent form was signed by all participants.

2.2 Methods

2.2.1 Plasma separation

Plasma was separated from heparinised anticoagulated whole blood by layering 6mls of heparin and 1ml of PBS over 10mls of Lymphoprep. This was centrifuged at 1000rpm for twenty minutes and then the plasma layer was aspirated and stored in two 1ml aliquots at -30°C until use.

2.2.2 Serum separation

Six mls of clotted blood was centrifuged at 1000rpm for ten minutes then the serum was aspirated from the tube. Serum was stored at 2-8°C in two 1ml aliquots.

2.2.3 Viral Load

Plasma viral load (VL) levels were measured for HIV-1 infected patients as part of routine clinical care. The VL test was performed by branched DNA amplification technology (HIV RNA 3.0 assay, *Bayer Healthcare*). The lower limit of detection of this test was 50 HIV-1 RNA copies/ml.

2.2.4 Lymphocyte subsets (LSS)

All patients had EDTA whole blood taken for estimation of lymphocyte subsets (LSS) and absolute count determination. LSS samples were stained with 10µl of pre-mixed Cyto-Stat TetraCHROME reagents (*Beckman Coulter*): CD3+ (T cells), CD4+ (helper T cells), CD8+ (cytotoxic T cells), CD19+ (B cells) and CD56+ (NK cells). 100µl whole blood was then added in an automated processor (*Beckman Coulter FP1000*), in which samples were lysed in an automated TQ Prep and 100µl of FlowCount absolute counting beads (*Beckman Coulter*) were added. LSS samples were processed by flow cytometry on a *Cytomics FC500 (Beckman Coulter)*.

2.2.5 Commercial ELISA for the detection of plasma APRIL

The human APRIL ELISA (*Bender med systems BMS2008*) was performed according to the manufacturer's protocol. Assays were performed on a fresh aliquot of heparinised plasma frozen and stored at -30°C. The APRIL standard curve was made up using double dilutions of kit standard from 50ng/ml to 0.78ng/ml and assayed in duplicate on the microtitre plate. Patient samples and controls were tested in

singlicate. Standard five and healthy control sera were used as internal quality control (IQC). Sample diluent was used as a blank (wells A1, A2). After sample diluent and samples were added to the wells of the 96 well microtitre plate, diluted biotin conjugate was added to all wells. The plate was incubated on a plate shaker for two hours at room temperature. Then the plate was washed three times using 300µl of supplied wash buffer. Streptavidin HRP was added to the plate and incubated for a further two hours on the plate shaker. After three washes, TMB substrate was added and the plate stopped by the addition of stop solution after about ten minutes when the top standard had developed a dark blue colour. Optical densities (OD) of wells were measured at 450nm on a spectrophotometer, (*Dynatech MRX, Thermolife Sciences*) using Revelation 4.22 software (*Dynex Technologies*).

2.2.6 *Commercial ELISA for the detection of plasma BAFF*

The BAFF Quantikine ELISA (*R&D systems*) was performed according to the manufacturer's protocol. The reagents were made up as instructed. BAFF ELISA assays were performed on a fresh aliquot of heparinised plasma frozen and stored at -30°C. The BAFF standard curve was made up using a preparation of double dilutions from 2000pg/ml to 31.2pg/ml to be assayed in duplicate on the microtitre plate. Patient samples were assayed in singlicate. Standard 250pg/ml and HC serum were used as QC. Sample diluent was used as blank (A1, A2). After sample diluent was added to each well, the samples were added to the wells of the microtitre plate and the plate put on a plate shaker for two hours at room temperature. The plate was washed four times in supplied wash buffer, conjugate was added and then the plate was further incubated for two hours on the plate shaker. After a further three washes, TMB substrate was added and the plate stopped after 30 minutes with the addition of stop solution. ODs were measured at 450nm on a Dynatech MRX spectrophotometer, (*Thermolife Sciences*) within 30 minutes using Revelation 4.22 software (*Dynex Technologies*).

2.2.7 *Commercial ELISA for the detection of plasma IL-7*

The *Diaclone IL-7* ELISA kit (cat. no. 850040096) was supplied by Gen-Probe Life Sciences and performed according to protocol. The reagents were made up as instructed and assays performed on a fresh aliquot of heparinised plasma stored at -

30°C. The IL-7 standard was made up using double dilutions from 200pg/ml to 3pg/ml to be assayed in duplicate on the microtitre plate. Patient samples were assayed in singlicate. Kit control (of supplied value) was used for quality control purposes. Sample diluent was used as blank (A1, A2). 100µl of diluted standard, control or patient samples were added to the wells of an anti-IL-7 coated microtitre plate. 50µl of biotinylated detection antibody was added to all wells. The plate was incubated at room temperature for two hours. The plate was washed three times in wash buffer, streptavidin-HRP conjugate was added and further incubated for 30 minutes on the plate shaker. After a further wash, 100µl of TMB substrate was added to each well and the reaction stopped after 10 to 15 minutes by the addition of 100µl stop solution. ODs were measured at 450nm on a Dynatech MRX spectrophotometer, (*Thermolife Sciences*) using Revelation 4.22 software (*Dynex Technologies*) within 30 minutes.

2.2.8 Assessment of vaccination responses by ELISA (Chapter IV)

In-house Enzyme linked Immunosorbent assays (ELISA) were set up to quantitate serum IgG and IgM antibodies to *S. pneumoniae* and IgG to tetanus toxoid in patient disease groups and healthy controls. Healthy control sera from the blood bank and from laboratory workers (n=83) were used to determine a normal range for IgM antibodies to *S. pneumoniae* since no reference preparation or normal range existed for this measurement. HIV-1 infection is associated with an increase in non-specific antibodies, which is a limitation of pneumococcal serology testing. These assays tested total IgM or IgG levels against pneumococcus and did not determine the avidity of the antibodies measured and therefore may overestimate antibody levels in HIV-1 infected individuals. For all ELISAs, pre and post vaccine sera (0, 4, 12, 24 weeks) were assayed on the same microtitre plate to minimize inter-assay variability. For quality control purposes, all plates had at least one high, an intermediate level and a low IQC sample added.

2.2.9 Determination of antibodies to *S. pneumoniae* by an in-house ELISA

Microtitre plates (*Maxisorp, Nunc, Fisher Scientific*) were coated with Pneumovax II 23-valent vaccine (*Sanofi Pasteur MSD*) in a sodium bicarbonate buffer at 4°C overnight. For the pneumococcal assays, patient samples, internal controls and

reference standards (IgG: SPRU standard, in-house IgM standard) were pre-incubated in LP4 tubes with cell wall pneumococcal polysaccharide antigen (*Statens Serum Institute*) in PBS/Tween 20/BSA for one hour at room temperature to adsorb non protective antibodies to cell wall polysaccharide. Samples were then further diluted and added in duplicate at four different dilutions (1/10, 1/50, 1/100 and 1/200). Plates were incubated at room temperature for one and a half hours on a plate shaker (*Titramax 1000, Heidolph*) and washed three times in 400µl of PBS/Tween (PBST) using an automated plate washer (*Denley Well wash 4, Thermo Life Sciences*). Plates were blotted using filter tissue to remove residual wash buffer and 100µl per well of an appropriate HRP labelled IgG or IgM conjugate (*Sigma Aldrich*) was added to individual plates and incubated for a further one and a half hours. After a final wash step in PBST, plates were developed using 100µl of OPD substrate and hydrogen peroxide in a substrate buffer. ODs were measured at 450nm on a Dynatech MRX spectrophotometer, (*Thermolife Sciences*) using Revelation 4.22 software (*Dynex Technologies*) with results calculated from a standard curve.

2.2.10 Determination of antibodies to Tetanus Toxoid by an in-house ELISA

Microtitre plates (*Maxisorp, Nunc, Fisher Scientific*) were coated with tetanus toxoid antigen (Tetanus Toxoid Adsorbed Third International Std, 98/552, *NIBSC*) in a sodium bicarbonate buffer and incubated overnight at 4°C. Plates were washed three times in 400µl of PBST using an automated plate washer (*Denley Well wash 4, Thermo Life sciences*) and blocked with 5% milk/PBST for one hour at 37°C to reduce non-specific binding. After a further wash cycle in PBST, samples, standard (*NIBSC 3rd International Standard anti-Human Ig*) and internal controls were diluted and added in duplicate (1/10, 1/100, 1/200 and 1/400) and added to the microtitre plates. Plates were incubated at 37°C for one and a half hours, washed in PBST and a HRP labelled IgG conjugate (*Sigma Aldrich*) was added in milk/PBST and incubated for a further one and a half hours. After a final three washes in PBST, plates were developed using OPD substrate and hydrogen peroxide in a substrate buffer. The reaction was stopped using sulphuric acid. ODs were measured at 450nm on a Dynatech MRX spectrophotometer, (*Thermolife Sciences*) using Revelation 4.22 software (*Dynex Technologies*) with results calculated from a standard curve.

2.3 Immunophenotyping by Flow Cytometry

2.3.1 Detailed B and T cell Immunophenotyping (Chapter III)

Four colour B cell and T cell subset analysis was performed in 120µl EDTA whole blood within 24 hours of venesection. Whole blood was washed twice in 2mls of 10% FCS/PBS and centrifuged at 400rpm to remove circulating immunoglobulin for analysis of B cells. Washed samples were then incubated with monoclonal antibodies for 20 minutes at room temperature. Monoclonal antibodies used to define human B cell subsets were: PE-cyanin 7 (PC7) conjugated anti-CD19, PE-conjugated anti-human IgD, Cy5-conjugated anti-human IgM, FITC-conjugated anti-CD27, PE-conjugated anti-CD21 and FITC-conjugated anti-CD38 (Table 3).

Table 3: B cell monoclonal antibody panel

<i>Tube</i>	<i>Antibody</i>	<i>Fluorochrome</i>	<i>Antibody Source</i>	<i>Volume (µl)</i>
1	Cells	NA		
2	IgG1	FITC	BD Pharmingen	10
	IgG1	PE	BD Pharmingen	10
	CD19	PC7	Beckman Coulter	10
3	CD27	FITC	Dako	1
	IgD	PE	Southern Biotech	0.025
	CD19	PC7	Beckman Coulter	10
	IgM	Cy5	Jackson Labs	0.25
4	CD38	FITC	BD Pharmingen	5
	CD21	PE	BD Pharmingen	5
	IgM	Cy5	Jackson Labs	0.25
	CD19	PC7	Beckman Coulter	10
5	CD27	FITC	Dako	1
	CD21	PE	BD Pharmingen	5
	CD19	PC7	Beckman Coulter	10
	IgM	Cy5	Jackson Labs	0.25

The expression of CD38 and CD28 on CD4 and CD8 T cell subsets was determined using FITC-conjugated anti-CD8/PE conjugated anti-CD38, Allophycocyanin-conjugated anti-CD3, PerCP-conjugated anti-CD4, FITC-conjugated anti-CD8 and PE-conjugated anti-CD28 (Table 4).

Table 4: T cell monoclonal antibody panel

<i>Tube</i>	<i>Antibody</i>	<i>Fluorochrome</i>	<i>Antibody Source</i>	<i>Volume (μl)</i>
1	Cells	NA		
2	CD8	FITC	Beckman Coulter	5
	CD38	PE	Beckman Coulter	5
	CD4	PerCP	BD Biosciences	5
	CD3	APC	BD Pharmingen	1
3	CD8	FITC	Beckman Coulter	10
	CD28	PE	Beckman Coulter	10
	CD4	PerCP	BD Biosciences	10
	CD3	APC	BD Pharmingen	1

After incubation, blood samples were lysed using the automated Beckman Coulter TQ Prep (*Beckman Coulter*) and then washed twice with 2mls of PBS by centrifugation at 400rpm for five minutes. 400 μ l of PBS were added to tubes for sample acquisition.

Cells were assessed using four-colour flow cytometry on a FACSCaliburTM (*Becton Dickinson*). Compensation on this flow cytometer was calibrated before use with an automated programme (FACsComp) using BD Calibrite beads (*Becton Dickinson*). Due to batch variability in IgM staining, B cell settings were further checked using single and dual stained fluorochromes on a healthy control sample. Data was analysed using CELLQUESTPROTM software (*Becton Dickinson*).

2.3.2 Analysis of B cell memory and maturational subsets by flow cytometry

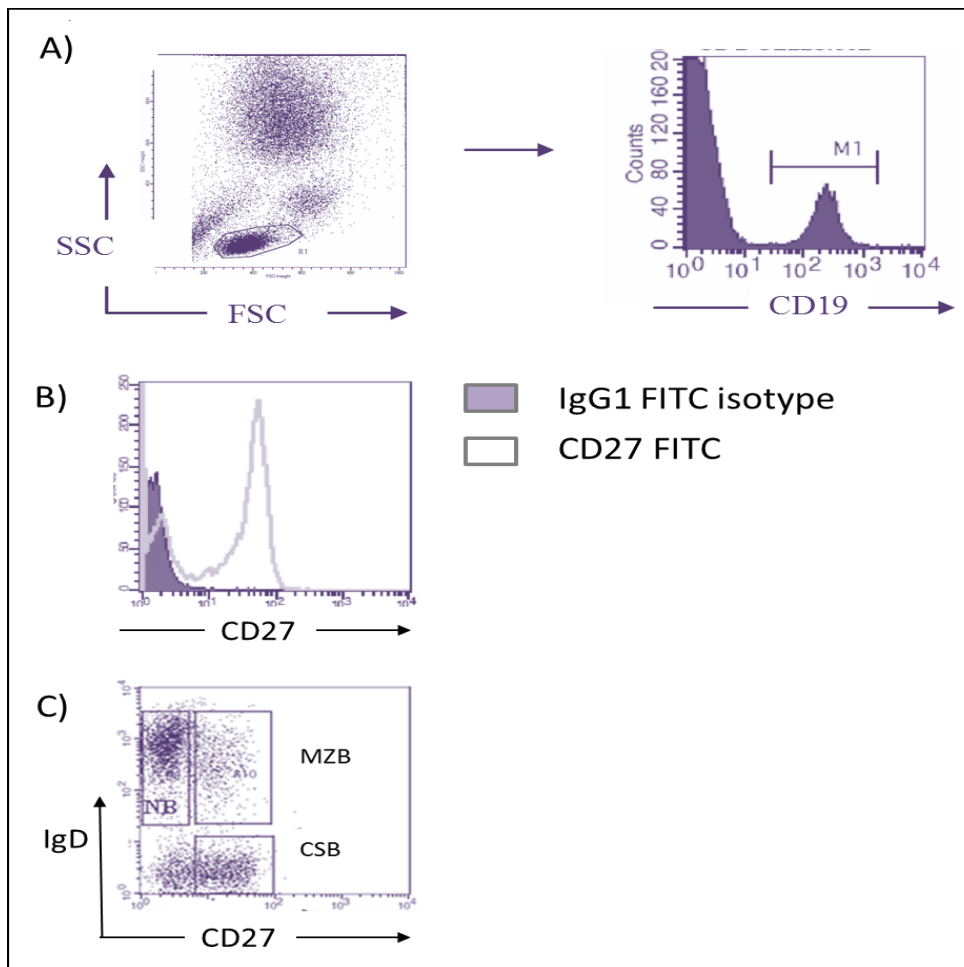


Figure 12: Gating strategy for CD27+ memory B cells

Sample flow cytometry plots of a representative healthy control. 12A) A lymphocyte gate was set according to FSc and SSc and then gates were set on CD19+ B cells. 5000 B cell events were collected. B) Isotype controls: IgG1 FITC, IgG1 PE were then used to set CD19+ quadrants. Histogram to show overlay of CD27 staining (open lined area) onto FITC isotype control staining (shaded area). C) B cells then analysed using CD27 FITC and IgD PE for B cell subset staining for: naïve B cells (NB), IgM memory B cells (MZB) and class switched memory B cells (CSB).

IgM memory B cells were defined as $CD19+CD27+IgM^{hi}IgD^{lo}$ or $CD19+CD27IgM^{hi}CD21^{hi}$ B cells (Fig.12). There was a very high level of agreement between the two sets of markers (correlation co-efficient = 0.90). Data for IgM memory B cells is represented by the $CD19+CD27+IgM^{hi}IgD^{lo}$ panel to avoid

repetition and to allow us to display the switched CD19+CD27+IgM-IgD- memory B cell data.

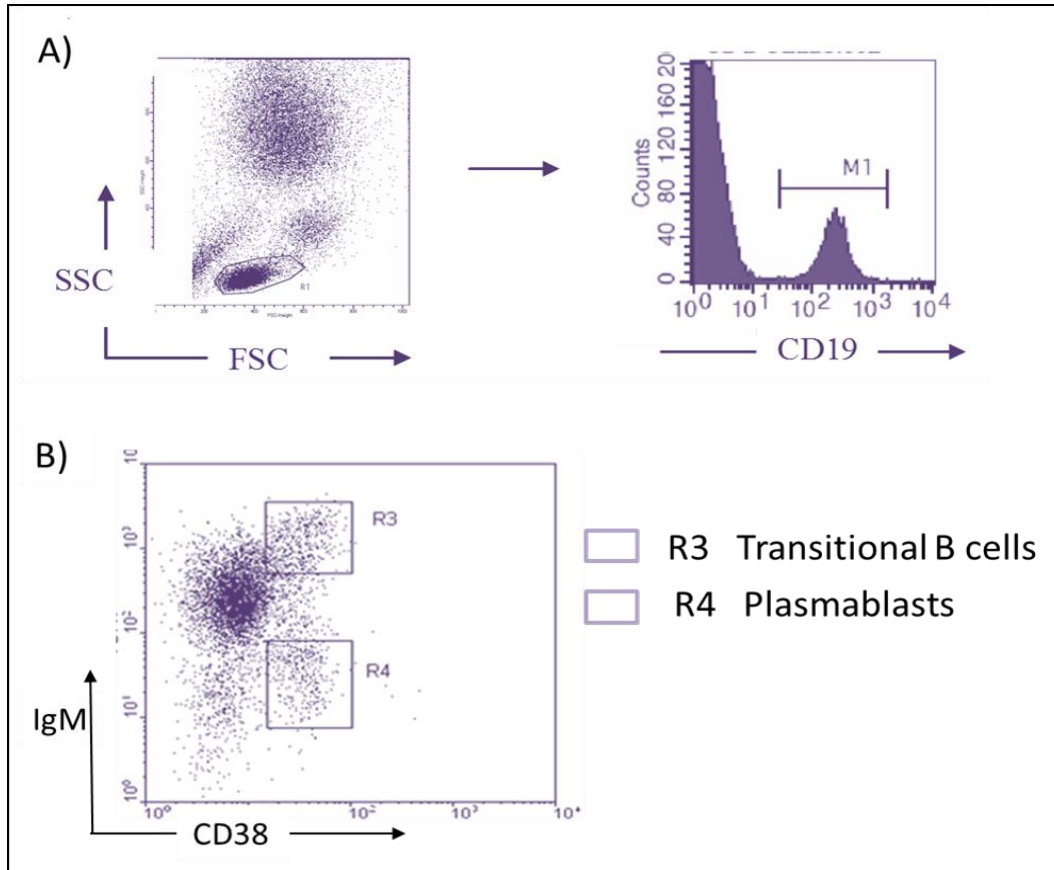


Figure 13: Gating strategy for transitional B cells

Transitional CD19+CD21^{lo}IgM^{hi}CD38^{hi} B cells were defined according to the Freiburg protocol [78]. Dot plot displays a representative CVID patient with elevated transitional B cells. A) A lymphocyte gate was set according to FSc and SSc and then gates were set on CD19+ B cells. 5000 B cell events were collected. B) Transitional B cells [R3] CD19+IgM⁺⁺CD38⁺⁺ and plasmablasts [R4] CD19+IgM-CD38⁺⁺ were then gated using differential expression of IgM and CD38.

2.3.3 Analysis of tissue like B cells by flow cytometry (*CD38^{lo}CD21^{lo}* and *CD27^{lo}CD21^{lo}* phenotyping)

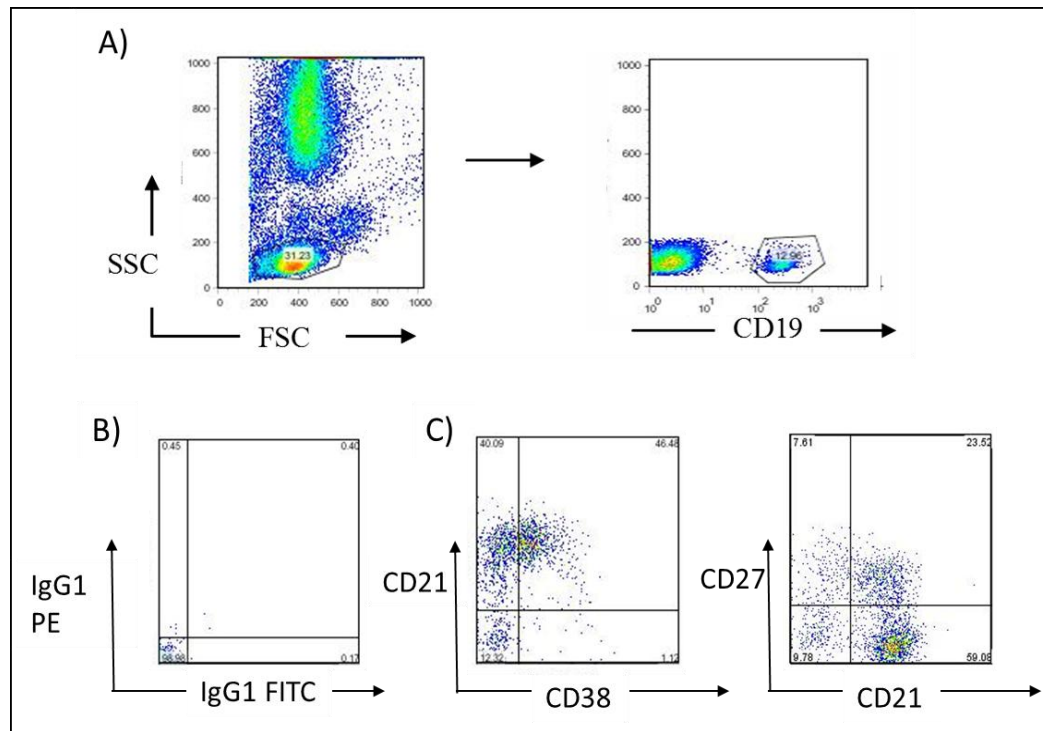


Figure 14: Gating strategy for tissue like B cells using CD21/CD38 or CD27/CD21

Sample flow cytometry plots of a representative untreated HIV-1 infected patient. 14A) A lymphocyte gate was set according to FSc and SSc and then gates were set on CD19+ B cells. 5000 B cell events were collected. B) Isotype controls: IgG1 FITC, IgG1 PE were then used to set CD19+ quadrants. C) Tissue like B cells were then analysed using CD21 and CD38 FITC (*CD38^{lo}CD21^{lo}*) or using CD27 and CD21 (*CD27^{lo}CD21^{lo}*).

Since the expansion of tissue like B cells in HIV-1 infection was described, data from the FI study was re-analysed to examine these cells in all disease groups (Fig.14).

2.3.4 Analysis of peripheral blood T cells by flow cytometry (Chapter IV)

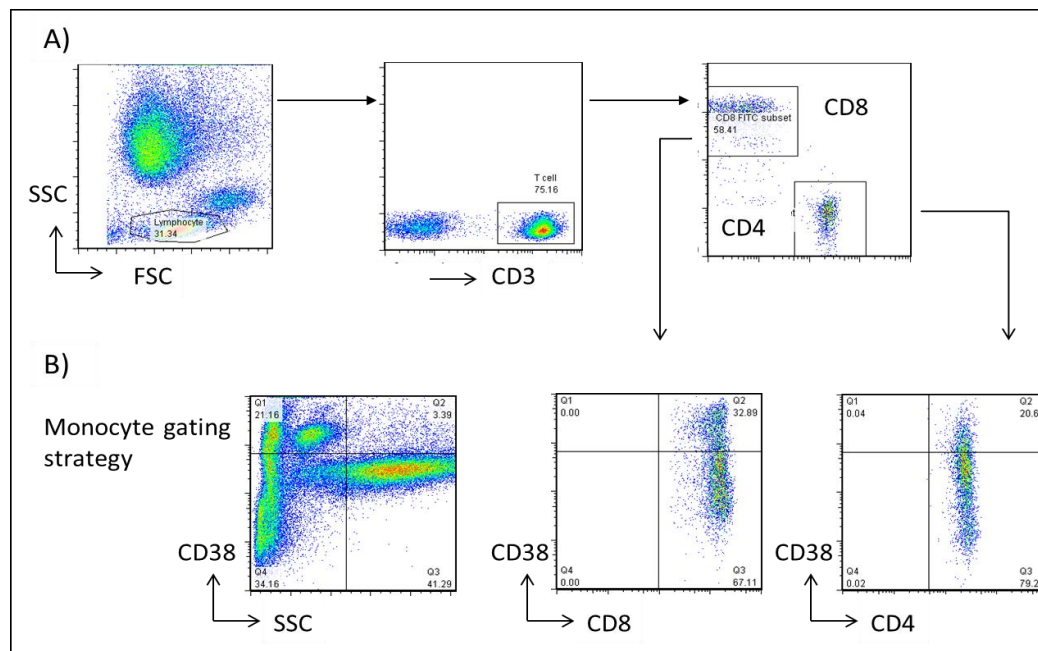


Figure 15: CD38 monocyte gating strategy

15A) Viable lymphocytes were determined using forward versus side-scatter gating and T cells were then analysed using side-scatter versus CD3 gating. Ten thousand events were acquired on the T cell gate and the results are expressed as a percentage of CD3 events. CD4 and CD8 T cells were evaluated using a CD3 gate. To select for CD8 T cells with high CD38 expression, monocytes were used as a positive control population (Tilling, Kinloch et al. 2002; Steel, John et al. 2008). B) Using an un-gated plot, a quadrant gate was drawn between the granulocyte and monocyte populations. This quadrant was then used to distinguish CD8+CD38 high+ T cells gated on FSC v SSC, then CD3. A representative HIV-1 infected patient with elevated CD8+CD38+ T cells is shown.

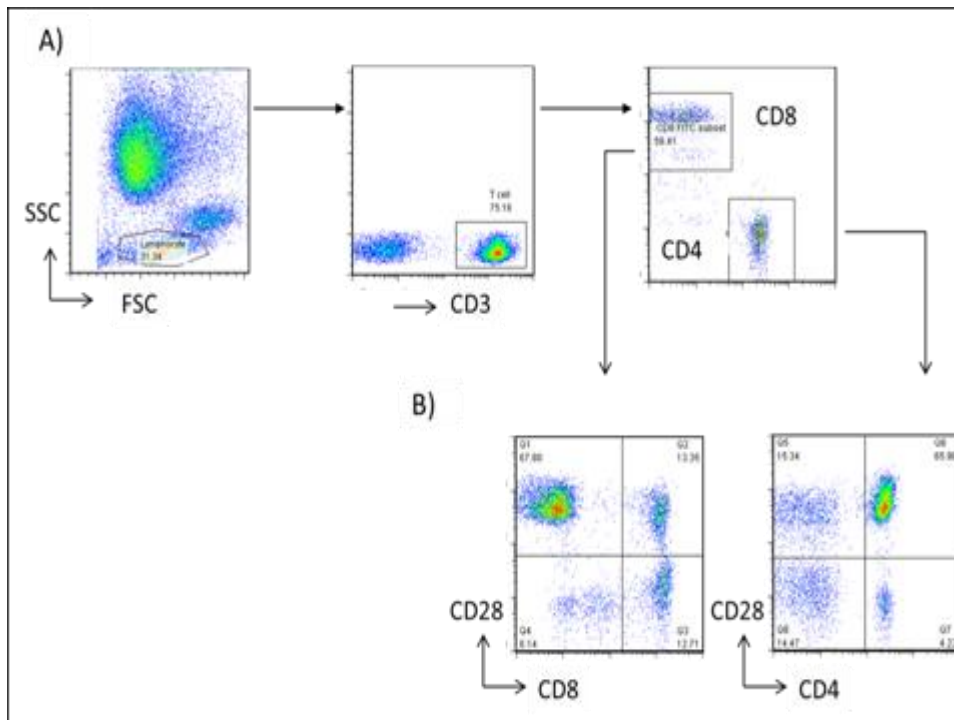


Figure 16: CD28 T cells gating strategy

16A) Viable lymphocytes were determined using forward versus side-scatter gating and T cells were then analysed using side-scatter versus CD3 gating. Ten thousand events were acquired on the T cell gate and the results are expressed as a percentage of CD3 events. CD4 and CD8 T cells were determined using a CD3 gate. B) The expression of CD28 on CD4 or CD8 T cells was evaluated. A representative healthy control is shown.

2.3.5 *B cell chemokine receptors, tissue like B cells and T_{FH} cell immunophenotyping by flow cytometry*

Five colour B cell and T cell subset analysis was performed within 24 hours of venesection. Analysis of B cell subsets were performed on 150µl whole anticoagulated (EDTA) whole blood. B cell tubes were washed twice by the addition of 2mls of 10% FCS/PBS, centrifuged at 400rpm for 5mins and the supernatant aspirated using a Pasteur pipette to remove circulating free immunoglobulin. Tubes were then incubated with monoclonal antibodies for 30mins at room temperature. Monoclonal antibodies used to define human B cell subsets were: PE-cyanin 7 (PC7) conjugated anti-CD19, PE or FITC-conjugated anti-human IgD, PerCP-conjugated anti CD10, APC-conjugated anti-CD27, PE or FITC-conjugated anti-CD21, PE-conjugated CD11c and PE-conjugated FCLR4 (see Table 5). For analysis of B cell chemokine receptors, FITC-conjugated BCMA and FITC-conjugated BAFF-R were used in combination with the B cell phenotype markers CD19, CD27, IgD and CD10. Due to poor staining of directly conjugated TACI monoclonal antibodies, TACI measurement was performed with biotin conjugated TACI instead. A biotin-labelled isotype matched control tube was also stained (see Table 5).

The expression of T_{FH} cells was determined using 120µl of whole blood stained with: APC-CY7 labelled CD3, PC7 labelled CD4, FITC-conjugated anti-CD45RO or CD57, Alexafluor-647 labelled CXCR5 and PE conjugated anti-ICOS (Table 6). An isotype control tube containing CD3 APC-CY7, CD4 PC7 and isotype matched PE, FITC and Alexafluor-647 antibodies was used for gating.

Table 5: B cell chemokine receptor monoclonal antibody panel

<i>Tube</i>	<i>Antibody</i>	<i>Manufacturer</i>	<i>Volume (μl)</i>
1 19 ISO	IgG1K-FITC	BD Pharmingen	5
	IgG2bK PE	BD Pharmingen	5
	IgG1 PerCP	BD Pharmingen	5
	IgG1K APC	BD Pharmingen	5
	CD19 PC7	Beckman Coulter	8
2 BCMA	BCMA FITC	Alexis Biochemicals	1
	IgD PE	Southern Biotech	1
	CD10 PerCP	Exbio	10
	CD27 APC	BD Pharmingen	5
	CD19 PC7	Beckman Coulter	8
3 BAFFR	BAFF-R FITC	BD Pharmingen	5
	IgD PE	Southern Biotech	1
	CD10 PerCP	Exbio	10
	CD27 APC	BD Pharmingen	5
	CD19 PC7	Beckman Coulter	8
4 FCLR4	CD21 FITC	Beckman Coulter	10
	FCLR4 PE	Biologend	20
	CD10 PerCP	Exbio	10
	CD27 APC	BD Pharmingen	5
	CD19 PC7	Beckman Coulter	8
5 CD11c	CD21 FITC	Beckman Coulter	10
	CD11c PE	Beckman Coulter	20
	CD10 PerCP	Exbio	10
	CD27 APC	BD Pharmingen	5
	CD19 PC7	Beckman Coulter	8
6 TACI ISO	IgG2 FITC	BD Pharmingen	5
	IgG2aK-biotin	Biologend	4
	IgG1 PerCP	BD Pharmingen	5
	IgG1 APC	BD Pharmingen	5
	CD19 PC7	Beckman Coulter	8
7 TACI biotin	IgD FITC	Beckman Coulter	1
	TACI-biotin	Biologend	2
	CD10 PerCP	Exbio	10
	CD19 PC7	BD Pharmingen	8
	CD27 APC	Beckman Coulter	5

Table 6: T_{FH} monoclonal antibody panel

<i>Tube</i>	<i>Antibody</i>	<i>Manufacturer</i>	<i>Volume (µl)</i>
1 T cell ISO	IgG2Ak FITC	BD Pharmingen	5
	IgG1 PE	BD Pharmingen	5
	IgG2bK Alexafluor 647	BD Biosciences	5
	CD4 PC7	Beckman Coulter	8
	CD3 APC-CY7	BD Biosciences	5
2 CD45RO ICOS	CD45RO FITC	BD Pharmingen	10
	ICOS PE	BD Pharmingen	20
	CXCR5 Alexafluor 647	BD Pharmingen	5
	CD4 PC7	Beckman Coulter	8
	CD3 APC-CY7	BD Biosciences	5
2 CD57	CD57 FITC	BD Pharmingen	10
	ICOS PE	BD Pharmingen	20
	CXCR5 Alexafluor 647	BD Pharmingen	5
	CD4 PC7	Beckman Coulter	8
	CD3 APC-CY7	BD Biosciences	5

After incubation, all blood samples (except the TACI stained tube and TACI isotype tube), were lysed using Beckman Coulter TQ Prep, and then washed twice with 2mls of PBS. 300µl of PBS was added to cell pellets for acquisition on the LSRII flow cytometer.

The two TACI tubes per sample were lysed by the addition of 2mls of BD FACsLyse (1:10 dilution) (*Becton Dickinson*), incubated for 20 minutes and washed twice in PBS. Supernatant was aspirated and the pellet resuspended in 100µl of PBS. Streptavidin-PE (*Sigma Aldrich*) was added at an appropriate concentration (varied per batch) and incubated for a further 20 minutes. After a wash in PBS, samples were made up to 300µl of PBS for acquisition.

Acquisition of stained samples was performed using five-colour flow cytometry on a LSRIITM flow cytometer (*Becton Dickinson*) using FACsDIVA software (*Becton-Dickinson*). PMT voltages were optimised using SpheroTM rainbow calibration particles (*Becton-Dickinson*) on a daily basis. Compensation settings for each panel were obtained using whole blood tubes stained with single fluorochromes or cells

alone and then automatic compensation settings were calculated using FACSDIVA software. Settings were checked using healthy control blood samples.

All data was analysed off-line using FLOWJOTM software (*Tree-Star, CA*). Viable lymphocytes were examined using forward- *versus* side-scatter gating and then a FSC A v FSC H gate to exclude doublets. B cells were then analysed using side-scatter *versus* CD19 gating. Ten thousand events were acquired on the B cell CD19+ gate and the results are expressed as a percentage of CD19+ events. Isotype controls were used to set CD19+ quadrants. For T_{FH} analysis, T cells were analysed using a CD3 v SSc gate, then CD3+CD4+ T cells were selected. 50,000 CD4 events were acquired and the results expressed as the percentage of CD3+CD4+ T cells or as the percentage of CD3+CD4+CD45RO+ T cells (for T_{FH} T cells).

2.3.6 Analysis of B cell chemokine receptors by flow cytometry: Gating strategy for BAFF-R, BCMA and TACI (Chapter VI)

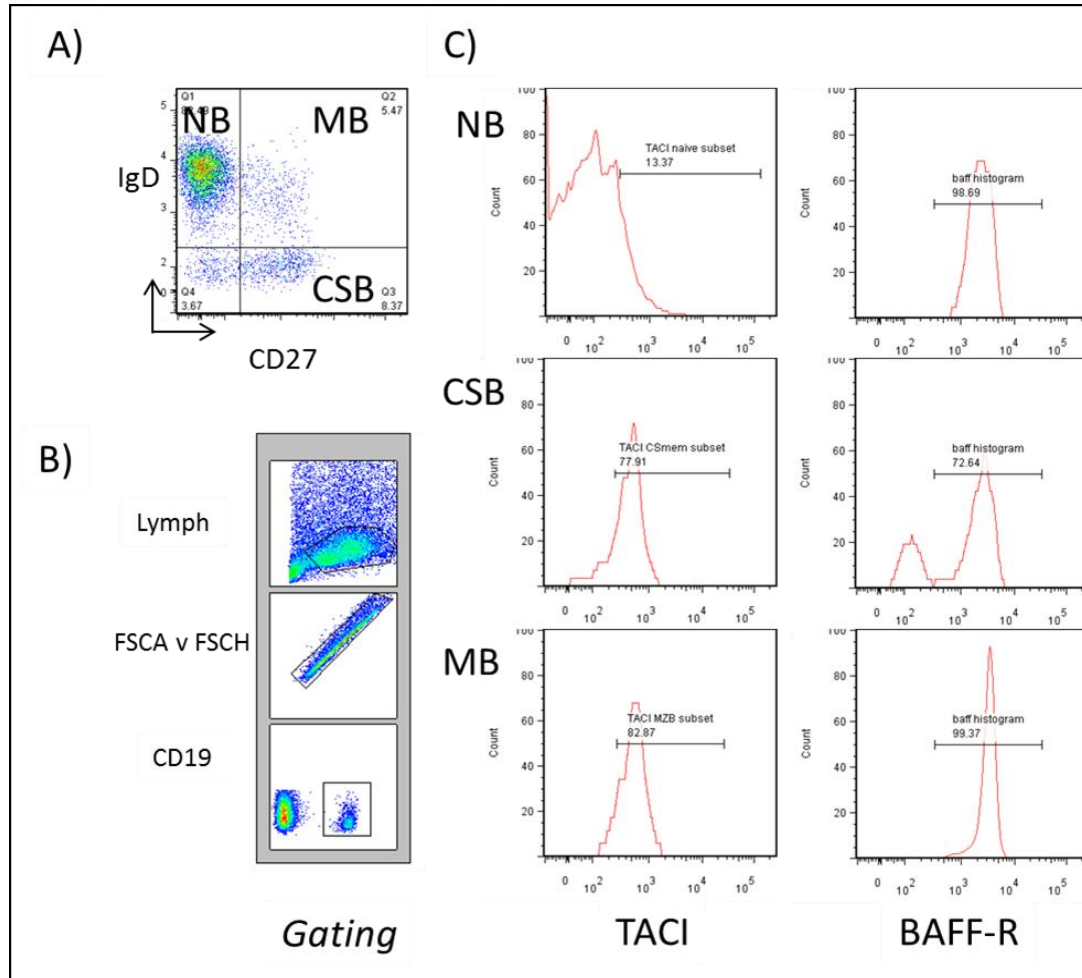


Figure 17: Gating strategy for expression of TACI and BAFF-R by B cell subsets.

Sample flow cytometry plots. 17A) Dot plot to show representative B cell subsets staining: naïve B cells (NB), IgM memory B cells (MB) and class switched B cells (CSB). **B)** A lymphocyte gate was set according to FSc and SSc, doublets were excluded using an FScA v FScH gate, and then gates were set on CD19+ B cells. 10,000 B cell events were collected. **C)** Representative histograms to show expression of TACI and BAFF-R by B cell subsets. Numbers on graph show percentage expression by each B cell subset on graph. Histogram gated on a HIV-1 infected patient using an isotype matched control.

2.3.7 Gating strategy for Tissue like B cells using additional markers CD11c and FCLR4 (Chapter V)

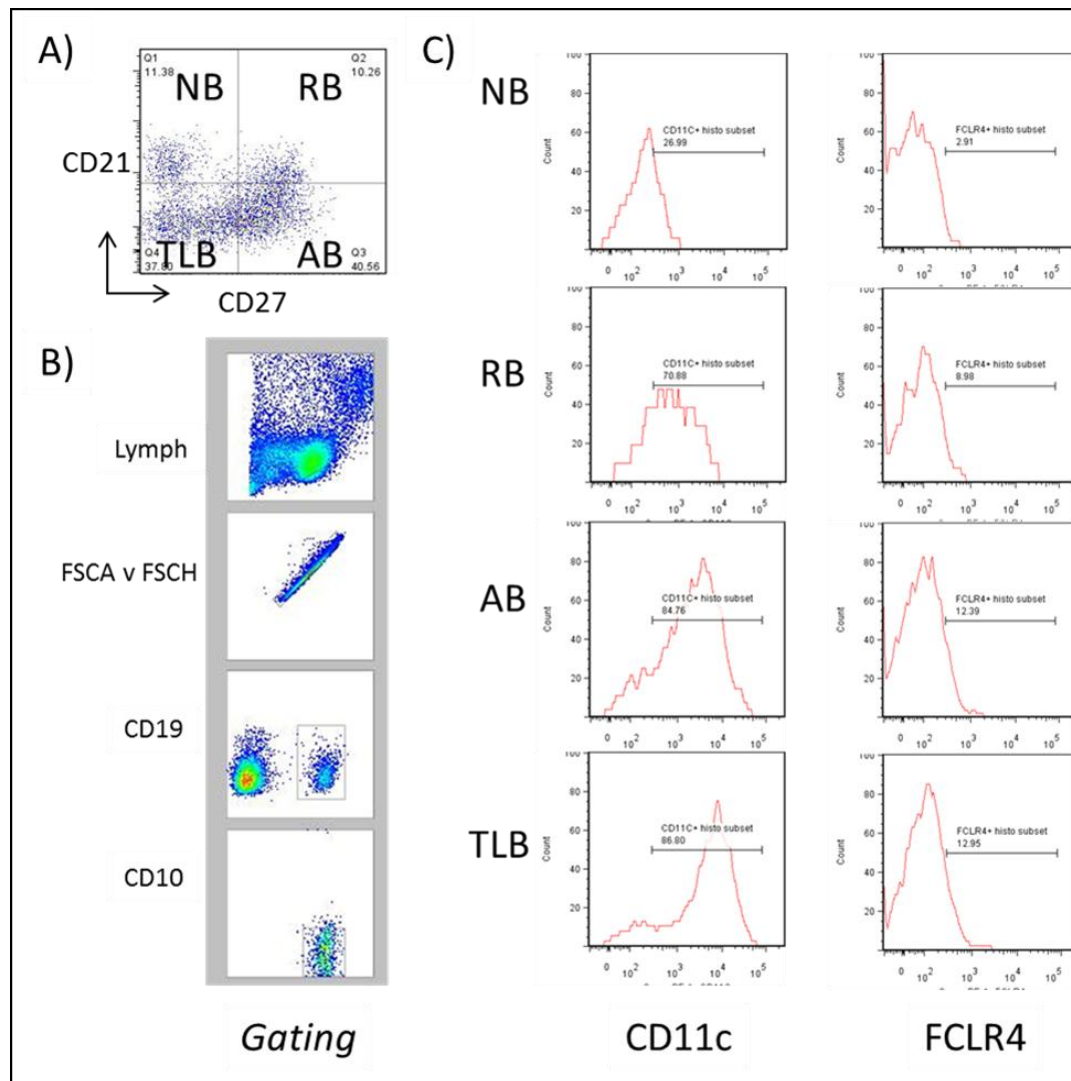


Figure 18: Gating strategies for tissue like B cells using CD11c and FCLR4

Sample flow cytometry plots. 18A) Dot plot to show representative B cell subsets staining of an untreated HIV-1 infected patient: naïve B cells (NB), resting memory B cells (RB), and activated memory B cells (AB) and tissue like B cells (TLB). B) A lymphocyte gate was set according to FSc and SSc, doublets were excluded using FScA v FSCH gate, and then gates were set on CD19+ B cells. 10,000 B cell events were collected. CD10+ B cells were excluded. C) Representative histograms to show expression of CD11c and FCLR4 by B cell subsets. Numbers on graph show percentage expression by each B cell subset on graph. Histogram gated using an isotype matched control.

The expression of the tissue like B cell subset was expressed as a percentage of CD10 negative mature B cells (see Fig.18). CD10 is expressed by transitional B cells which have other phenotypic characteristics with tissue like B cells e.g. low expression of CD21 and CD27 and therefore must be excluded from analysis. The expression of FCLR4 and CD11c were expressed as a percentage of CD19+CD10- B cells and as a percentage of B cell subsets: CD19+CD10-CD21+CD27- naïve B cells, CD19+CD10-CD21+CD27+ resting memory B cells, CD19+CD10-CD21-CD27- tissue like B cells or CD19+CD10-CD21-CD27+ activated memory B cells as measured by Moir *et al.* MFI analysis was also examined to assess the intensity of antigen density on various B cell subpopulations. Gating was defined according to the isotype tube, set at <1% staining in the quadrant sector.

2.3.8 Analysis of T_{FH} cells by flow cytometry

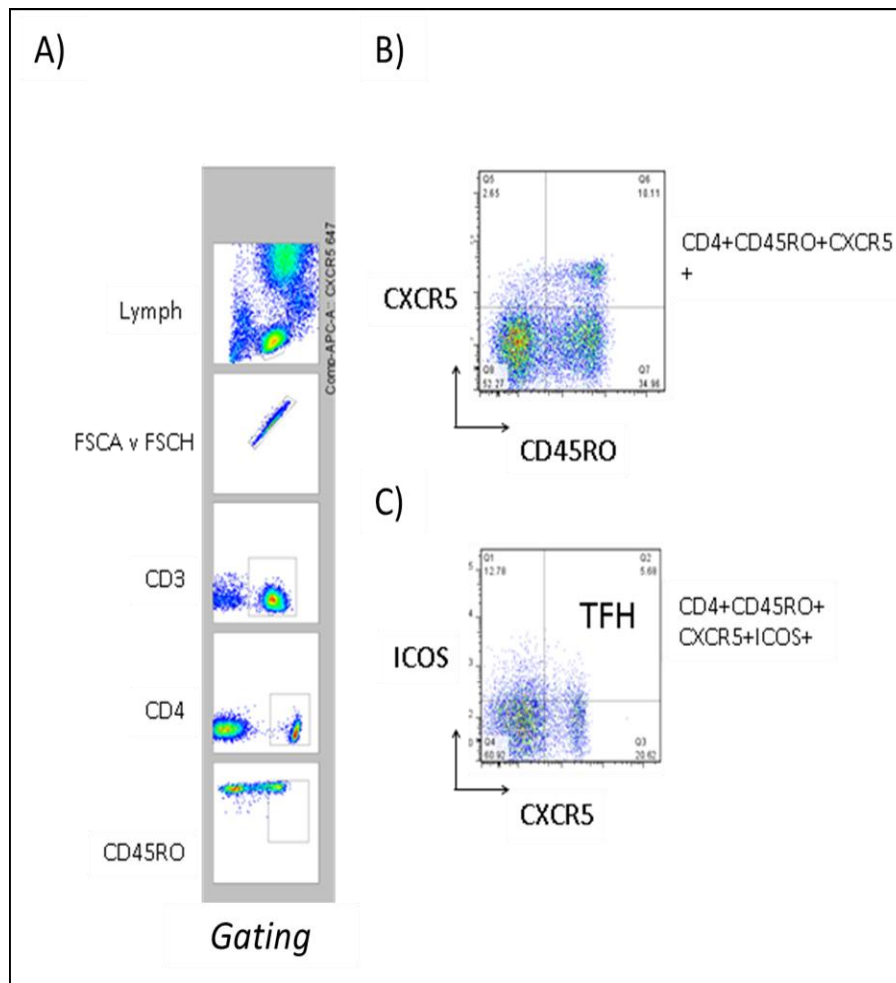


Figure 19: Gating strategy for T_{FH} cells

Sample flow cytometry plots. T_{FH} cells were determined as the percentage of ICOS+CXCR5+CD45RO+CD3+ CD4 T cells. In addition, the percentage of CXCR5+CD4 T cells and CD45RO+CXCR5+CD4 T cells was determined. 19A) A lymphocyte gate was set according to FSc and SSc, doublets were excluded using an FScA v FScH gate, then gates were set on CD3+ T cells, then CD4+ T cells. 50,000 CD4 events were acquired. B) Using cells gated on CD3+CD4+, CD45RO+CXCR5+ T cell numbers can be determined (upper right quadrant). C) Using CD3+CD4+CD45RO+ gating, CD4+CD45RO+CXCR5+ICOS+ T_{FH} cells are determined (upper right quadrant) Numbers on dot plot show percentage expression by each T cell subset on graph. T cells gated using an isotype matched control.

2.4 Statistical Outcomes

Non parametric statistical tests are used for populations that are not normally distributed or those populations which are not assumed to be normally distributed. The Mann-Whitney U test was used to analyse non-parametric data. The Mann-Whitney U test is used to test whether observations (median values) in one sample are larger than observations in the other sample. Spearman's rank correlation coefficient was used to test to see whether there was an association (statistical dependence) between two non-parametric variables. Chi Square test with Yates' correction for small sample sizes is used to test the difference between a sample and another established or hypothesized distribution.

The results for T and B cells subsets, HIV viral load, tetanus and pneumococcal serology were expressed as median and inter quartile ranges (IQR). Differences between patient groups were tested for statistical significance using the Mann-Whitney U test. Univariate correlations between different variables within a group were assessed using Spearman rank tests. Partial regression analysis was used to correct for the influence of viral load on the relationship between CD4 T cell counts and different B cell subsets.

A Chi square test with Yates' correction for small sample size was used to compare the proportion of HIV-1 patients on ART who achieved IgM and IgG pneumococcal vaccine responses, with healthy controls. The Mann Whitney U test was used to test our hypothesis that B cell memory proportions influenced post vaccine responses (IgM memory B cells and pneumococcal IgM levels: switched memory B cells and pneumococcal/tetanus IgG levels). All other secondary variables (CD4 T cell counts, viral load, markers of immune activation) which potentially could influence post vaccine antibody levels underwent Bonferroni analysis. All statistical calculations were performed with SPSS v14 software. GraphPad Prism software was used to display a selection of graphs.

3. Chapter III: Transitional, memory B cell numbers and T cell activation status of patients with untreated and treated HIV-1 infection, CVID and asplenia.

3.1 Introduction

Since the discovery of CD27 as a memory B cell marker in 1999, analysis of B cell memory subsets by flow cytometry has become of interest in patients with both primary and secondary immune deficiency. Antigen experienced CD19+CD27+memory B cells are divided into two main populations (Warnatz, Denz et al. 2002; Weller, Braun et al. 2004). Switched CD19+CD27+IgM-IgD- B cells are involved in T cell-dependent immune responses, secrete IgG and IgA antibodies and maintain long-term serological memory. IgM memory CD19+CD27+IgMhighIgDlowCD21high B cells are important in T cell-independent immune responses and secrete high-affinity IgM in the early phase of infection to inhibit microbial replication in blood (Shi, Agematsu et al. 2003). This cell population is believed to be the circulating counterpart of splenic marginal zone B cells and responds to the 23-valent polysaccharide pneumococcal vaccine (Weller, Braun et al. 2004). Unlike switched memory B cells, IgM memory B cells do not promote long-term protective humoral immune responses and it has been proposed that this B cell subset be regarded as natural effector B cells which bridge innate and adaptive immune responses (Weller, Reynaud et al. 2005).

CD19+CD38++IgM++CD10+ transitional B cells are immature B cells that have exited the bone marrow to mature in the periphery. In healthy individuals, transitional B cells are found at very low numbers (0 to 4% of B cells) in the peripheral blood. Increased numbers of transitional B cells are a characteristic feature of several inherited humoral immunodeficiency conditions and HIV infection (Cuss, Avery et al. 2006; Malaspina, Moir et al. 2006) and are associated with decreased memory B cells (Cuss, Avery et al. 2006). Expansion of transitional B cells may reflect increased immune activation in HIV-1 infection. The heterogeneity of common variable immunodeficiency (CVID) has led to attempts to classify this condition to define homogeneous patient groups (see General Introduction, Chapter I for further details).

The most widely used classification schemes (Freiburg, Paris and EURO Class) assess the composition of memory B cell subsets (Warnatz, Denz et al. 2002; Piqueras, Lavenu-Bombled et al. 2003; Wehr, Kivioja et al. 2008). A number of studies have found associations with defects in B cell memory composition and clinical complications of CVID including respiratory disease (bronchiectasis and granulomatous lung disease) (Carsetti, Rosado et al. 2005). See General Introduction (Chapter I) for a detailed account of B cell maturation pathways and B cell classification systems.

In HIV-1 infection, invasive pneumococcal infection is an important cause of morbidity even in HIV patients on antiretroviral therapy (ART) (Barry, Zetola et al. 2006). Serological memory after exposure to pathogens or vaccination is maintained by plasma cells and memory B cells (Bernasconi, Traggiai et al. 2002; Traggiai, Puzone et al. 2003). Intrinsic defects in B cell function result in a failure of this cell population to respond to accessory CD4 T cell help (Moir, Malaspina et al. 2001; Moir, Ogwaro et al. 2003) and inhibition of Ig isotype class switching by HIV viral proteins may also contribute to the development of impaired humoral immune responses seen in HIV-1 infection (Qiao, He et al. 2006). Memory B cell (CD19+CD27+) counts are reduced in HIV-1-infected individuals (De Milito, Morch et al. 2001; Nagase, Agematsu et al. 2001; Chong, Ikematsu et al. 2004) and are associated with reduced measles and tetanus antibody concentrations (De Milito, Nilsson et al. 2004). Plasma levels of IgM and IgG pneumococcal antibody are reduced in HIV-1 infection and the capacity of PBMC to produce IgG and IgM pneumococcal antibody following *in vitro* B cell polyclonal stimulation is impaired (Titanji, De Milito et al. 2006). Loss of CD19+CD27+ memory B cells may be also an additional factor responsible for impaired humoral immunity and poor vaccine responses in HIV infection.

Patients that have congenital asplenia or have undergone splenectomy due to trauma, lose the splenic compartment which is believed to be crucial for the maturation of IgM memory B cells and subsequent production of IgM antibodies important for protection against encapsulated bacteria (Kruetzmann, Rosado et al. 2003; Weller, Braun et al. 2004). Splenectomy has long been associated with impaired antibody responses, especially of the IgM class, to pneumococcal vaccination post operation

(Hosea, Brown et al. 1981; Hosea, Burch et al. 1981; Giebink, Le et al. 1984). Pneumococcal vaccination is a standard preoperative procedure prior to a scheduled splenectomy because *S.pneumoniae* is a common cause of postoperative infections (Ammann and Diamond 1978). Splenectomised individuals present with low or absent IgM memory B cell numbers (Carsetti, Pantosti et al. 2006), therefore splenectomised patients were tested as disease controls for this assay.

3.2 Aims

- 1) To measure the distribution of transitional, naive and memory B cell subsets in patients with HIV-1 infection and to compare these with other patient groups at increased risk of invasive pneumococcal disease (CVID and splenectomised patients).

- 2) To apply the Paris classification for PID to the B cell compartment of untreated and treated HIV-1 infected individuals.

- 3) To see whether there was an association between memory B cell percentages and respiratory complications in patients with CVID.

3.3 Patient Cohort

Peripheral blood B and T cell phenotyping was performed on 29 HIV-1 infected untreated patients, 55 HIV-1 treated patients on ART (FI study), 28 CVID patients, 8 individuals with a history of splenectomy and 17 healthy control laboratory workers from the Immunology laboratory. Demographic and flow cytometry data were not available for occupational health donors (Table 7). The results shown for controls are for laboratory staff only. We studied 36 patients with CVID to assess whether memory B cell subsets were associated with respiratory complications. 28 patients were from the described cohort above and an additional eight patients were included, also diagnosed by ESID criteria. Bronchiectasis was defined clinically on chest CT scan according to standardised clinical criteria as part of clinical care. Pulmonary function tests were performed in all patients (FEV1, FVC, KCO and FEV1/FVC ratio) and analysed by a clinician, Dr. P Kelleher. Airflow limitation was defined by a respiratory clinician as FEV1/FVC <70% and FEV1 <80%.

Table 7: Patient Demographics and immune parameters (median and IQR)

	HC (n=17)	HIV ART- (n=29)	HIV ART+ (n=55)	CVID (n=36)	SPLEN (n=8)
<i>Patient Demographics (Cells/ul)</i>					
Age	31 (28-40)	38 (35-44)	46 (41-52)	50 (37-61)	40 (33-56)
Viral Load	NA	48,187 (7,993–155,233)	<50	NA	NA
Time on ART (days)	NA	NA	1,013 (547–1,760)	NA	NA
M/F	12/5	27/2	51/4	11/25	3/5
CD4	788(553-918)	374(188-472)	409(279-556)	660 (410-854)	1435(905-2323)
CD8	388(280-516)	925(711-1280)	793(624-1296)	347(179-711)	713(500 -904)
CD19	171(116-219)	111 (59-167)	249(167-311)	170 (56-250)	686(462-1053)
<i>B cell subsets (% CD19)</i>					
CD27+ Mem	31.0(22.8-42.5)	18.6(11.2-30.1)	16.9(10.8-26.6)	9.6 (4.6 -23.8)	10.6(7.9-19.0)
IgM Mem	14.9 (11.6-23.1)	6.6 (3.7-14.0)	5.5 (2.9-8.6)	6.5 (2.9-20.2)	3.3 (1.6-8.1)
CS Mem	14.9 (11.2-23.0)	14.3 (7.4-20.1)	10.7 (6.4-19.5)	2.3 (0.7-4.7)	7.8 (3.9-11.9)
Trans B	0.8 (0.4-1.6)	7.5 (3.7-12.3)	2.6 (1.4-3.9)	2.0 (0.9-6.0)	2.1 (0.9-6.8)

**Note:* Data for HC, HIV-1 patients not on ART (HIV ART-), patients on ART (HIV ART+), CVID and splenectomised patients (SPLEN). NA (not applicable).

From Table 7, HIV-1 patients on ART were a median age of 46 years old and were significantly older than controls and untreated HIV-1 patients ($p < 0.001$). The CVID patient group was also older than healthy controls ($p < 0.01$) and ART naïve patients ($p < 0.05$). Very few HIV-1 infected patients were female. CD19+ B cell counts were significantly lower in the untreated HIV-1 cohort in comparison with healthy controls ($p < 0.01$). CD4 T cell counts were significantly lower in untreated HIV-1 infected patients compared to healthy controls, CVID and HIV-1 patients on ART (all $p < 0.01$). Memory B cell (CD19+CD27+) percentages were significantly reduced in both HIV patient groups, CVID and splenectomised patients compared with controls (all $p < 0.01$), see following text for detailed B cell subset analysis.

3.4 Results

3.4.1 Impact of ART on B cell memory and maturational subsets by flow cytometry

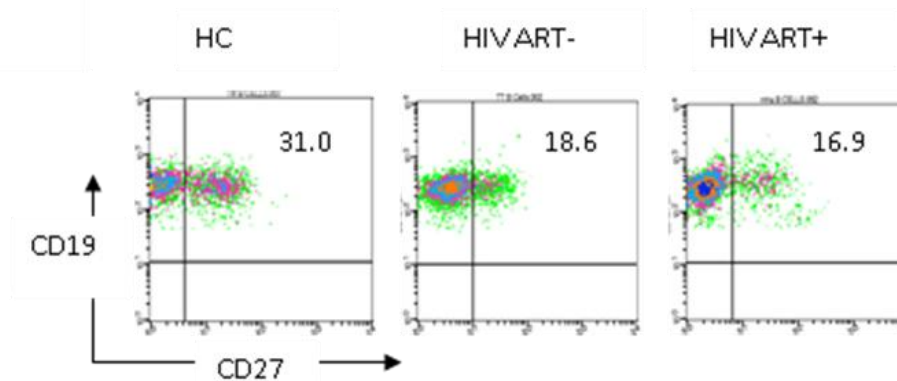


Figure 20: Flow cytometry dot plots showing CD19+ CD27+ total memory B cells in HC and HIV-1 infected patients.

Representative plots of CD19+CD27+ memory B cells in healthy controls (HC) (n=17), untreated HIV-1 infected individuals (HIV ART-) (n=29) and HIV-1 ART treated patients (HIV ART+) (n=55). Numbers in upper right quadrant indicate median values for each group as a percentage of CD19+ B cells, using an isotype matched control. The Mann-Whitney U test was used to analyse non-parametric data.

As shown in Table 7 and Fig. 20, there was a significant reduction in CD19+CD27+ memory B cell populations in both untreated HIV-1 infection and HIV-1 infected patients on ART compared to healthy controls ($p < 0.001$ for both groups). In addition, as expected, total memory B cell percentages were also significantly decreased in patients with COVID and splenectomised individuals (see Table 7), ($p < 0.01$). These findings were consistent with the literature.

3.4.2 Analysis of IgM memory B cell subsets

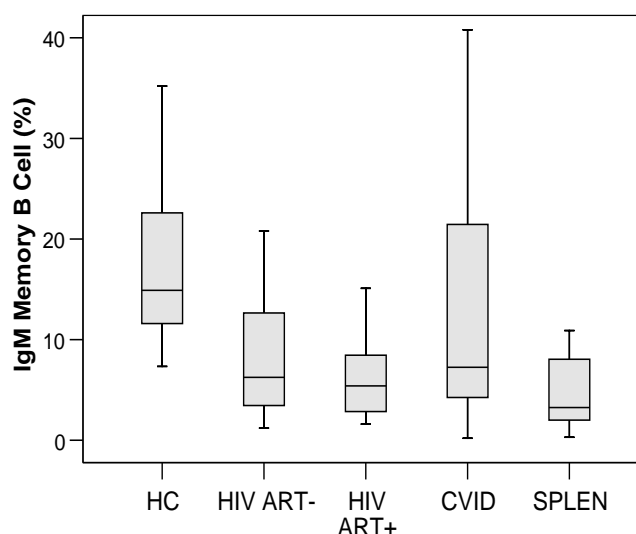
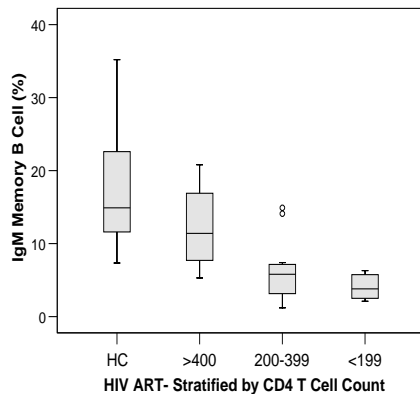


Figure 21: IgM memory B cell percentages in HIV-1, CVID, splenectomised patients and healthy controls.

Box and whisker plot displaying median and IQR IgM memory B cells (CD27+IgD+IgM+) as a percentage of CD19+ B cells, for healthy controls (HC), (n=17), untreated HIV-1 infected individuals (HIV ART-) (n=29), HIV-1 ART treated patients (HIV ART+) (n=55), CVID patients (n=28) and splenectomised individuals (SPLEN) (n=8). Gates were set using an isotype matched control. The Mann-Whitney U test was used to analyse non-parametric data.

The median percentage of IgM memory B cells were significantly reduced in both untreated HIV-1 infected patients and ART treated patients ($p < 0.001$, $p < 0.001$) in comparison with healthy controls (10,10.8 v 22.0 cells/ μ l) (Fig. 21, Table 7). The median range for IgM memory B cells in the healthy control group, (n=17) was consistent with previous publications (Piqueras, Lavenu-Bombled et al. 2003). ART treatment did not restore IgM memory B cell populations in HIV-1 infected patients despite significantly increased absolute B cell counts. Although there was considerable heterogeneity in IgM memory B cell percentage in the CVID group, overall, patients with CVID had significantly reduced circulating IgM memory B cells compared to healthy controls. IgM memory B cells mature in the spleen and consistent with the literature, individuals that had undergone a splenectomy had the most marked loss of IgM memory B cells (Fig. 21).

A)



B)

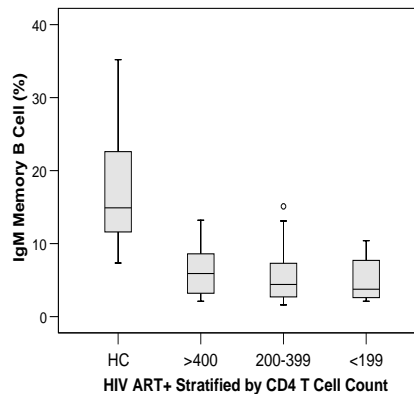


Figure 22: IgM memory B cell percentages stratified by CD4 count in HIV-1 patient groups vs. HC.

Box and whisker plots displaying median and IQR IgM memory B cells (CD27+IgD+IgM+) as a percentage of CD19+ B cells. Gates were set using an isotype matched control. 22A) HC (n=17) and untreated HIV-1 patients (n=29), 22B) HC (n=17) and HIV-1 patients on ART (n=55). The Mann Whitney U test was used to analyse non-parametric data.

HIV-1 patient groups were stratified by CD4 T cell counts to assess the effect of progressive infection on IgM memory B cell percentage. In the untreated HIV-1 patient group (Fig. 22A), there was a significant decrease in median IgM memory B cells in patients with a reduced CD4 count of 200-399 or under 200 cells/ μ l in comparison with those patients with a higher CD4 count >400 cells/ μ l (6.2% in 200-399 group v 11.4% in the group >400cells/ μ l, $p=0.002$). Correcting for viral load, there was a significant association between IgM memory B cell percentage and CD4 T cell count ($r = 0.51$, $p=0.01$) in the HIV ART- patients by partial regression analysis.

In the ART treated HIV-1 patient group (Fig. 22B), stratification by CD4 T cell count did not show any significant differences between the percentage of IgM memory B cells in the HIV-1 ART+ treated group. IgM memory B cell percentages were significantly lower in all HIV patients groups, even in patients with CD4 T cell greater than 400 cells/ μ l (Fig. 22B), compared to healthy controls ($p<0.001$), suggesting ART did not restore IgM memory B cell percentages.

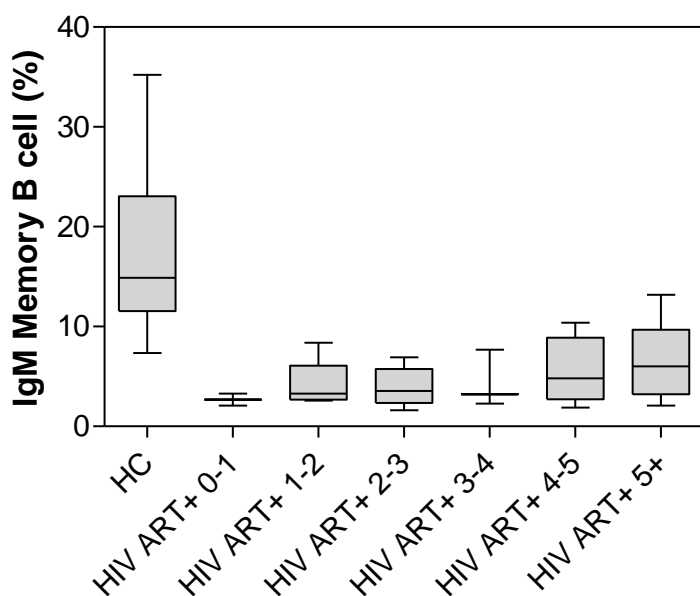


Figure 23: IgM memory B cell percentages in HIV-1 infected patients stratified by time in years since commencing successful ART.

Box and whisker plot displaying median and IQR IgM memory B cells (CD27+IgD+IgM+) as a percentage of CD19+ B cells. Gates were set using an isotype matched control in HC (n=17) and HIV ART+ patients (n=50) stratified into: <1year on ART (HIV ART+0-1,n=2), 1-2yrs on ART (HIV ART+1-2, n=4), 2-3yrs on ART (HIV ART+2-3, n=8), 3-4yrs on ART (HIV ART+3-4, n=3), 4-5yrs on ART (HIV ART+4-5, n=7) and >5yrs on ART (HIV ART+5+, n=27). The Mann-Whitney U test was used to analyse non-parametric data.

After data from this chapter was published, a later publication suggested that the reduction observed in the IgM memory B cell population of untreated HIV-1 infected individuals is normalised after commencing ART therapy (Moir, Malaspina et al. 2008). The median time on ART for our patients was 1013 days, suggesting this finding was not consistent with our data. We retrospectively stratified available data from 50 HIV patients on ART according to duration since successful ART had been achieved. From Fig. 23, it can be seen that most of the HIV-1 patients on ART in this cohort had been on ART for four years or longer (34/50), yet IgM memory B cell percentages in these patients were still significantly reduced compared to healthy controls ($p<0.0001$). Therefore, loss of IgM memory B cells in HIV-1 infection were not normalised by treatment despite greater than four years of successful ART.

3.4.3 Analysis of class switched memory B cell subsets

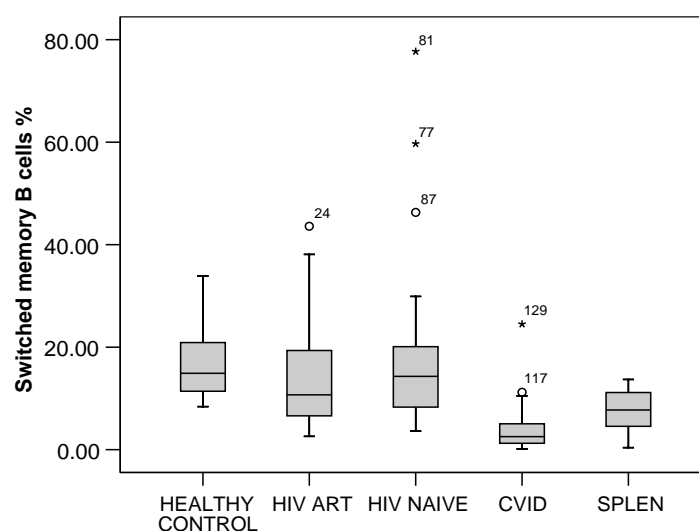
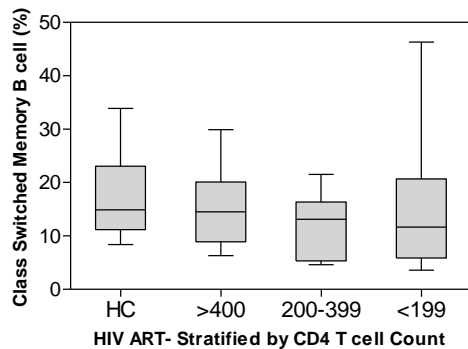


Figure 24: Percentages of class switched memory B cells in HC, untreated HIV-1 patients, HIV-1 ART+ patients, CVID and splenectomised individuals.

Box and whisker plot displaying median and IQR class switched memory B cells (CD27+IgD-IgM-) as a percentage of CD19+ B cells, for healthy controls (n=17), untreated HIV-1 infected patients (HIV NAIVE) (n=28), treated HIV-1 infected patients (HIV ART) (n=55), CVID patients (n=29) and splenectomised individuals (SPLEN) (n=8). Gates were set using an isotype matched control. The Mann-Whitney U test was used to analyse non-parametric data.

There was no significant reduction overall in the median percentage of class switched memory B cells in both HIV patient groups in comparison with healthy controls (Fig. 24). As expected, the CVID patient control group had a significantly reduced median percentage of switched memory B cells ($p < 0.001$). A reduction in class switched memory B cells was found in the splenectomised group in comparison with the healthy control population ($p < 0.001$), however, this was a small group of eight patients, with heterogeneous disease: ITP, sarcoid and trauma, possibly in addition to a loss of the IgM memory B cells, these patients were more likely to have additional reductions in the class switched compartment. Additionally it is possible that some underlying conditions (ITP, sarcoid etc.) may be associated with reductions in the class switched memory B cell compartment.

A)



B)

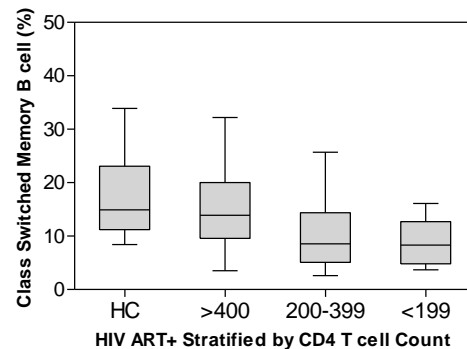


Figure 25: Switched memory B cell percentages stratified by CD4 count in HIV patient groups vs. HC.

Box and whisker plots displaying median and IQR class switched memory B cells (CD27+IgD-IgM-) as a percentage of CD19+ B cells. 25A) HC (n=17) and untreated HIV-1 patients (n=29), 25B) HC (n=17) and HIV-1 patients on ART (n=55). Gates were set using an isotype matched control. The Mann Whitney U test was used to analyse non parametric data.

Switched memory B cell percentages were not reduced overall in HIV-1 infected untreated individuals (Fig. 25A). Stratification of untreated HIV-1 patients according to CD4 T cell counts showed that in untreated HIV infection, disease progression was not associated with lower class switched memory B cells in this cohort. Strikingly, stratification of HIV-1 patients on ART with CD4 T cell counts less than 400 cells/ μ l also showed a significant reduction in switched memory B cells compared with patients with higher CD4 T cell numbers (8.9% v 13.8% $p = 0.002$) and healthy controls (8.9% v 14.9% $p < 0.001$), (Fig. 25B). The loss of the switched memory compartment in a proportion of HIV-1 patients on ART may be associated with inadequate immune reconstitution and persistent suppression of the CD4 T cell count on therapy.

3.4.4 Analysis of Transitional B cells

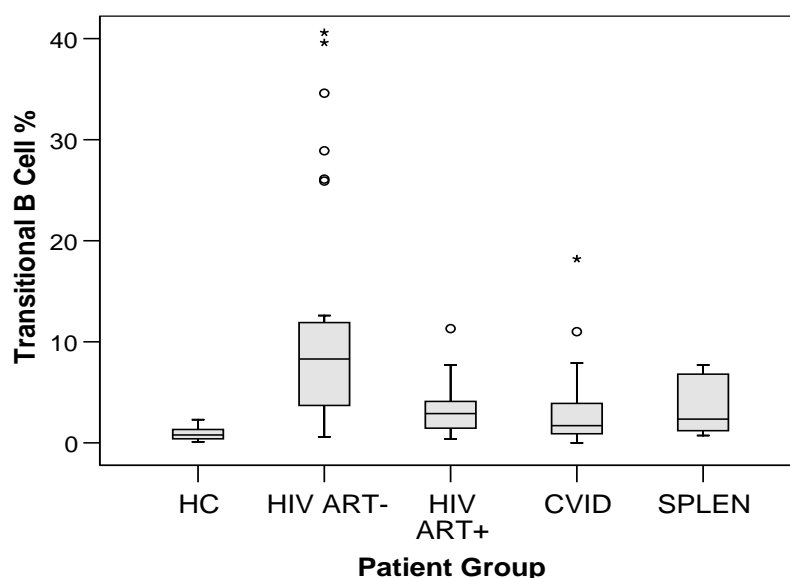


Figure 26: Transitional B cell percentages in HIV untreated and HIV treated patients and control groups.

Box and whisker plot displaying median and IQR transitional B cells (CD19+CD38++IgM++) as a percentage of CD19+ B cells, for healthy controls (HC) (n=17), untreated HIV-1 infected individuals (HIV ART-) (n=29), HIV-1 ART treated patients (HIV ART+) (n=55), CVID patients (n=28) and splenectomised individuals (SPLEN) (n=8). Gates were set using an isotype matched control. The Mann-Whitney U test was used to analyse non-parametric data.

The percentage of transitional B cells was significantly raised in all HIV patient groups, CVID ($p=0.02$) and splenectomised individuals ($p<0.03$) compared to healthy controls (Fig. 30). In the healthy control group, less than 2% of B cells were transitional B cells (as measured by CD38+IgM++), which is consistent with previous studies. The most striking increase in transitional B cells was seen in the untreated HIV-1 patient group ($p<0.0001$), yet HIV-1 patients in the ART treated group still displayed an increase in transitional B cell numbers ($p<0.0001$). In untreated and treated HIV-1 infection, no correlation between transitional B cell percentage and CD4 count was found. On analysis, CVID patients showed a correlation between transitional B cell and CD27+ memory B cell percentage ($p<0.04$) however, this was not true for both HIV-1 infected groups.

3.4.5 *Heterogeneity of B cell subsets in CVID and HIV-1: use of a Classification*

System to categorise patients.

Considerable heterogeneity in the CVID patient group has led to classification of this group on the basis of impaired B cell memory differentiation (Warnatz, Denz et al. 2002; Piqueras, Lavenu-Bombled et al. 2003). We analysed the distribution of B cell subsets in HIV-1 infected individuals according to the Paris classification for CVID (Piqueras, Lavenu-Bombled et al. 2003). The Paris scheme classifies patients into three subgroups according to total memory B cells, class switched memory B cells and/or IgM memory B cell levels as a percentage of CD19+ B cells (Piqueras, Lavenu-Bombled et al. 2003). Patients with less than 1% of B cells were excluded from analysis. The MBO group was defined as <11% total CD19+CD27+ memory and had the greatest defect in B cell differentiation. The MB1 group classified patients with <8% class switched memory B cells and >8% IgM memory B cells. The MB2 group defined those patients with normal-high numbers of memory B cells that did not fit into MBO or MB1. We added the MZB- group to include those patients with <8% IgM memory B cells, yet normal class switched memory B cell numbers.

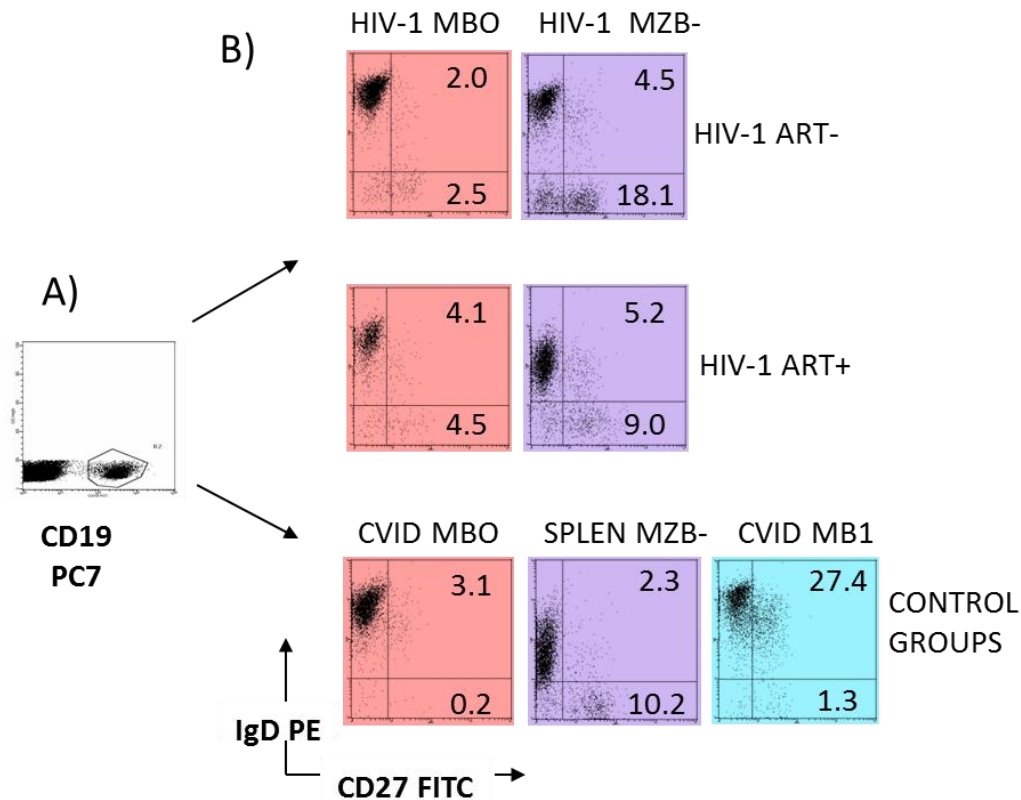


Figure 27: Dot plots to show HIV-1 patients classified according to a modified Paris B cell classification for CVID

27A) Cells gated on FSc and SSc, then on CD19 B cell gate. B) Representative plots of patient samples categorised in the MBO, MB1 or MZB- groups as described. MB2 group (normal memory B cells) not shown. IgM memory B cells (upper right quadrant) and class switched memory B cells (lower right quadrant) in HC (n=17), untreated HIV-1 infected individuals (HIV ART-) (n=29), HIV-1 ART treated patients (HIV ART+) (n=55) and control groups: CVID (n=28) and splenectomised patients (SPLEN) (n=8). Numbers in upper right and lower right quadrant indicate median values for each group as a percentage of CD19+ B cells, using an isotype matched control.

Classifying the untreated and treated HIV patients using a modification of the Paris scheme (Piqueras, Lavenu-Bombled et al. 2003) showed that individual HIV-1 patients had marked defects in B cell subsets similar to those patients with CVID (see Fig.27). Despite no overall median group reduction in class switched memory B cells compared to HC, using the Paris scheme, 17/55 (31%) of HIV-1 patients on ART and 7/29 (24%) of untreated HIV-1 infected individuals had marked reductions in class switched B cell percentages (less than 8% of total B cell population). Most of these patients with a reduction in the switched memory B cell subset also had a loss in the IgM B cell subset and would meet the criteria for the CVID patient group (MBO)

with the greatest impairment in B cell differentiation (Table 8). 24% of untreated HIV-1 infected individuals and 27% of HIV-1 infected individuals on ART were thus classed as MBO (see Table 8). 41% of HIV-1 infected untreated individuals and 49% of HIV-1 infected individuals on ART had a reduction in the IgM memory B cell subset only (modified MZB- subset) (Table 8), compared to 1/17 of the HC group and 1/28 of the CVID group. Just 34% of untreated HIV-1 individuals and 20% of HIV-1 infected individuals on ART had normal overall levels of memory B cells (>11%).

Table 8: Modified classification scheme (no. of subjects and percentage)

GROUP	MBO	MB1	MB2	MZB-
HIV ART- (n=29)	7 (24%)	0 (0%)	10(34%)	12 (41%)
HIV ART+ (n=55)	15 (27%)	2 (4%)	11 (20%)	27 (49%)
HC (n=17)	0 (0%)	0 (0%)	16 (94%)	1 (6%)
CVID (n=28)	16 (57%)	9 (32%)	2 (7%)	1 (4%)

3.4.6 Memory B cells stratified according to respiratory disease in CVID patients

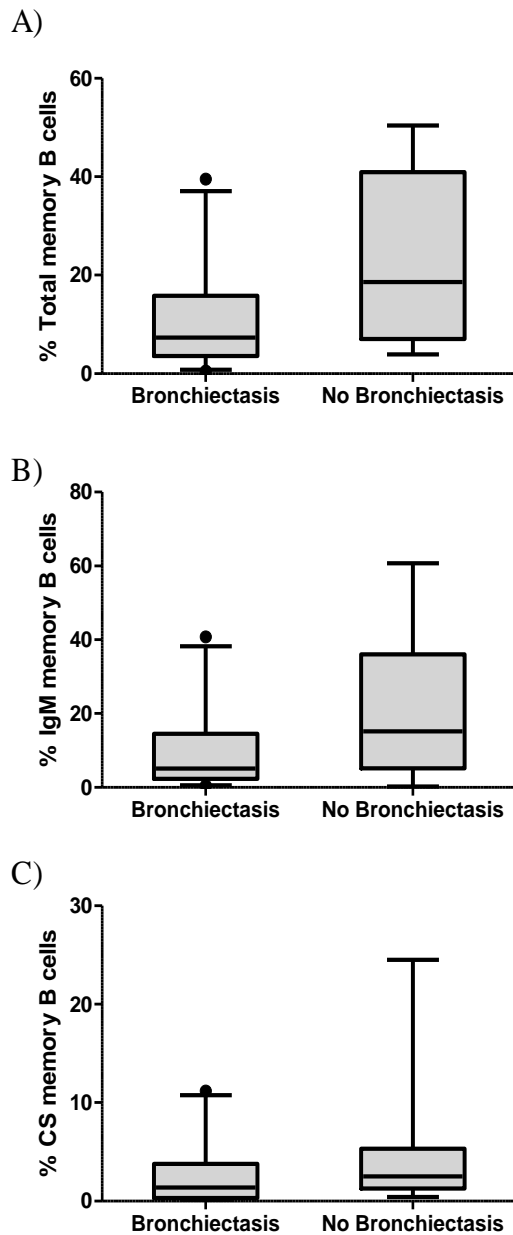


Figure 28: Memory B cells in CVID patients with or without bronchiectasis

Box and whisker plots displaying median memory B cells as a percentage of CD19⁺ B cells in 36 patients diagnosed with CVID. 23 patients were diagnosed with bronchiectasis (Bronchiectasis), whilst 13 patients had no bronchiectasis (No Bronchiectasis). 28A) Total memory B cells (CD19⁺CD27⁺) in patients with or without bronchiectasis. 28B) IgM memory B cells (CD19⁺CD27⁺IgD⁺) in patients with or without bronchiectasis. 28C) Class switched memory B cells (CD19⁺CD27⁺IgD⁻) in patients with or without bronchiectasis. The Mann Whitney U test was used to analyse non parametric data.

Stratifying CVID patients with bronchiectasis, total memory B cell percentages were significantly lower in patients with bronchiectasis compared to patients with no diagnosis of bronchiectasis ($p=0.04$). Evaluating IgM memory B cell percentages, there was a trend towards reduced IgM memory B cells in those CVID patients with bronchiectasis but this did not reach significance ($p=0.07$). Class switched memory B cell percentages were low but not significantly different between CVID patients with or without bronchiectasis (Fig. 28C).

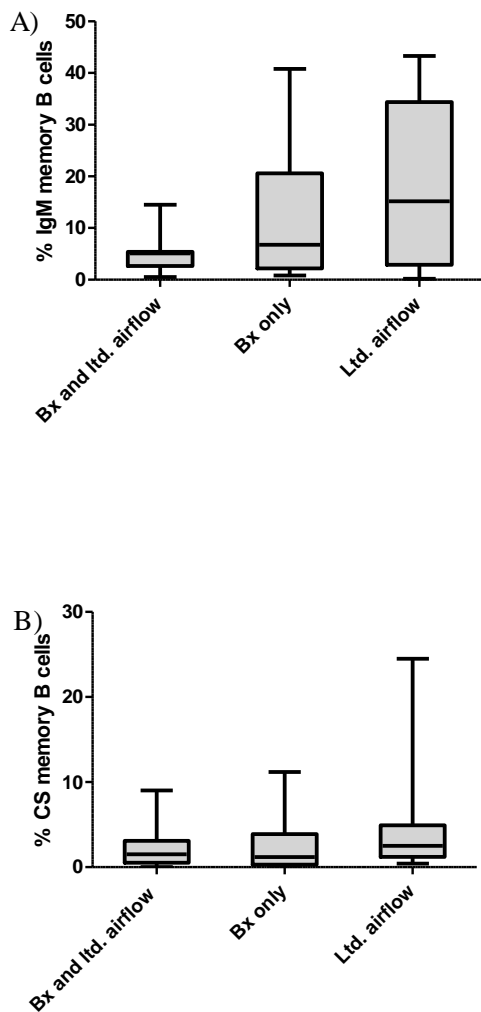


Figure 29: Levels of memory B cell subsets in CVID patients with bronchiectasis and or airflow limitation.

Box and whisker plots displaying median memory B cells as a percentage of CD19+ B cells in 32 patients diagnosed with CVID and respiratory complications. Patients stratified into: Bronchiectasis (Bx) and airflow limitation (Ltd. Airflow) (n=9), Bronchiectasis only (n=12) and airflow limitation only (n=11). 29A) IgM memory B cells (CD19+CD27+ IgD+). 29B) Class switched

memory B cells (CD19+CD27+ IgD-). The Mann-Whitney U test was used to analyse non-parametric data.

From Fig.29A, it could be seen that IgM memory B cells were reduced in CVID patients with both bronchiectasis and airflow limitation compared with no evidence of bronchiectasis, however this reduction was not statistically significant ($p=0.07$), possibly due to small cohort sizes in these groups. Further clinical data is required. The percentage of class switched memory B cells was no different whether CVID patients' had bronchiectasis and/or limited airflow, suggesting a limited role for these cells in protection against bronchiectasis (Fig.29B).

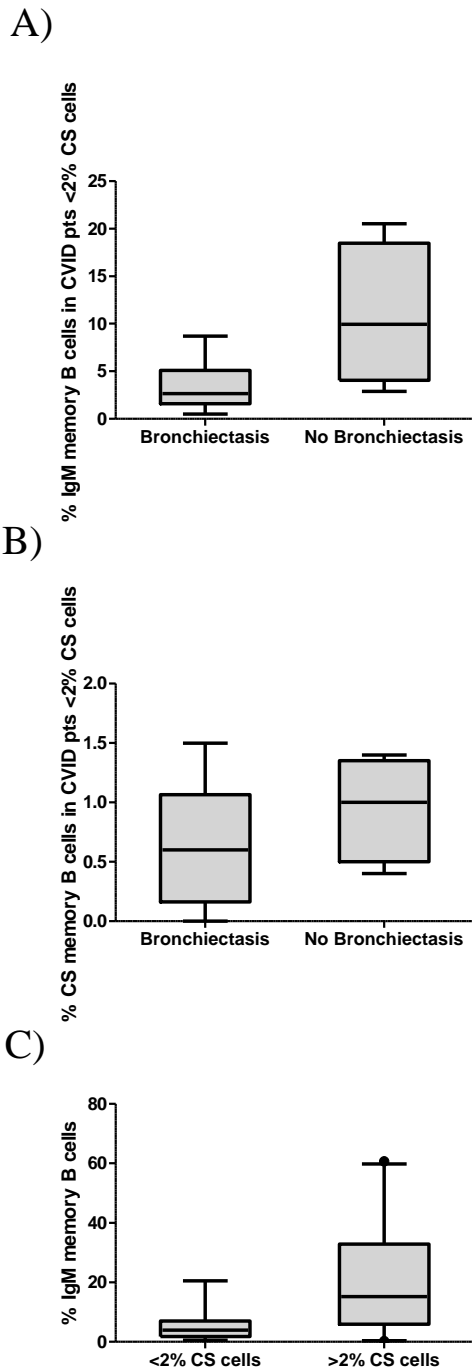


Figure 30: EURO Class classification and bronchiectasis in CVID smB- patients

Box and whisker plots displaying median memory B cells as a percentage of CD19+ B cells. CVID patients with <2% class switched memory B cells were classified as smB- (n=16) using EURO Class and according to bronchiectasis (n=12) or no bronchiectasis (n=4). Fig.30A) IgM memory B cell percentage and bronchiectasis in smB- patients, 30B) CS memory B cell percentages and bronchiectasis in smB- CVID patients and 30C) IgM memory B cells levels in smB- CVID (n=16) patients versus smB+ CVID patients (n=20) (defined as >2% CS B cells). The Mann-Whitney U test was used to analyse non-parametric data.

Decreased IgM memory B cells were significantly associated with bronchiectasis in smB- patients ($p < 0.05$), see Fig. 30A. This was a small cohort of patients, however, increasing the cut-off of smB- group from 2% to 2.5% included 20 patients: bronchiectasis ($n=13$), no bronchiectasis ($n=7$), differences in IgM memory B cell numbers and the presence of bronchiectasis were still significant ($p < 0.05$). No difference was found between class switched memory B cell numbers and the development of bronchiectasis using a cut-off of 2% or 2.5%. Fig. 30C shows that IgM memory B cell percentages were significantly lower in smB- CVID patients compared to smB+ CVID patients ($p < 0.05$).

3.5 Discussion

3.5.1 *B cell Immunophenotyping in HIV-1 infection, CVID and splenectomy*

In this study, B cell subsets were measured in healthy controls, HIV-1 infection, CVID and individuals who had undergone splenectomy. Total memory B cells were reduced in patients with CVID, HIV-1 and in splenectomised individuals. As expected, IgM memory B cells were reduced in splenectomised patients and patients with CVID. In addition, IgM memory B cell counts were reduced in HIV-1 infection and not restored by ART. IgM memory B cell defects were severe in some patients with HIV-1 infection, suggesting functional hyposplenism similar to that seen in splenectomised individuals. Class switched memory B cells were reduced in 25% of HIV infected individuals and patients with CVID or splenectomy. Transitional B cells were increased in all patient groups compared to healthy controls (untreated and treated HIV-1 infection, CVID and individuals with splenectomy). HIV-1 infected individuals therefore displayed numerous B cell abnormalities. Applying the Paris classification scheme for PID, ~25% of HIV-1 infected individuals would fit the criteria for the MBO group, which includes CVID patients with the most severe deficiency of memory B cell subsets.

3.5.2 *B cell memory in splenectomised individuals*

The splenectomised patients' peripheral blood samples were used primarily as controls for low IgM memory B cell numbers. Individuals with splenectomy were found to have significantly lower IgM memory B cells, compared to healthy controls, and consistent with previous reports (Carsetti, Pantosti et al. 2006). Interestingly, there were some IgM memory B cells present in some patients, suggesting the presence of splenunculi (small portions of spleen outside the splenic area) or some residual/long-lived IgM memory B cells were present (possibly due to a short time since splenectomy). It is possible that some IgM memory B cells are able to mature elsewhere in the lymphoid system. In contrast, a later study found reduced IgM memory B cells in splenectomised patients but six patients diagnosed with hereditary spherocytosis were reported to have normal numbers of IgM memory B cells. This led the authors to suggest that extra splenic sites may be important for IgM memory B cell maturation in patients at least with this disease (Wasserstrom, Bussel et al. 2008). However, on inspection of the tabular data in the Wasserstrom paper, IgM memory B

cell subsets were reduced in the hereditary spherocytosis patients but the data was statistically underpowered due to small cohort size. Individuals splenectomised due to autoimmunity were also shown to make a protective IgG serotype response against Pneumovax vaccination, despite diminished post vaccine IgM anti-pneumococcal levels and low peripheral IgM memory B cells (Wasserstrom, Bussel et al. 2008). This study is interesting and suggests some additional factors are important in the immune response against encapsulated bacteria. However, the study did not consider the possibility that long lived pre-existing antigen specific plasma cells in the bone marrow or pre-existing long-lived mature memory B cells may have mounted this antibody immune response or provided some plasticity in the case of an recently absent spleen. Clinically, we currently measure IgG antibodies to pneumococcus carbohydrate in the laboratory so there is an understanding that there must be some class switching of pneumococcal antigen specific IgM memory B cells to long-living IgG producing pneumococcal specific plasma cells during the T cell independent immune response (Weill, Weller et al. 2009). Unfortunately, our current work only examined eight patients with splenectomy so we were unable to test pre and post vaccination IgG and IgM responses to Pneumovax in these patients.

In addition, we found class switched memory B cells were slightly reduced in our splenectomised patients, as reported elsewhere (Kruetzmann, Rosado et al. 2003; Weller, Braun et al. 2004; Wasserstrom, Bussel et al. 2008). However, this current work studied only eight patients, with a diverse reason for absent spleen and so these findings could not be examined in greater detail here. The findings of an additional reduced class switched memory B cell compartment in splenectomised patients may suggest the spleen has some important role in B cell maturation or maturation of all memory B cell subsets. Conversely, reduced class switched B cells may be reduced due to the pre-existing factors that required scheduled splenectomy in some cases such as pre-existing autoimmune conditions such as ITP.

3.5.3 Loss of IgM memory B cells reported in other diseases causing functional hyposplenism

Loss of IgM memory B cells has subsequently been reported in patients with various forms of functional hyposplenism: Inflammatory Bowel Disease (IBD) (Di Sabatino, Rosado et al. 2005), including Crohn's disease (Di Sabatino, Rosado et al. 2008) and

Coeliac disease (Di Sabatino, Rosado et al. 2007), providing additional evidence that the spleen has an important role in IgM memory B cell maturation and/or survival.

3.5.4 Total CD27+ B cell memory in HIV-1 infection

We have confirmed the loss of CD27+ memory B cells in HIV-1 infection as reported by others (De Milito, Morch et al. 2001; Moir, Malaspina et al. 2004; Titanji, Chiodi et al. 2005) in our cohort of 29 untreated and 55 treated HIV-1 infected patients and extended this work to include the measurement of two memory B cell subsets with discrete immune functions (Hart, Steel et al. 2007). Two later papers from the same group reported normalisation of B cell counts and subpopulations after ART, yet this referred primarily to transitional, naïve B cells and tissue like B cell proportions (Moir, Malaspina et al. 2008; Moir, Buckner et al. 2010). That work conceded that although CD27+ memory B cells are increased in number and seem to be less activated (fewer CD21^{low} activated memory B cells), these are not restored by ART to levels seen in healthy individuals (Moir, Malaspina et al. 2008; Moir, Buckner et al. 2010).

3.5.5 IgM memory B cells in HIV-1 infection

IgM memory B cells are significantly reduced in our cohort untreated and treated HIV-1 infection compared to healthy controls (Hart, Steel et al. 2007). 70% of our HIV-1 patient cohort had a loss of peripheral IgM memory B cells similar to that observed in splenectomised patients. This is consistent with a small previous study consisting of five HIV infected patients with recent seroconversion illness, which found a reduction of the IgM memory B cell subset in these patients, which then normalised after one year of ART (Titanji, Chiodi et al. 2005). In September 2006, the same group also reported a non-statistically significant reduction of IgM memory B cell percentages in a small number of chronically HIV infected patients (CHI) and patients that had seroconverted within the last three to six months (Primary HIV Infection (PHI)) compared to healthy controls (Titanji, De Milito et al. 2006). These small studies contrasted slightly with our findings; however, this is likely to be due to sample size and the stage of disease of both cohorts. The PHI patients as described by the Titanji group had suffered from an acute seroconversion illness as they entered the study; in contrast none of our patients had a recent seroconversion illness. In addition, since symptomatic patients with PHI are often put onto ART early, it is possible that it

is easier to restore immune function in this cohort. Our study evaluated a larger cohort of patients in each group: 29 HIV-1 untreated individuals and 55 HIV-1 infected patients on ART. In addition, our ART treated group had been on ART therapy for a median time of five years, which is considerably longer than the CHI patients in these studies. The longer period of time on ART gives more potential for immune recovery, and adds weight to our finding that the IgM memory B cell subset is not restored by ART therapy.

In untreated HIV infection, consistent with these findings (Hart, Steel et al. 2007) later publications studying the peripheral blood of HIV infected adults (Subramaniam, Metzger et al. 2009) (D'Orsogna, Krueger et al. 2007) confirmed our findings of a loss of IgM memory B cell compartment. However, in one study of gut samples early after HIV infection, a selective loss of IgM memory B cells was not found (Levesque, Moody et al. 2009). In addition, a recent paper did not report a significant reduction of IgM+CD27+ B cells before and after successful ART (Moir, Buckner et al. 2010), these findings may reflect the complexity and heterogeneity of HIV disease, however, in our observations staining for IgM+ is poor without the addition of IgD, which is much clearer to differentiate and is the consensus marker used to label IgM memory B cells (Piqueras, Lavenu-Bombléd et al. 2003; D'Orsogna, Krueger et al. 2007; Vodjgani, Aghamohammadi et al. 2007; Jacobsen, Thiebaut et al. 2008; Wehr, Kivioja et al. 2008) and so close comparisons cannot be made.

We have shown the loss of the IgM memory B cell subset in untreated HIV-1 patients to be significantly associated with a reduced CD4 T cell count. This finding has been confirmed by others in adults (D'Orsogna, Krueger et al. 2007) but not in vertically acquired HIV-1 infection in children (Jacobsen, Thiebaut et al. 2008). The IgM memory B cell subset could therefore be an important indicator of progressive disease in adult HIV-1 infection, especially since IgM memory B cell populations are not depleted in long term non progressors (LTNP) (Titanji, Chiodi et al. 2005; Titanji, De Milito et al. 2006) .

In treated HIV infection, the loss of IgM memory B cell (CD19+CD27+IgD+) B cell subset was found to extend to vertically acquired HIV-1 infected children on successful ART (Jacobsen, Thiebaut et al. 2008). This loss of IgM memory B cells

was found to be irrespective of HIV-1 disease duration or CD4+ T cell count, suggesting early onset of B cell humoral dysregulation in these patients. No reduction in class switched memory B cells was observed. An Australian study of adult HIV infected individuals did not find a loss of the IgM memory compartment in ART treated individuals unlike their untreated cohort, again this difference may be accounted for by cohort size and time on ART (D'Orsogna, Krueger et al. 2007).

3.5.6 Switched Memory B cell loss in HIV-1 infection

We also reported a loss in the switched memory B cell subset in a proportion of our untreated and treated HIV-1 patients (25%). Later in 2007, (D'Orsogna, Krueger et al. 2007) confirmed our findings of a loss of the switched memory B cell compartment in HIV-1 infection, which was restored by ART in their study. Loss of the class switched memory B cell subset was not found in HIV-1 vertically infected children (Jacobsen, Thiebaut et al. 2008). Loss of the switched memory B cells in a proportion of untreated HIV-1 infected patients in our study was associated with a reduced CD4 T cell count. Jacobsen *et al* (Jacobsen, Thiebaut et al. 2008), hypothesised that normal class switched memory B cell populations in infants compared to the low class switched memory B cell seen in some adults in our study and D'Orsogna may be due to age related differences or splenic output in HIV-1 infection since GC are only depleted at a late stage of HIV infection (Wilkins, Davis et al. 2003). The hypothesis that the GC function may be impaired at a late stage of HIV infection supports our findings since we only found a reduction of class switched memory B cells in a proportion of treated HIV-1 infected individuals that had a reduced CD4 T cell count (<400 cells/ μ l) suggestive of advanced disease and incomplete immune restoration.

Switched memory B cells are dependent on T cells for isotype switching and activation, therefore, a loss of these cells may be due to lack of cognate CD4 T cell help or intrinsic B cell defects in B cell function associated with the HIV virus. Indeed, there is some evidence that HIV-1 viral proteins such as Nef may interfere with T cell dependent B cell function, by reducing expression of the T cell ligand CD40 on B cells and suppression of class switch recombination (Qiao, He et al. 2006). Xu *et al* showed that intercellular conduits protruding from HIV-1 infected macrophages transfer Nef to B cell germinal centres (Xu 2009). This was associated

with impaired class switching to IgG2 and IgA (Xu 2009) and may be a mechanism for impaired numbers of class switched memory B cells. In addition, since we and others were also able to show a slightly reduced class switched memory B cell compartment in addition to the established loss of IgM memory B cells in splenectomised individuals, we cannot discount the possibility that this similar reduction in class switched memory B cells in a proportion of HIV-1 infected individuals may be a similar structural defect associated with a disturbed splenic or lymphoid environment.

3.5.7 Phenotyping in CVID

CD27⁺ memory B cells were found to be reduced in CVID as found in previous work (Brouet, Chedeville et al. 2000; Jacquot, Macon-Lemaitre et al. 2001; Warnatz, Denz et al. 2002). However, similar to the disease itself, the B cell compartment of CVID is heterogeneous, leading to attempts to sort these patients into groups based on B cell memory subsets. Most of these classification schemes have focussed on class switched memory B cells and transitional B cells or CD21^{low} B cells. Consistent with previous work, we found a significant reduction of class switched memory B cells in this cohort of patients with CVID, reductions of IgM memory B cells in some patients and expansions of transitional B cells in a subset of patients. We were able to classify this cohort using the Paris classification and found 57% of patients fitted the MBO criteria, 32% were classified into MB1 and 7% of patients had normal levels of memory B cells. In addition, one patient (4%) had reduced IgM memory B cells and normal class switched memory B cells, which we denoted MZB⁻. This may be associated with functional hyposplenism in this patient.

There is still limited data on the prognostic efficacy and stability of B cell flow cytometry classification schemes in CVID, probably related in part to the short time frame since these schemes were introduced and a lack of long term data on these immunophenotyped individuals, for example, patients may progress from MB2 to MBO.

The Freiburg scheme, (2002), classified CVID patients on the basis of class switched PBL numbers and CD21^{low} B cells (Warnatz, Denz et al. 2002). Using this scheme, three papers subsequently reported significantly reduced overall percentages of class

switched memory B cells in CVID and attempted to correlate these findings with clinical phenotypes (Ko, Radigan et al. 2005; Alachkar, Taubenheim et al. 2006; Vodjani, Aghamohammadi et al. 2007). Ko *et al* found decreased class switched memory B cells in CVID were associated with a reduced IgG level, reduced antibody response to pneumococcal immunisation and clinically increased autoimmunity and granulomatous disease (Ko, Radigan et al. 2005). Alachkar *et al* reported reduced class switched memory B cells were clinically associated with bronchiectasis, splenomegaly and autoimmunity and concluded that class switched memory B cell counts were more informative than Ig levels for risk of recurrent bacterial infection and bronchiectasis (Alachkar, Taubenheim et al. 2006). Vodjani *et al* (Vodjani, Aghamohammadi et al. 2007) also found a higher rate of bronchiectasis in patients with reduced class switched B cells and a higher rate of autoimmunity in Group 1 patients with increased CD21^{low} B cells (Group 1a), consistent with data published in the Freiburg paper (Warnatz, Denz et al. 2002). Again, since the Freiburg scheme did not evaluate IgM memory B cell counts, so close comparisons between respiratory complications in CVID cannot be made with this current study or previous work.

Sanchez-Ramon *et al* (2008), reported higher switched memory B cells in females and using their own cut-off, ($\leq 0.55\%$ of B cells), they found reduced numbers of switched memory B cells were an independent risk factor of autoimmune disease, granulomas and splenomegaly but no link to bronchiectasis (Sánchez-Ramón, Radigan et al. 2008). This cohort displayed increased overall mean levels of IgM memory B cells however there was huge variability in IgM memory B cell percentage in this cohort, suggesting again the wide heterogeneity of CVID disease.

Some data has now been published evaluating the recent 2008 EURO Class scheme in 313 French subjects with CVID (Mouillot, Carmagnat et al. 2010). This study by Mouillot *et al* also evaluated the T cell classification scheme for CVID published by Giovannetti *et al* (Giovannetti, Pierdominici et al. 2007), which demonstrated that reduced naïve CD4⁺ T cell counts in some CVID patients was associated with increased disease severity, splenomegaly and reduced thymic output. The Mouillot study grouped PID patients into those suffering from infections without any further CVID complications and CVID patients with infections, lymphoproliferative, autoimmunity or digestive disorders (Mouillot, Carmagnat et al. 2010). Only 50% of

the infection only CVID group were found to have abnormalities in the B cell/T cell compartments compared to 75-90% of the CVID patients with other clinical manifestations, suggesting combined B and T cell analysis may be useful in differentiating patients at risk of further complications and for research purposes into cause of disease. The Mouillot paper reported an increase in CD21^{low} B cells in their patient groups with lymphoproliferative, autoimmune complications and chronic enteropathy, but not in the infection only group (Mouillot, Carmagnat et al. 2010). In addition to an increase in CD21^{low} B cells, patients with lymphoproliferative or autoimmune complications reported a decrease in regulatory T cells (as reported previously, (Fevang, Yndestad et al. 2007) and increased T cell activation (CD4+HLA-DR+). The association between CD21^{low} B cells and Tregs has been reported in another paper (Arumugakani, Wood et al. 2010). Transitional B cells were not found to be higher overall nor associated with a clinical phenotype.

3.5.8 Loss of memory B cells in CVID is associated with bronchiectasis

Evaluating a larger cohort of CVID patients for lung function and bronchiectasis, we found that CVID patients with bronchiectasis had a significantly lower total memory B cell count compared to those CVID patients without bronchiectasis. Subdividing the total memory B cell compartment into IgM memory B cells and class switched memory B cells, class switched B cell percentages were no different between patients with or without bronchiectasis. There was a trend for the percentage of IgM memory B cells to be reduced in those patients with bronchiectasis, consistent with a previous report (Carsetti, Rosado et al. 2005), however this narrowly missed significance.

The recent EURO Class classification scheme does not measure IgM memory B cells, which have been associated with bronchiectasis and respiratory infections in CVID patients and so our results cannot be compared with papers evaluating EURO Class. However, some CVID researchers believe that those patients with low IgM memory B cells also always have low class switched memory B cells and so measurement of class switched memory B cells is sufficient in a classification scheme. Therefore, we evaluated a larger cohort of patients using the EURO Class schemes to see if the EURO Class scheme was able to identify those patients with bronchiectasis and low IgM memory B cells. Examining CVID patients with less than 2% of class switched memory B cells (the cut-off used in EURO Class for class switched memory

percentage), we found significantly reduced IgM memory B cell percentages in CVID patients with bronchiectasis. Those CVID patients without bronchiectasis, despite low class switched B cells, had significantly higher IgM memory B cell percentages. This suggests that the presence of IgM memory B cells may be protective, despite extremely low class switched B cells. Non class switched IgM memory B cells may therefore play an important role in host defence against bronchiectasis and small airway inflammation and the EURO Class classification scheme should consider the incorporation of IgM memory B cells. This may be especially important in those individuals with CVID demonstrated to have extremely low IgM memory B cells because replacement Ig does not contain pneumococcal IgM antibodies if these are absent and this may leave patients at additional risk of respiratory disease. Limitations of this study include a highly selective patient group, retrospective analysis of chest CT scans and confounding factors such as time since CVID diagnosis, asthma, compliance with physiotherapy and inhalers.

3.5.9 Increased Transitional B cell percentages in HIV-1 infection

Expansions of immature transitional B cell populations were shown to be significantly increased in both HIV untreated and HIV treated patients. Transitional B cell expansions in untreated and treated HIV-1 infected individuals were not found to be associated with CD4+ T cell counts in this study. During the last few years, other studies examining B cell defects in HIV infection have also reported similar findings (Cuss, Avery et al. 2006; Malaspina, Moir et al. 2006). In HIV-1 vertically infected children, transitional B cells were not shown to be increased in one study (Jacobsen, Thiebaut et al. 2008). Prior to this, expansions of transitional B cells had only been reported in primary immune deficiency states such as CVID (Warnatz, Denz et al. 2002) where they are associated with lymphadenopathy (Wehr, Kivioja et al. 2008) and autoimmunity (SLE) (Sims, Ettinger et al. 2005).

Transitional B cells represent B cells at an immature stage of B cell development, that are found at increased frequencies in cord blood, are increased in the peripheral blood of infants and decline with age in the healthy host, representing approximately <1-10% of peripheral B cells (Weill, Weller et al. 2009). Since the work in this chapter was published in 2007, recent work has extended the characterisation of transitional B cells. Recent studies examining B cell repopulation following HSCT or B cell

depletion therapy using Rituximab have shed some light on transitional B cell maturation (Anolik, Barnard et al. 2007; Anolik, Friedberg et al. 2007), (Palanichamy, Barnard et al. 2009; Suryani, Fulcher et al. 2010). In man, CD24 and CD38 have been used to differentiate transitional B cells into T1 (CD38+++CD24+++), T2 (CD38++CD24++) and possibly a T3 population (Palanichamy, Barnard et al. 2009), consistent to the T1-T3 subsets in mice.

Recently, transitional B cells have been split into two distinct populations using CD21 expression (CD21^{low} or CD21^{high}) (Suryani, Fulcher et al. 2010). CD21^{low} transitional B cells were believed to be a precursor of CD21^{high} transitional B cells. The authors provide some evidence for this: XLA patients have less than 1% peripheral B cells, yet of these CD21^{low} transitional B cells are the predominant population, with little or no CD21^{high} transitional B cells present. In addition, their studies of patients following HSCT show the CD21^{low} transitional B cells are the first B cell population to repopulate the periphery (Suryani, Fulcher et al. 2010). CD21^{low} transitional B cells also displayed a higher amount of auto-reactivity as determined by secretion of IgM ANA antibodies (Suryani, Fulcher et al. 2010). However, it is likely that the CD21^{low} transitional B cells reflect the T1 subset and CD21^{high} transitional B cells describe the T2 subset characterised previously.

It is thought transitional B cells are immature B cells that have left the bone marrow and homed to the spleen or other secondary lymph organs to mature under the influence of B cell growth factors such as BAFF and APRIL into mature B cells. Increased levels of BAFF have been found in HIV-1 infection and are thought to be associated with polyclonal activation (Stohl, Cheema et al. 2002), polyclonal Ig class switch recombination (He, Qiao et al. 2006) and B cell exhaustion leading to impairment of the humoral immune response (De Milito, Morch et al. 2001; Rodriguez, Valdez et al. 2003). Newly discovered genetic defects in the TACI/BAFF pathway in CVID suggest these chemokines play a key role in loss of B cell function and lack of specific antibodies (Knight, Radigan et al. 2007). In HIV-1 infection, high levels of BAFF secretion is associated with reduced CD19⁺ CD27⁺ memory B cells (De Milito, Morch et al. 2001) and with disease progression (Rodriguez, Valdez et al. 2003). Whether this chemokine is able to affect B cell homeostasis is yet to be fully determined, however, this increased production of BAFF may be a mechanism for

increased bone marrow output of transitional B cells in HIV infection and needs further investigation.

This increase in immature transitional B cells may suggest a maturational defect of B cells and may be associated with a parallel reduction in mature IgM memory B cells, as hypothesized by us and others as seen in a subset of CVID patients (Wehr, Kivioja et al. 2008). The ontogeny of IgM memory B cells is not clear, since one proposal suggests the subset arise during the differentiation of switched germinal centre B cells (Weller, Braun et al. 2004). However, more think the IgM memory B cell population are derived directly from transitional B cells and there is some emerging evidence to support this. CpG stimulation has been shown to induce *in vitro* maturation of cord blood transitional B cells into IgM memory B cells, suggesting a direct maturational link between the two cell types (Capolunghi, Cascioli et al. 2008).

3.6 Conclusions

In conclusion, evidence from ourselves and others suggests the loss of the IgM memory B cell subset in HIV-1 infection, however, this is not universally found in every study to be significant because IgM memory B cell percentages are variable, even in health (Weill, Weller et al. 2009). I believe grouping patients into median levels masks a huge variation in individual memory B cell percentages, as found in healthy individuals and CVID. Use of cut off levels as used in classification schemes for CVID or using reference ranges may be more clinically important. Doing so, unmasked 25% of HIV infected individuals with extremely low class switched memory B cells, despite no overall median reduction in the group. In addition reported differences in IgM memory B cell subsets is likely to reflect the huge heterogeneity of disease duration, (i.e. time exposed to VL before treatment), time to progression and the huge variation of disease complications seen in HIV infected individuals.

In the potential era of early ART, which may be able to reduce extensive B cell dysregulation, measuring IgM memory B cells and class switched memory B cells and employing a classification scheme may predict which patients may be susceptible to invasive infection with encapsulated bacteria. Identifying these HIV-1 infected

patients as having additional secondary immune deficiency may allow for some application of prophylactic treatment (e.g. antibiotics kept at home) and closer clinical follow up, which would reduce morbidity and mortality and be less expensive than treating invasive disease in these patients. If as evidence by ourselves and others suggests, memory B cell subsets are able to predict invasive disease in HIV-1 infection, follow up clinical data could then be published to further add weight to this data. Since current ART does not restore memory B cell levels, this measurement could be done at one time point in treated individuals, for example, at the time ART is started.

4. Chapter IV: Functional serological vaccination responses in HIV-1 infected patients on ART and healthy controls

4.1 Introduction

Patients with HIV-1 infection have a higher risk of disease and are at risk of more severe disease than the healthy population. Therefore, where possible, vaccination is pre-indicated for vaccine-preventable infections. Invasive pneumococcal infection and bacterial community acquired pneumonia (BCAP) are important causes of morbidity and mortality even in HIV patients on antiretroviral therapy (ART) (Barry, Zetola et al. 2006; Pedersen, Lohse et al. 2010) and so vaccination is recommended. Protective levels of specific microbial antibody titres minimise the risk of invasive infections such as invasive pneumococcal disease, which is known to be a significant cause of morbidity and mortality in HIV-1 infected patients.

In 1984, shortly after AIDS had been described, the first case of pneumococcal vaccination failure in a HIV infected patient was reported (Simberkoff *et al*, 1984), however, the efficacy of pneumococcal immunisation in HIV-1 patients receiving ART is still controversial (Dworkin, Ward et al. 2001; Lopez-Palomo, Martin-Zamorano et al. 2004). Studies evaluating the relative risk of pneumonia, ART, CD4 status and vaccination efficacy have produced conflicting results, a confounding factor may be that HIV-1 infected individuals are also frequently associated with other documented risk factors for acquiring pneumonia: alcohol abuse, smoking, intravenous drug use, liver disease and low socio-economic status (Madeddu, Laura Fiori et al. 2010; Pedersen, Lohse et al. 2010).

Plasma levels of IgM and IgG pneumococcal antibody are reduced in HIV-1 infection and the capacity of PBMC to produce IgG and IgM pneumococcal antibody following *in vitro* B cell polyclonal stimulation is impaired (Titanji, De Milito et al. 2006). Serological memory after exposure to pathogens or vaccination is maintained by plasma cells and memory B cells (Bernasconi, Traggiai et al. 2002; Traggiai, Puzone

et al. 2003). Serological memory can also be assessed by measuring baseline antibody levels and response to vaccination. Memory B cell (CD19+CD27+) counts are reduced in HIV-1-infected individuals (De Milito, Morch et al. 2001; Nagase, Agematsu et al. 2001; Chong, Ikematsu et al. 2004) and are associated with reduced measles and tetanus antibody concentrations (De Milito, Nilsson et al. 2004). Loss of CD19+CD27+ memory B cells may also be an additional factor responsible for impaired humoral immunity and poor vaccine responses in HIV infection. See General Introduction (Chapter I), for a detailed description of HIV-1 and principles of general vaccination.

4.1.1 *Immunisation against S. pneumoniae in HIV-1 infection*

To prevent infection in those at high risk of disease, antibiotic prophylaxis and/or vaccination is given. Immunisation against tetanus, *S.pneumoniae* and other infectious pathogens is an effective strategy in preventing disease transmission in susceptible patient groups but requires extremely high vaccine coverage and early vaccination.

Over 90 capsular serotypes of *S.pneumoniae* have been described; greater than 80% of those causing invasive pneumococcal disease are covered by the unconjugated 23-valent polysaccharide pneumococcal vaccine (Pneumovax II) which is recommended to be given in most at risk groups by the British Health Protection Agency (HPA). In addition, the British Department of Health (Health 2007), BHIVA (Geretti, Brook et al. 2008) and the American Advisory Committee on Immunisation Practices (ACIP) recommend that HIV infected individuals receive pneumococcal vaccination to prevent opportunistic infections by *S. pneumoniae* (Masur, Kaplan et al. 2002). The Centers for Disease Prevention (CDC) immunisation guidelines (2007-2008) advise administering the vaccine to all HIV infected individuals regardless of CD4 count or treatment status (Prevention 2007).

However, immunisation efficacy studies of the unconjugated pneumococcal vaccine (Pneumovax II) in HIV infected adults are limited and have found modest protection overall, depending on the cohort studied, ART treatment or methods used to assess vaccine efficacy (Gebo, Moore et al. 1996; Nielsen, Kvinesdal et al. 1998; Breiman, Keller et al. 2000; Dworkin, Ward et al. 2001; Lopez-Palomo, Martin-Zamorano et al. 2004; Rodriguez-Barradas, Goulet et al. 2008). A four-fold rise in pneumococcal titre

post vaccination is considered protective, as long as an adequate threshold of protection is reached. Antibody responses to pneumococcal vaccination are reduced in ART-naive HIV patients with CD4 T cell counts <500 cells/ μ l (Rodriguez-Barradas, Musher et al. 1992). Viral load may also be important, high viral load has been associated with low pneumococcal vaccine effectiveness (Teshale, Hanson et al. 2008).

There is possibly a difference between efficacy of the polysaccharide pneumococcal vaccine for HIV patients not on ART between ethnic groups and in differing geographical locations. To date, the only randomised pneumococcal vaccination study on untreated HIV infected individuals was performed in Uganda with mixed findings. The initial study found no effect in preventing pneumococcal disease in any groups and in fact seemed to suggest an increase in pneumonia amongst the vaccinated group (French, Nakiyingi et al. 2000). However, a six year follow up study showed a 16% reduction in mortality in the vaccinated group and no further increase in pneumonia (Watera, Nakiyingi et al. 2004). Preliminary findings from the Ugandan study were partially supported by Northern American studies, showing protection in the white cohort but poor vaccine efficacy in the African American black population (Breiman, Keller et al. 2000) and black HIV infected patients represent one of the highest burden of disease, however, no causative etiological factor has been found.

Few polysaccharide pneumococcal vaccine studies have been performed specifically on ART treated individuals (Dworkin, Ward et al. 2001; Lopez-Palomo, Martin-Zamorano et al. 2004) and none have been randomised, due to the nature of retrospective data collection. CD4 counts may be an important factor to consider regardless of treatment status: one study found efficacy only with a CD4 count >200cells/ μ l (Gebo, Moore et al. 1996) and another >500cells/ μ l (Dworkin, Ward et al. 2001), leading to a conclusion that pneumococcal vaccination should be performed before profound immunosuppression has occurred (Dworkin, Ward et al. 2001).

4.1.2 Tetanus Toxoid Immunisation in HIV infection

Spores of *Clostridium tetani* are commonly found in soil or mammalian intestinal tracts. Disease is caused in an unvaccinated host by the introduction of the gram negative bacterium through small wounds or surgery, releasing a neurotoxin

(tetanospasmin) which causes tetany (spasms, skeletal rigidity, respiratory failure) and has a 30% fatality rate overall. Vaccination with inactivated tetanus toxoid is effective and form part of the British childhood vaccination schedule, following a repeat booster, immunity is now considered life-long.

In the pre- ART era, it was discovered that HIV infected patients responded poorly to immunisation with tetanus toxoid and other common recall T cell dependent antigens. Impaired immunisation responses (tetanus IgG titres and tetanus specific lymphoproliferation) are associated with advanced disease (AIDS) or lower CD4 counts in untreated patients (Janoff, Hardy et al. 1991), especially under 300 cells/ μ l (Kroon, van Dissel et al. 1994). In the ART treated population, tetanus toxoid response studies are limited and difficult to compare due to differences in assays used to measure protection: ELISpots, lymphocyte proliferation tests, delayed hypersensitivity tests (DTH) and IgG serum antibody titres are all used as correlates of *in vivo* protection to tetanus toxoid protein. Antibodies provide clinical protection by neutralising the tetanus toxoid capsule. For clinical use, achieving a serum IgG antibody titre of >0.15 IU/mL is considered protective in the short term (Simonsen, Bentzon et al. 1986).

In one study of ART treated individuals, in comparison with healthy controls, HIV-1 infected patients responded with lower serum antibody titres against tetanus toxoid, even when corrected for CD4 counts (Lange, Lederman et al. 2003). The maintenance of a long lived serological antibody response to protein antigens such as tetanus toxoid is proposed to be mediated by on-going polyclonal activation of memory B cells (Bernasconi, Traggiai et al. 2002). In addition, the patient nadir CD4⁺ T cell count and baseline numbers of the co-stimulatory molecule CD28 on peripheral blood CD4 T cells predicted the response to tetanus toxoid immunisation whereas current CD4 T cell count did not (Lange, Lederman et al. 2003). This study concluded that patients commencing ART therapy during advanced HIV disease had a greater likelihood of impaired immune restoration.

4.2 Aims

- 1) To measure the baseline levels of IgG and IgM pneumococcal and IgG tetanus antibody levels in patients with untreated and treated HIV-1 infection and to compare these with healthy controls.
- 2) To assess the serological response to vaccination in healthy controls and HIV-1 infected individuals on ART.
- 3) To measure percentages of specific memory B cell subsets against the baseline antibody level or response to specific vaccination in HIV-1 infected individuals on ART.
- 4) To measure T cell activation markers in HIV infection, CVID and splenectomised patients and correlate these with vaccination responses.

4.3 Patient Cohort

Serological microbial antibody levels were performed on 55 HIV-1 treated patients on ART as part of the Functional Immunity Study. 29 HIV-1 infected untreated patients and 83 healthy controls had baseline antibody levels measured. The 55 HIV-1 patients on ART, 29 HIV-1 infected untreated patients and 17 healthy controls had T and B cell phenotyping performed as measured in Chapter II. Sera collected from 66 blood bank donors were used in addition to healthy control data to calculate normal reference intervals.

4.4 Results

4.4.1 Serological Specific Microbial Titres

Table 9: Baseline serum pneumococcal and tetanus antibody levels in HCs and HIV patient groups (median and IQR).

	Pneumo IgM (U/ml)	Pneumo IgG (U/ml)	Tetanus IgG (IU/ml)
HC (n=83)	30.0 (15-44.8)	88.0 (53.0-225.0)	2.7 (0.6-4.0)
HIV ART-ve (n=29)	21.0 (13.8-37.3)	43.0* (22.3-120.8)	0.4* (0.1-0.9)
HIV ART+ve (n=55)	17.0 (11.0-38.0)	29.0* (18.3-61.5)	0.4* (0.1-0.8)

Healthy control sera reference intervals were derived from sera from 83 blood bank donors. A significant reduction was seen in baseline pneumococcal IgG and tetanus IgG in the HIV-1 untreated and HIV-1 treated patient groups in comparison with the healthy control group *(p=0.005) (Table 9). Tetanus IgG levels were strikingly reduced in both HIV cohorts. IgM pneumococcal median values in untreated and treated HIV-1 infection were reduced compared to healthy controls at 21U/ml and 17U/ml vs. 30 U/ml but this did not reach statistical significance. The Mann Whitney U test was used to analyse non parametric data.

No associations were found with peripheral CD4 counts, VL, or B cell counts for any baseline antibody level. No association between transitional B cells levels and tetanus and pneumococcal baseline serology was found in HIV-1 patients (data not shown). Pneumococcal IgG levels did not correlate with memory B cell subsets in both HIV-1 patient groups.

4.4.2 Associations between serum antibody levels and memory B cell percentages

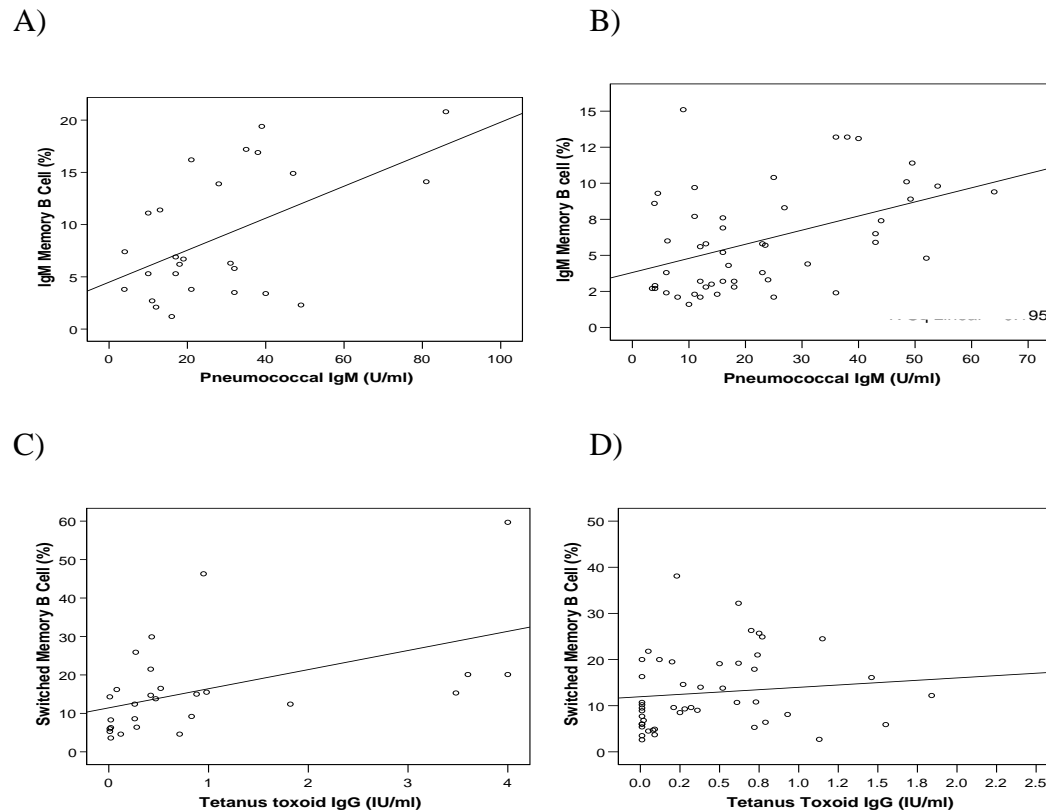


Figure 31: Scatter plots to evaluate associations between memory B cells and baseline serum antibody levels in HIV-1 infection.

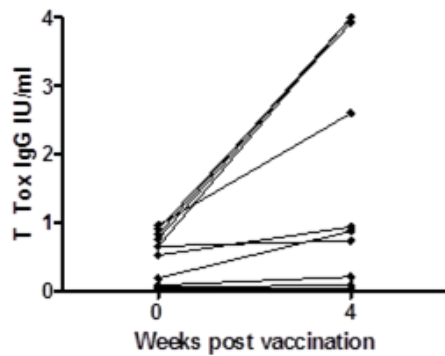
A) Associations between IgM memory B cell percentages and pneumococcal IgM levels in untreated HIV-1 infection (n=29) were analysed using Spearman's rank correlation coefficient. B) Associations between IgM memory B cell percentages and pneumococcal IgM levels in HIV-1 treated patients (n=55). C) Associations between switched memory B cell percentages and tetanus toxoid IgG levels in untreated HIV-1 infection (n=29). D) Associations between switched memory B cell percentages and tetanus toxoid IgG levels in treated HIV-1 infection (n=55).

In both HIV-1 infected patient groups, IgM memory B cells were significantly correlated to pneumococcal IgM antibody titres. Greater proportions of IgM memory B cells were associated with higher levels of serum pneumococcal IgM (Fig. 31A and B). Correlations between IgM memory and pneumococcal IgM in untreated HIV infection were $p=0.002$, $r=0.39$ and in the HIV-1 infected patients on ART $p=0.011$, $r=0.36$. The association between switched memory B cell percentages and tetanus toxoid IgG was also significant (Fig. 31C and 31D), ($p<0.001$, $r=0.58$ for HIV-1 untreated group and $r=0.30$, $p<0.034$ for HIV-1 treated group). HIV-1 patients with

the lowest switched memory B cells had the lowest serum tetanus toxoid antibody levels.

4.4.3 Functional Serological Vaccination Responses in HIV ART+ patients

A)



B)

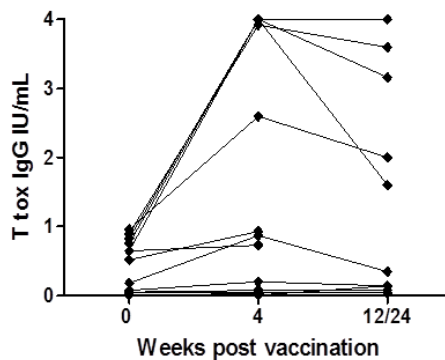


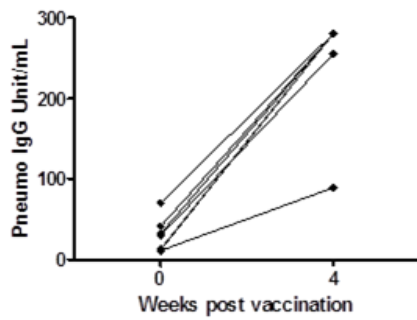
Figure 32: Post immunisation tetanus toxoid IgG responses in HIV-1 patients on ART.

Data is shown for 14 HIV ART+ patients vaccinated with tetanus toxoid. A) Pre and post vaccine tetanus responses at 0-4 weeks and B) Pre and post vaccine tetanus responses at 0 weeks, 4 weeks and 12 or 24 weeks. Some patients were not tested at either 12 or 24 weeks so these groups were combined. A successful vaccination response was defined as a 4 fold rise in antibody titre and a minimum post vaccine target level of 2.6 IU/ml (50th centile of the HC group).

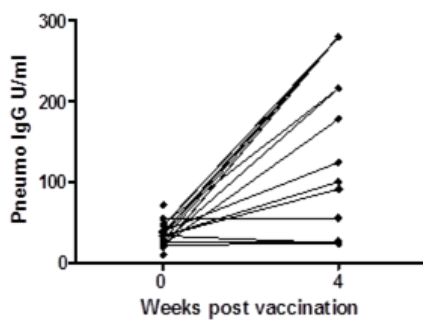
No data was shown for healthy control tetanus toxoid levels post vaccination because all healthy control patients tested had protective levels of tetanus toxoid antibody at baseline. Tetanus toxoid vaccination is part of the Childhood Immunisation schedule

and all healthy controls tested had previously responded successfully to the vaccine. Therefore, no healthy controls were vaccinated with tetanus toxoid. Fourteen HIV-1 patients were vaccinated with tetanus toxoid as part of the Functional Immunity study. At four weeks, a bimodal distribution could be seen (Fig. 32A), with 8/14 patients failing to respond at all to tetanus toxoid vaccination, however, some HIV-1 patients on ART did produce high levels of tetanus toxoid at this time point. At three to six months post tetanus toxoid immunisation, an impaired response was seen in 10/14 patients (Fig. 32B) and it can be seen that several patients had made an initial response and then antibody levels had declined, suggesting short term immunity. Interestingly it could be seen that those HIV-1 patients with a tetanus toxoid antibody level $>0.5\text{IU/mL}$ at baseline were those likely to respond to vaccination, at least in the short term (4 weeks) (see Fig. 32B).

A)



B)



C)

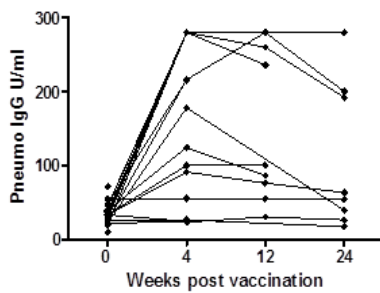
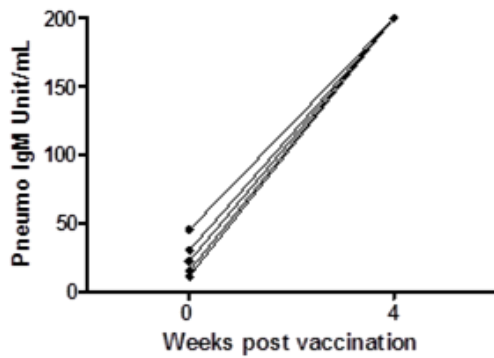


Figure 33: Post immunisation Pneumovax II IgG vaccination data for 18 HIV-1 patients on ART and 8 healthy controls with no history of respiratory disease.

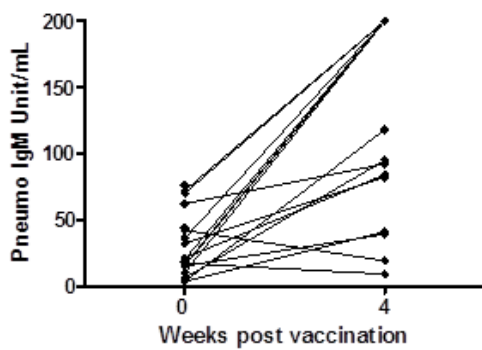
Data is shown for 18 HIV ART+ patients. A) Healthy control pre and post pneumococcal IgG vaccination responses at 0-4 weeks B) HIV-1 ART pre and post pneumococcal IgG vaccination responses at 0-4 weeks. C) HIV-1 ART treated patients pre and post pneumococcal IgG vaccination responses at 0, 4, 12 and 24 wks. A successful vaccination response was defined as a 4 fold rise in antibody titre and a minimum post vaccine target level of 225 U/ml (75th centile of the HC group).

From Fig.33A and 33B, whilst 7/8 healthy controls vaccinated with Pneumovax II achieved a protective post immunisation IgG titre of 225U/ml at 4 weeks, most of the HIV patients had an impaired serological vaccination response (10/18 patients) by failing to achieve this antibody level. This difference was statistically significant ($p < 0.05$). Anti-pneumococcal IgG titres declined in one of the healthy controls at 3 to 6 months (data not shown). Two HIV-1 ART+ patients who had achieved an initial post vaccine target level of 225U/ml failed to maintain this three to six months later (Fig.33C).

A)



B)



C)

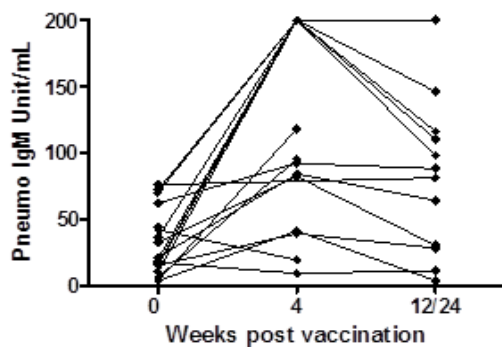


Figure 34: Pneumovax II IgM vaccination data for 8 healthy controls with no history of respiratory disease and 19 HIV ART treated patients

Data is shown for 19 HIV ART+ patients. Fig.34 A) Healthy control pre and post pneumococcal IgM vaccination responses at 0-4 weeks, Fig.34B) HIV-1 ART pre and post pneumococcal IgM vaccination responses at 0-4 weeks and Fig.34 C) HIV-1 ART treated patients pre and post pneumococcal IgM vaccination responses at 0, 4, 12 and 24 wks. A successful vaccination response was defined as a 4 fold rise in antibody titre and a minimum post vaccine target level of 200 U/ml.

Strikingly, at 4 weeks post immunisation, only 7/19 HIV-1 patients achieved an IgM anti-pneumococcal vaccine titre of >200U/ml, in comparison to the healthy control group, all of whom achieved a post vaccine titre of at least 200 U/ml (Fig. 34A and 34B) ($p<0.02$). At 3 or 6 months post immunisation, all but one of the HIV-1 patients that responded to vaccination showed a decline in post vaccine IgM titres, indicating that the antibody response was not sustained (Fig.34C). IgM data was not available for several of the healthy control group and so statistical parameters could not be tested.

4.4.4 Association between vaccination responses and memory B cell percentage at baseline

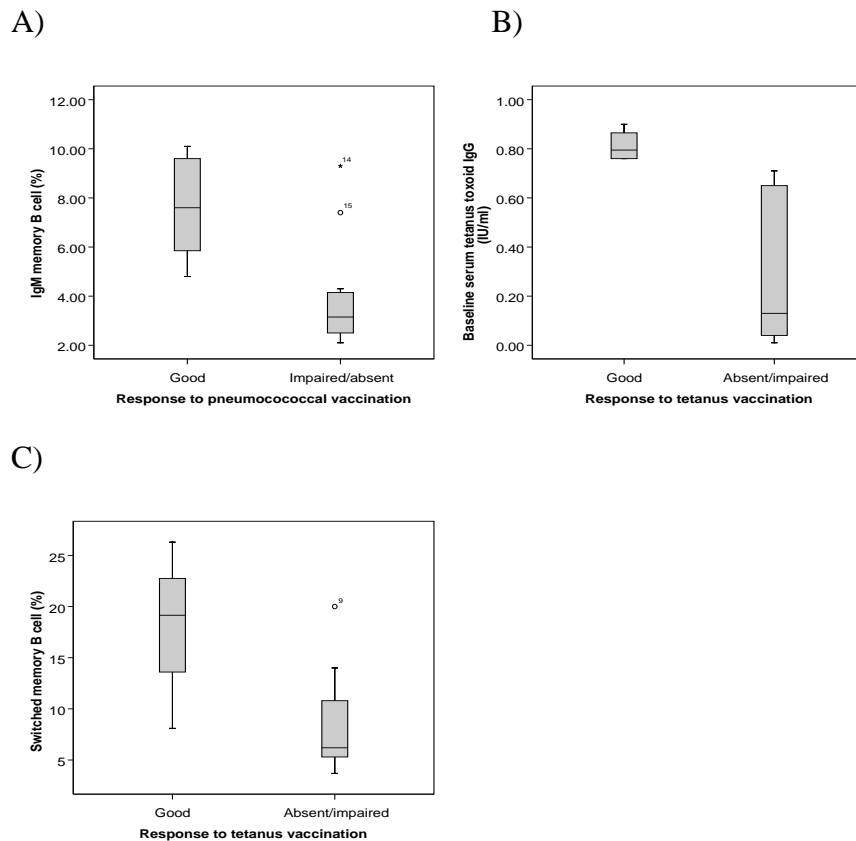


Figure 35: Immune parameters which influence IgM pneumococcal and IgG tetanus antibody responses in HIV-1 patients on ART.

Fig 35A) IgM memory B cell percentage in HIV-1 patients with good (n=7) and impaired/absent (n=12) IgM pneumococcal Ab responses. B) Baseline serum tetanus toxoid Ab concentration in HIV-1 patients with good (n=4) and impaired/absent (n=10) IgG tetanus Ab responses. C) Class-switched memory B cell percentage in HIV-1 patients with good and impaired/absent IgG tetanus Ab responses. Impaired/absent responses were classed as less than a 4 fold rise in post vaccine antibody titre and failure to reach post immunisation target antibody titres. Pneumococcal IgM responses target value >200U/ml, Tetanus IgG immunisation responses target value 2.6IU/ml. The Mann-Whitney U test was used to analyse non-parametric data.

Patients were categorised according to whether they had had a good or Absent/impaired response to immunisation and then median percentages of memory B cell subsets were compared (Fig.35A and Fig. 35C). HIV-1 infected patients on ART who responded well to pneumococcal vaccination (>200U/ml anti-pneumococcal

IgM) had significantly higher IgM memory B cell percentages ($p=0.003$), than those patients who did not achieve the post vaccine target level of 200U/ml: median percentages of IgM memory B cells were 7.6% in the good responder group versus 3.2% in the absent/impaired response group (Fig.35A).

Patients with high switched memory B cell percentages were also more likely to respond to tetanus vaccination (Fig. 35C). Since all tetanus immunised HIV patients in this study should have been historically immunised with tetanus toxoid as part of the British Vaccination Schedule, levels of recall antibody at baseline was compared for Good and Absent/Impaired responders. The baseline serum tetanus toxoid antibody level strongly predicted response to vaccination: patients with a high baseline level of tetanus toxoid ($>0.80\text{IU/mL}$) all responded well to vaccination (Fig. 35B). Unlike IgM memory B cell percentages, baseline serum pneumococcal antibody levels did not predict response to vaccination (data not shown).

4.4.5 Analysis of T cell Immunophenotyping

T cell immunophenotyping was performed primarily to establish whether there was a relationship between vaccine responses and levels of CD8+ CD38+ cells (immune activation) or CD4+ CD28- cells (CD4 T cell costimulation).

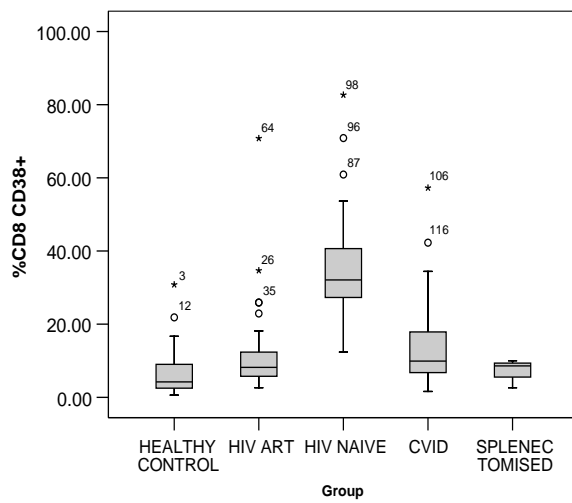
Table 10: T cell phenotype in HIV-1 infected patients and control groups (median and IQR)

T cell Phenotype %	HC (n=17)	HIV ART- (n=29)	HIV ART+ (n=55)	CVID (n=28)	SPLEN (n=8)
CD8+CD28-	18.5 (12.3-28.8)	70.7 (60.3-81.0)	48.0 (36.6-62.7)	35.3 (20-51.3)	17.9 (9.9-41.3)
CD4+CD28-	0.1 (0.0-0.5)	9.6 (3.1-22.9)	6.7 (2.0-12.0)	0.1 (0.0-0.5)	0.2 (0.1-0.3)
CD8+CD38+	4.2 (2.5-12.2)	32.1 (24.4-42.5)	8.2 (5.7-12.9)	9.9 (6.5-18.5)	8.6 (4.1-9.7)
CD4+CD38+	16.5 (13.5-23.9)	27.7 (20.9-34.2)	17.3 (11.3-24.9)	21.4 (14.8-32.5)	20.3 (7.2-27.3)

4.4.6 Expression of CD38+ on CD4 and CD8 T cells in HIV infection

From Table 10 above and Fig.36, using the Mann-Whitney U test for non parametric data, CD38 expression on CD8 T cells was significantly increased in the HIV naïve patients compared to the healthy control group ($p < 0.001$), whereas this increase was less pronounced in the HIV patients on ART ($p = 0.017$). Individuals with CVID also showed a statistically significant rise in the percentage of CD8 cells expressing CD38 ($p = 0.011$). There was no difference in CD8+CD38+ T cell percentages between splenectomised patients and healthy controls ($p = 0.52$). The expression of CD38 on CD4 T cells was significantly increased in the HIV naïve group ($p = 0.011$) (Fig. 36B) but not in the HIV ART treated group ($p = 0.78$) or the other disease controls. ($p = 0.22$, $p = 0.89$, respectively). No correlation was found between baseline CD8+CD38+ or CD4+CD38+ T cell counts and response to Pneumococcal or Tetanus toxoid vaccination.

A)



B)

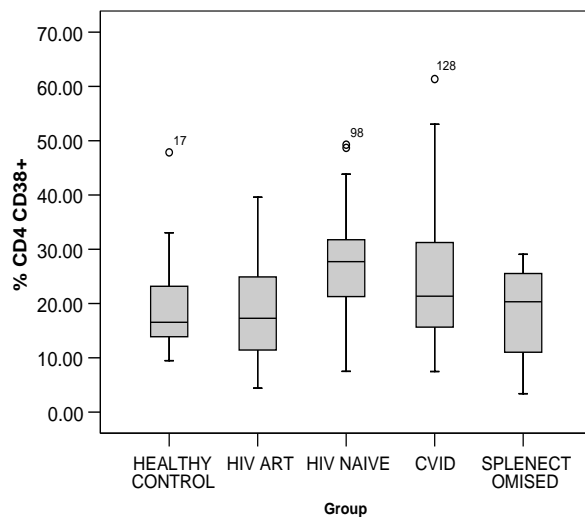
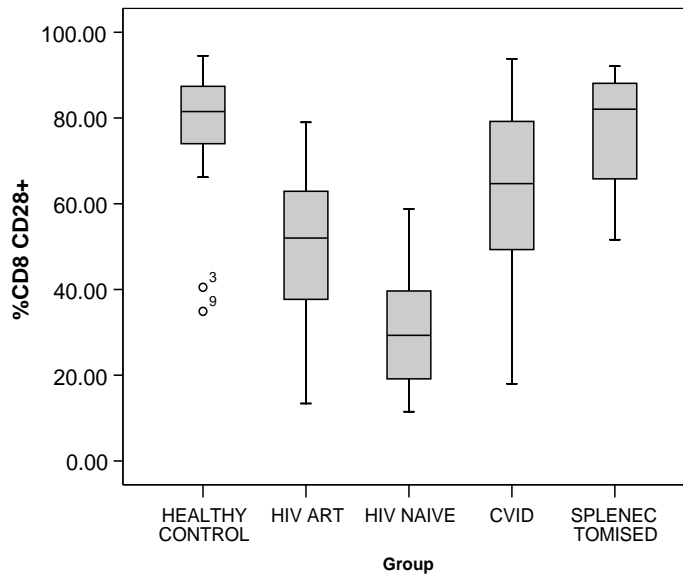


Figure 36: Expression of CD38 on T cells in HIV-1, CVID, splenectomised patients and healthy controls

Box and whisker plots displaying median and IQR CD38⁺ as a percentage of Fig.36A) CD8 T cells and Fig.36B) CD4 T cells for healthy controls (n=17), untreated HIV-1 infected patients (HIV NAÏVE) (n=29), treated HIV-1 infected patients (HIV ART) (n=55), CVID patients (n=28) and splenectomised individuals (n=8). Gates were set using CD38 monocyte strategy (see Materials and Methods). The Mann-Whitney U test was used to analyse non-parametric data.

4.4.7 Expression of CD28 on CD8 and CD4 T cells in HIV-1 infection

A)



B)

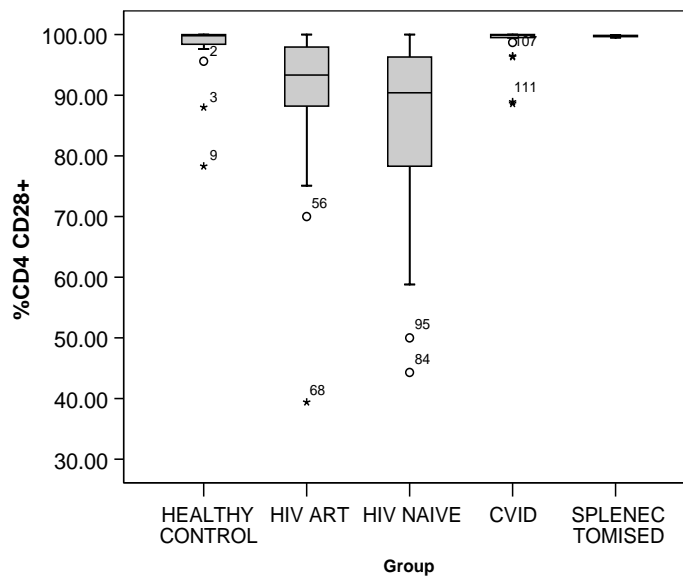


Figure 37: Expression of CD28 on T cells in HIV-1, CVID, splenectomised patients and healthy controls

Box and whisker plots displaying median and IQR CD28+ as a percentage of Fig.37A) CD8 T cells and Fig.37B) CD4 T cells for healthy controls, untreated HIV-1 infected patients (HIV NAÏVE) (n=29), treated HIV-1 infected patients (HIV ART) (n=55), CVID patients (n=28) and splenectomised individuals (n=8). Gates were set using isotype matched controls. The Mann-Whitney U test was used to analyse non-parametric data.

From Fig. 37 and Table 10, it can be seen that there was a striking increase in the percentage of CD8 T cells that had lost the expression of CD28 in HIV ART treated patients ($p < 0.001$), HIV ART naïve patients ($p < 0.001$) and CVID patients ($p = 0.02$) in comparison with healthy controls. The percentage of CD8⁺ CD28⁻ T cells in the peripheral blood of splenectomised patients was no different to the healthy control group. This was a small group of patients ($n = 8$), however, these findings suggest no difference in T cell costimulation via CD28 in splenectomised patients. In addition, the HIV untreated and ART treated groups also showed a pronounced loss of CD28 expression on CD4 T cells (both $p < 0.001$) (Fig.37B). This reduction was confined to the HIV cohort since the disease controls (CVID or splenectomised patients) did not show any loss in CD28 expression on CD4 T cells ($p = 0.68$, $p = 0.81$ respectively).

No significant correlations were found between baseline CD4⁺CD28⁺ or CD8⁺CD28⁺ T cell counts and response to Pneumococcal or Tetanus toxoid vaccination.

4.5 Discussion

In this chapter, levels of IgG pneumococcal antibodies were statistically lower in patients with untreated and treated HIV-1 infection. IgM pneumococcal levels were reduced in both HIV groups but this did not reach significance. Tetanus toxoid IgG was found to be reduced in both HIV groups. Vaccination in HIV-1 infected individuals to tetanus toxoid or pneumococcus was shown to be impaired. Low pre-existing antibodies and impaired vaccination responses were associated with a loss of memory B cell subsets. High levels of T cell immune activation were seen in patients with HIV-1 infection but this was not associated with responses to vaccination.

4.5.1 *Low specific Ig levels to pneumococcus and tetanus toxoid antigens in HIV-1 infection*

In this study, both HIV-1 untreated and HIV-1 treated patients were found to have significantly reduced levels of specific IgG antibodies to a common protein vaccine antigen (tetanus toxoid) and to a polysaccharide vaccine antigen (Pneumovax II). These antigens are T cell dependent and T cell independent respectively, indicating defects in both arms of the humoral immune response in HIV-1 disease. The findings are consistent with reports in the literature that HIV-1 infected patients have a defect in antibody production to these specific antigens (Janoff, Hardy et al. 1991; Kroon, van Dissel et al. 1994; Nielsen, Kvinesdal et al. 1998; Weiss, 1995 #340; De Milito, Nilsson et al. 2004; Titanji, Chiodi et al. 2005; Titanji, De Milito et al. 2006) and to other antigens such as naturally acquired measles virus (De Milito, Nilsson et al. 2004), HIV-1 gp41 (De Milito, Nilsson et al. 2004), influenza vaccination (Kroon, van Dissel et al. 2000; Malaspina, Moir et al. 2005) or varicella vaccination in children (Bekker, Scherpbier et al. 2006). A non-statistical reduction in pneumococcal IgM antibody levels was seen in both HIV-1 patient groups compared to healthy controls. Unfortunately, serotype specific analysis could not be performed; this assay may have been sensitive enough to show more subtle differences between HIV-1 infection compared to healthy individuals. No associations were found between baseline antibody levels to Pneumovax II or tetanus toxoid and peripheral CD4 counts in the HIV patients in this study in both patient groups. This is in contrast to some other work, Malaspina *et al*, found a correlation between CD4 counts and influenza memory B cell response (Malaspina, Moir et al. 2005).

4.5.2 *Impact of ART on specific antibody levels in HIV-1 infection*

Impaired IgG antibody levels to pneumococcal and tetanus antigens in the ART treated cohort suggest ART has not been able to restore specific antibody levels in our cohort. This was consistent with recent work suggesting that ART therapy started during chronic infection (Titanji, De Milito et al. 2006) or even started sooner in primary HIV-1 infection (Titanji, Chiodi et al. 2005) is unable to improve measles or pneumococcal serological memory. Antigen specific pools of memory B cells may therefore be eliminated at some time point in primary HIV-1 infection (Cagigi, Nilsson et al. 2010). Many HIV-1 infected patients had impaired baseline tetanus toxoid levels. Since these patients should have been vaccinated, a loss of pre-existing antibodies already reflects a loss of protective tetanus specific memory at some time point and this then cannot be reversed by ART.

4.5.3 *Importance of pneumococcal levels in protection against pneumococcus*

These findings confirm low IgG pneumococcal levels in patients with HIV-1 infection, which may be a risk factor for invasive pneumococcal disease. Despite a reduction in baseline pneumococcal IgM levels in HIV-1 infected individuals on ART, this was not statistically significant. This may be due to small cohort sizes studied or more detailed serotype specific analysis may be required. Recent studies highlighting the importance of IgM pneumococcal IgM antibodies have been published. Pneumococcal IgM antibodies have been shown to be better at opsonising *S.pneumoniae* than pneumococcal IgG antibodies (Park and Nahm 2011). In addition, natural IgM antibodies with specificity for polysaccharide antigens can be found after immunization. Natural polyreactive IgM antibodies are known to cross-react with blood group antigens and produce ANA staining patterns against filament proteins. Natural antibody is reported to play a role in the early control of invasive bacterial infection in mice (Baxendale, Johnson et al. 2008) since natural IgM antibodies were able to provide some protection against all pneumococcal polysaccharide capsular serotypes in a mouse challenge model. However, natural IgM antibody is not thought to be as effective as mutated serotype specific antibody produced by IgM memory B cells, in one study, a mutated pneumococcal serotype specific IgM derived from human IgM memory cells was shown to be almost 100% protective against live

pneumococcal challenge in mice (Zhong, Burns et al. 1999). This suggests a serotype specific analysis may be more relevant because the pneumococcal IgM measured in this study may include both natural low affinity antibodies and high affinity serotype specific pneumococcal antibodies.

4.5.4 *Low memory B cells are associated with low baseline specific antibody levels in untreated and treated HIV-1 infection*

We extended our study to also examine discrete memory B cell percentages and response to prior tetanus toxoid or pneumococcal vaccination/prior exposure. In both untreated and treated HIV infection, IgM memory B cells but not switched memory B cells were significantly correlated to pneumococcal IgM antibody titres. As hypothesized, a depletion of IgM memory B cells was associated with decreased levels of serum pneumococcal total IgM. This agrees with recent studies in the literature regarding healthy individuals and patients with primary immune deficiency. IgM memory B cells have been shown to be necessary for pneumococcal antibody production (Kruetzmann, Rosado et al. 2003; Weller, Braun et al. 2004) and in CVID, patients who had normal numbers of IgM memory B cells were less likely to have clinical bronchiectasis (Carsetti, Rosado et al. 2005) and were most likely to respond to vaccination (Goldacker, Draeger et al. 2007).

Consistent with our findings that low IgM memory B cell percentages predict an impaired response to Pneumovax in HIV-1 infection, low IgM memory B cell numbers also predict susceptibility to HIV-associated cryptococcosis (infection caused by *C.neoformans*) (Subramaniam, Metzger et al. 2009). *C.neoformans* is another encapsulated bacteria, where IgM antibody is also thought to act as an opsonin to clear infection (Subramaniam, Metzger et al. 2009).

The association between discrete memory B cell percentages and tetanus toxoid IgG was also examined. As hypothesized in this study, the association between class switched memory B cell percentages and tetanus toxoid antibody levels in the HIV untreated and ART treated group were significant. A depletion of class switched memory B cells correlated with lower baseline tetanus antibody levels in HIV-1 infected patients. This is consistent with a study of healthy controls by Lanzavecchia *et al*, who found tetanus specific memory B cell numbers correlated with tetanus

specific antibody production (Lanzavecchia, Bernasconi et al. 2006). Class switched memory B cells contribute to the plasma cell pool and continued production of tetanus toxoid antibody in a T cell dependent humoral manner and this finding again suggests a lack of cognate T cell help or an intrinsic B cell defect is associated with HIV-1 infection and has implications for vaccine design.

4.5.5 *Vaccination responses in HIV patients on ART are impaired*

We showed that vaccine responses to pneumococcal and tetanus toxoid were impaired in HIV-1 infected patients on ART. Poor vaccination responses confirm that our findings of poor serological memory in the HIV ART+ group and confirm this poor response to common antigens is a defect and not due to a possible lack of prior antigen exposure. Tetanus toxoid is a T cell dependent antigen and pneumococcus is a T cell independent antigen, therefore defects extend to both arms of the humoral response. The impairment varied in severity and we were able to show that this severity correlated with a loss of specific memory B cell subsets in these patients. Baseline CD4 T cell, nadir CD4 count, CD19 B cell counts, transitional B cell percentage did not influence post vaccine pneumococcal IgM, IgG or tetanus toxoid post vaccine levels.

Baseline tetanus toxoid levels predicted the response to tetanus toxoid vaccination, suggesting pre-existing T cell dependent recall responses may be protected in a subset of patients. This is important and suggests maintenance of long-lived serological memory may be attainable in some patients if vaccination is performed before HIV-1 infection. However, all our patients should have been vaccinated during the Childhood Immunisation Schedule; one adult booster is considered to give lifelong protection. Although a small study, those patients in our study which had no pre-existing protective antibody levels to tetanus toxoid were not able to generate an antibody response upon vaccination, which suggests again an intrinsic irreversible B cell defect. This has implications for susceptibility for *C. tetani* infection and may have implications for vaccination with other protein neo antigens. The mechanisms for this loss is unclear, however, early ART is able to preserve humoral immunity and vaccination responses in vertically infected children (Pensiero, Cagigi et al. 2009) and it is thought possible to induce T cell dependent immunisation responses very

early on in primary HIV-1 infection (Cagigi, Nilsson et al. 2010) and so our findings may reflect patients started on ART during established primary or chronic infection, at which time irreversible T cell dependent humoral defects have already occurred.

Pre-existing pneumococcal IgG or IgM antibody levels did not predict the response to pneumococcal vaccination. This may be due to cohort size or more likely reflects other factors such as a mixed exposure history to *S.pneumoniae*. Unlike tetanus toxoid, to which individuals should have some serological protection prior to HIV infection as part of the childhood vaccination schedule, low or absent pneumococcal antibody level at baseline may reflect poor prior exposure to *S.pneumoniae*. Pneumococcal immunisation was not part of the childhood British Vaccination Schedule historically, therefore any pre-existing baseline antibodies from prior vaccination history or natural antigen exposure would be varied in this small cohort of patients (n=19). Prior receipt of pneumococcal vaccine in the last five years was an exclusion criterion, however, some patients may have had a vaccine as part of clinical care prior to this, others may have been variably naturally exposed to *S.pneumoniae* or patients may have variable levels of natural IgM antibodies with low affinity for pneumococcus.

4.5.6 Impaired vaccination responses in HIV patients on ART is associated with loss of specific memory B cell subsets

Consistent with our findings that low levels of specific memory B cell subsets were associated with low levels of pre-existing antibodies, low levels of specific memory B cell subsets were associated with impaired response to vaccination. A successful tetanus toxoid vaccination was associated with higher baseline class switched memory B cell percentages. A successful pneumococcal vaccination response as defined by a four-fold rise in specific IgM antibody titre was associated with a higher percentage of IgM memory B cells. This suggests both low antibody levels and poor immunisation responses are dependent on levels of memory B cell subsets. The work in this chapter examined HIV-1 patients on ART for a median time of >4 years. Immunisation responses and memory B cell percentages in untreated primary HIV-1 infection may be very different, one study reported normal numbers of memory B cells but with an abnormal phenotype and lower antibodies against pneumococcal

antibodies, some believe this indicates an intrinsic B cell defect occurring early in HIV-1 infection (Cagigi, Nilsson et al. 2010).

4.5.7 T cell Immunophenotyping

T cell subset work was primarily performed to assess the possible impact of T cell immune activation on vaccination responses. The influence of immune activation or co-stimulatory molecules on the response to specific vaccination has not been thoroughly assessed previously.

4.5.8 Loss of CD28 expression on CD4 and CD8 T cells

We found a marked loss of CD28 expression by CD4 and CD8 T cells in ART naïve and treated HIV-1 infection, which is consistent with the literature. Some studies have suggested CD28 negative T cells represent chronically expanded clones that have impaired proliferation or are specific for a limited number of antigens (Weekes, Carmichael et al. 1999).

4.5.9 CD38 expression on CD4 and CD8 T cells

CD38 expression on CD8 T cells was greatly increased in the HIV ART naïve patients and to a lesser extent, those patients on ART, suggesting high levels of immune activation in HIV infection, which is consistent with the literature, since CD8+CD38 expression is an established marker of immune activation. Immune activation as part of an inflammatory immune response is thought to play a role in CD4 depletion in HIV-1 infection; therefore CD8+CD38+ expression on T cells is a possible marker of disease progression in untreated infection. In treated HIV infection, high levels of immune activation are associated with a failure to suppress viral replication on ART (Steel, John et al. 2008) and so immune activation may be involved in the regulation of immune restoration in patients on ART.

Individuals with CVID also showed a statistically significant rise in the percentage of CD8+ cells expressing CD38, suggesting increased immune activation in this group of primary immune deficient patients. The expression of CD38 on CD4 T cells was significantly increased in the HIV ART naïve group only, which is in agreement with the hypothesis that restoration of CD4 counts on ART is associated with lower immune activation and a reduction of CD38 expression.

4.5.10 Correlations between T cell immune activation or CD4+CD28+ T cells and response to vaccination

No correlation was found between baseline CD4+CD28+, CD8+CD28+ or CD8+CD38+ or CD4+CD38+ T cell counts and response to Pneumococcal or Tetanus toxoid vaccination. This suggests the response to T cell dependent or T cell independent vaccination is not influenced by T cell immune activation. This may be due to the cohort studied in this work, since these were HIV-1 infected patients on ART or more likely demonstrates other factors were more important, such as memory B cell subset percentage and pre-existing baseline specific antibody levels, as detailed above.

Few studies have examined CD28 in association with immunisation responses and B cell memory subsets. In one study, the expression of co stimulatory CD28 on CD4+ T cells was an independent predictor of response to immunisation with diphtheria/tetanus toxoid [71]. We did not find any association between CD4+CD28+ T cells and tetanus toxoid vaccination in the current study, however there were great differences in definitions of a successful immune response, measurement of tetanus toxoid antibody responses and differences in the HIV cohort studied. In the previous work that reported an association between nadir CD4 T cells and response to tetanus toxoid vaccination (Lange *et al*) [71], the cohort had been highly selected to measure only those patients with advanced disease (as demonstrated by extremely low nadir CD4 counts).

4.5.11 Limitations

A limitation of this work was the cohort size studied. Although the study aimed to recruit 100 patients, only 55 were recruited for vaccination and a proportion of these patients were given a placebo as part of the study design. In addition, this study examined total IgM and IgG antibody levels, a more complete analysis of serotype specific IgG levels and IgM levels would be useful for future work. I would also have liked to perform opsonic antibody killing assays (e.g. MOPA) to examine the function of the pneumococcal and tetanus antibodies produced in HIV-1 infection. In addition, IgA antibodies at mucosal surfaces may be additionally important to measure as these

may also be reduced in HIV-1 infection and may be functionally important. Finally, a pneumococcal vaccination study using Prevnar, the conjugated vaccine would be useful to see if this can reduce morbidity and mortality in HIV infection.

4.6 Conclusions

This study has confirmed low specific Igs to both T cell independent and T cell dependent microbial antigens in HIV-1 infection, even despite ART, suggesting an intrinsic defect in B cell function. This was confirmed by poor vaccination responses to both T cell dependent and T cell independent antigens in HIV-1 patients on ART. Low pre-existing antibodies and impaired vaccination responses were associated with a loss of memory B cell subsets. Our study has shown that serological response to vaccination is therefore dependent on both plasma cell function and memory B cell subsets percentages in HIV-1 infected patients on ART. This has important implications for treatment strategies and vaccination of HIV infected patients, measurement of class switched memory B cells and IgM memory B cells could be a useful clinical tool in assessing the functional humoral immune system in HIV-1 infection. As this study has shown, stratification of specific memory B cell percentages is an important parameter to study vaccine efficacy in HIV patients and may be able to predict an effective response to vaccination as agreed by others (D'Orsogna, Krueger et al. 2007). Data presented may account for the increased risk of invasive pneumococcal infection reported in patients with HIV-1 infection. Intrinsic defects in B cell function may contribute to clinically significant complications of HIV-1 infection and be responsible for impaired humoral immune responses to vaccination observed in this condition.

5. Chapter V: Measurement of tissue like B cell subsets in untreated and treated HIV-1 infected individuals and patients with CVID: associations with B cell dysregulation

5.1 Introduction

5.1.1 Tissue like B cells

We previously reported a loss of discrete IgM memory CD27⁺ memory B cell subsets in HIV infection (Hart, Steel et al. 2007) which has been confirmed by others. CD27 is a classical marker of B cell memory, however, B cell memory may not be exclusively contained within the CD27⁺ subset: small frequencies of IgG⁺ or IgA⁺ cells with other phenotypic and functional characteristics attributed to B cell memory such as ABCB1 transporter negative activity and evidence of somatic hypermutation have recently been identified in the CD27⁻ compartment of healthy individuals and in SLE (Wirhth and Lanzavecchia 2005; Fecteau, Cote et al. 2006; Ma, Pittaluga et al. 2006). These are generally reported to be IgG⁺ (~3.5%) or IgA⁺ (~1.5%) CD27⁻ cells and represent <5% of the peripheral B cell pool in healthy individuals (Fecteau, Cote et al. 2006; Ma, Pittaluga et al. 2006).

CD27 negative memory B cells were named tissue like B cells by Ehrhardt *et al* (Ehrhardt, Hsu et al. 2005) because they are found predominantly in the human lymphoid tissues: tonsils, lymph node, Peyer's patches and spleen in healthy individuals. Morphologically, tissue like memory B cells are comparatively large B cells with prominent nucleoli and a large cytoplasm enriched for mitochondria (Ehrhardt, Hsu et al. 2005).

5.1.2 Phenotype of tissue like B cells

An extensive phenotypic analysis of the tonsillar compartment of healthy individuals found that the tissue like B cell has decreased CD21 and CD27 expression. This B cell subset is positive for Fc receptor homolog 4 (FCLR4, also known as FcRH4 or IRTA1 receptor), a transmembrane molecule of the Fc receptor homologue family and

a potential immunomodulatory regulator of B cells. Tonsillar tissue like memory cells express CD11c, a adhesion marker associated with movement to mucosal tissues, upregulate activation markers such as CD70, express co-stimulatory molecules (CD86) and express higher levels of chemokine receptors CXCR3 and CCR6 compared to classical memory B cells and naïve B cells (Ehrhardt, Hsu et al. 2005). Tonsillar FCLR4+ B cells have similar levels of somatic hypermutation and immunoglobulin isotype switching to their FCLR4- memory B cell counterparts and secrete higher levels of Ig upon stimulation by T cell cytokines (but not to BCR ligation) indicating these are *bona fide* memory B cells (Ehrhardt, Hsu et al. 2005). One note of caution: previous phenotyping of the FCLR4+ IgD-CD38- tissue like memory B cell subset analysed in this study by Ehrhardt and colleagues will include a contaminating proportion of CD27+ classical class switched memory B cells, (CD27 positivity approximated as one third of the IgD-CD38- fraction in the Ehrhardt study) and so the functional results must be interpreted with caution. Further characterisation of the FCLR4+ B cell subset in tonsillar and peripheral blood is required.

5.1.3 Evidence of expanded tissue like B cell in HIV infection, CVID and in autoimmune disease

The appearance of an unusual CD19+ B cell population expressing decreased or negative levels of CD21 (CD21^{low}) in the peripheral blood of viraemic HIV infected individuals has been recognised since 1992 (Benedetto, Di Caro et al. 1992; Scott, Landay et al. 1993) but was poorly defined. CD21^{lo} B cells expanded in HIV infection were shown to have poor proliferation to B cell stimulation (CD40L, IL-2, IL-10, TLR9 signalling or BCR stimulation (Moir, Malaspina et al. 2001; Moir, Malaspina et al. 2004) and reduced ability to produce immunoglobulins after PWM stimulation (Benedetto, Di Caro et al. 1992), yet other studies have associated the expanded CD21^{low} B cell subset with spontaneous secretion of immunoglobulin during HIV viraemia (Moir, Malaspina et al. 2001). Crucially, recent work has shown that the CD21^{low} B cell fraction is heterogeneous and contains several different cell populations aberrantly expanded in conditions with disturbed B cell homeostasis. CD21^{low} cells have differing functions and variable expression of CD21: immature transitional B cells (CD21^{int}CD10⁺CD38⁺⁺IgM⁺CD24⁺), activated memory B cells (predominantly characterised in HIV infection: CD21^{int}CD27⁺CD10⁻) and most

recently, tissue like memory B cells (CD21dim/neg CD27dim/neg, CD38- FCLR4+, CD11c+) have been characterised to date.

Premature exhaustion of virus specific T cells in HIV viraemic individuals has recently been identified and attributed to increased immune activation (Day, Kaufmann et al. 2006; Trautmann, Janbazian et al. 2006). Similarities between upregulated markers of viral T cell immune exhaustion (CXCR3) and phenotypic markers of tissue like memory B cells reported by Ehrhardt, led researchers to recently report an expanded population of tissue like memory B cells in HIV viraemic individuals. These tissue like B cells are thought to be an exhausted dysfunctional memory B cell population and have been shown to be enriched for HIV specific responses (Moir, Ho et al. 2008). This tissue like population expanded in HIV infection was defined by the phenotype CD19+ CD27- CD21 (dim/negative) CD10-. Higher levels of FCLR4 inhibitory receptor, and higher levels of lymphocyte trafficking receptors CXCR3, CD11c and CCR6 expression were found, characteristics associated with FCLR4+ tonsillar tissue like B cells (Ehrhardt, Hijikata et al. 2008). The FCLR4 positive population seen in the HIV viraemics contained some CD27+ memory B cells in addition to the CD27- cell subset, these FCLR4+ CD27+ B cells are proposed to be the activated CD27+ memory B cell subset (Moir and Fauci 2009). This tissue like memory B cell subset may be functionally important: Cagigi and colleagues have reported the presence of a CD27- memory population producing class switched antibodies in HIV aviraemic individuals (Cagigi, Du et al. 2009), a function normally attributed to CD27+ memory B cells. The B cell phenotype of the CD27- population is not very well characterised and based on data presented by Moir, the Cagigi CD27- memory population may contain tissue like memory B cells. This indicates that tissue like B cells are able to produce class switched antibodies.

An expanded CD21low population has also been described in patients with other conditions associated with disturbed B cell homeostasis such as chronic hepatitis (Charles, Green et al. 2008), malaria, immune deficient conditions such as CVID (Warnatz, Denz et al. 2002; Warnatz, Wehr et al. 2002), Idiopathic CD4+ T cell lymphopenia, Wiskott-Aldrich syndrome (Park, Shcherbina et al. 2005) and autoimmune conditions such as SLE (Warnatz, Wehr et al. 2002; Wehr, Eibel et al.

2004) and RA (Isnardi, Ng et al. 2010). Again, this population has been poorly defined functionally and phenotypically and as such did not fit into known subsets of B cell differentiation. A lack of multi-parametric flow cytometry and variable gating/different B cell phenotypic markers used by different research groups to characterise B cell subsets has caused confusion to such an extent that it has been unclear whether some early papers were describing the more recently characterised transitional B cell subset (Sims, Ettinger et al. 2005) which also has decreased CD21 expression and is also expanded in HIV, CVID and SLE (Malaspina, Moir et al. 2006).

During my experiments, two recent studies of CVID and RA patients further described the phenotype of this CD21^{low} population as CD27^{lo} CD21^{lo} CD86^{hi} CD95^{hi} CD11c^{hi} and decreased expression of CXCR5, CCR5, CD62L and CCR7. FCLR4 was possibly expressed on these cells in one study, however only one dot plot was shown. Upregulation of CXCR3, CD72 and SIGLEC inhibitory genes and high levels of CD22 and CD85 inhibitory receptors were found on peripheral CD21^{low} B cells of RA and CVID patients (Isnardi, Ng et al.). The phenotype of these CD21^{low} B cells is highly consistent with the description of tissue like B cells found in the tonsils and described in HIV infection.

However, unlike the class switched tissue like B cells predominant in the small tissue like compartment of healthy donors, the tissue like B cell population reported in CVID and RA was an unmutated, non-class switched B cell population. One study of CVID patients termed these tissue like B cells ‘an innate like B cell population’ with tissue homing capacity, based on the finding that these cells resemble innate murine B1 cells whilst expressing appropriate chemokine receptors to migrate to inflammatory sites. Further to this, they found the tissue like B cell subset are enriched in the BAL of CVID patients with interstitial lung disease and are found in the synovial fluid of patients with RA (Rakhmanov, Keller et al. 2009).

5.2 Aims

- 1) To measure the frequency of tissue like B cells in untreated and treated HIV-1 infected patients, CVID and healthy controls.
- 2) To look for an association in HIV infection and CVID between numbers of tissue like B cells and classical memory B cells.
- 3) To examine whether there is an association between tissue like B cells and response to vaccination in HIV infection.

5.3 Methods

5.3.1 *Study One*

The Functional Immunity study was described as before: HIV-1 infected patients were recruited from the Kobler Clinic, St Stephen's Centre, Chelsea and Westminster Hospital. These comprised 29 HIV-1 infected untreated patients and 55 HIV-1 patients on ART that had been recruited into a randomised trial to assess the efficacy of ART treatment on pneumococcal and tetanus toxoid vaccination. Inclusion criteria for this trial included an undetectable viral load for ≥ 2 consecutive months. Patients were matched for nadir CD4 counts and immunised with Pneumovax II (T cell independent carbohydrate antigen), tetanus toxoid (T cell dependent protein antigen) or placebo. The antibody response to vaccination was assessed at screening, baseline, 4 weeks, 12 weeks and 24 weeks post immunisation. 29 CVID patients and 8 individuals with a history of splenectomy were recruited from the Respiratory Clinic at the Royal Brompton Hospital. 17 healthy laboratory workers from the Immunology laboratory were immunophenotyped as healthy controls.

5.3.2 *Study Two*

HIV-1 infected patients were recruited from the Kobler Clinic, St Stephen's Centre, Chelsea and Westminster Hospital. These comprised 14 HIV-1 infected untreated patients and 14 HIV-1 patients on ART. 24 CVID patients were recruited from the Respiratory Clinic at the Royal Brompton Hospital. 11 healthy laboratory workers from the Immunology laboratory were also tested to act as control subjects.

5.3.3 *Study One: Phenotypic measurement of tissue like B cells using four colour flow cytometry*

In this chapter, I evaluated two gating strategies for four colour flow cytometry based on the panel from the Functional Immunity study (Study One) and on two published phenotypes.

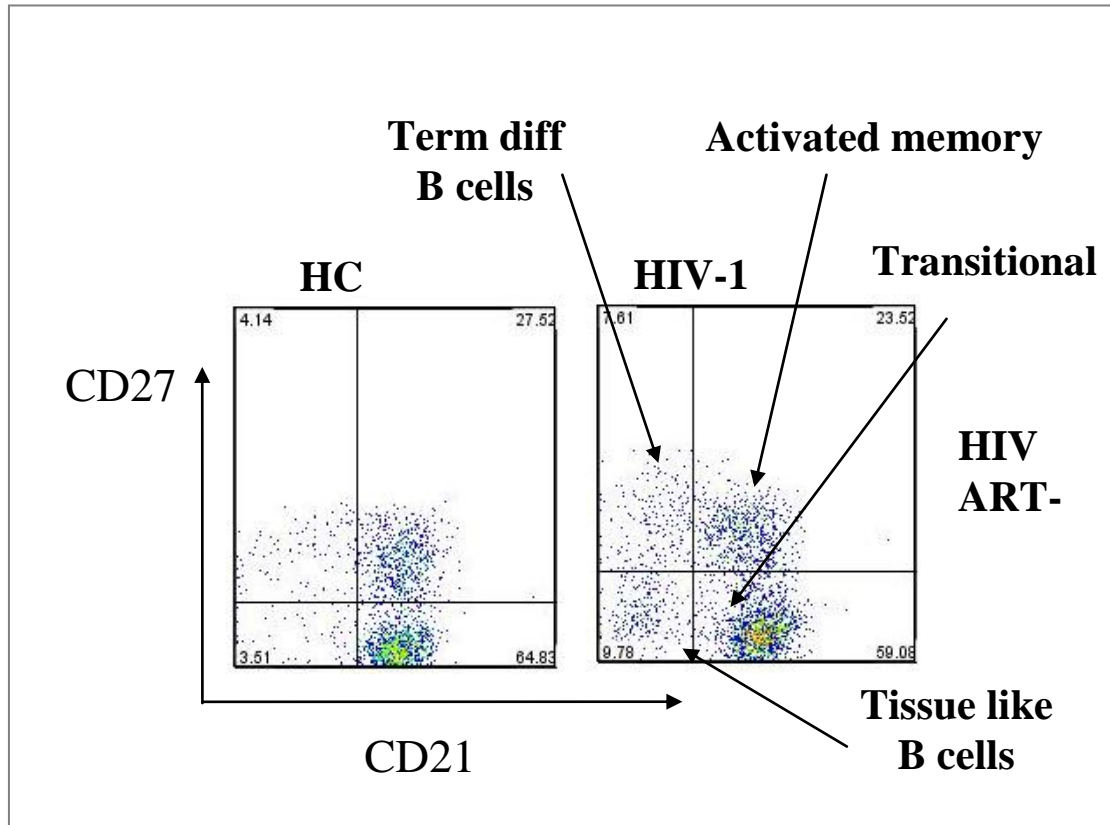


Figure 38: CD27^{lo}CD21^{lo} B cell phenotyping in a representative healthy control (HC) and an untreated HIV-1 infected individual.

Analysis of CD27^{lo}CD21^{lo} B cells was performed using a modified version of gating by Moir *et al* (Moir, Ho et al. 2008). Moir *et al* have used CD21/CD27/CD10 gating analysis in several publications to measure CD21^{low} B cells. In their papers, the CD21^{low} and CD21^{negative} population is included, as shown in the representative plots (Fig.38). CD10⁺ transitional B cells are normally excluded from analysis by depletion before staining or by excluding CD10⁺ B cells. CD10⁺ B cells make up less than 2% of B cells in the healthy population. Since this antibody cocktail did not include CD10, I evaluated where the transitional B cell population appeared on a

CD27 v CD21 dot plot (see Fig.38). Consistent with other reports, the expanded transitional B cell population seen in some HIV viraemic individuals express CD21 at an intermediate level and therefore can be gated out by the analysis.

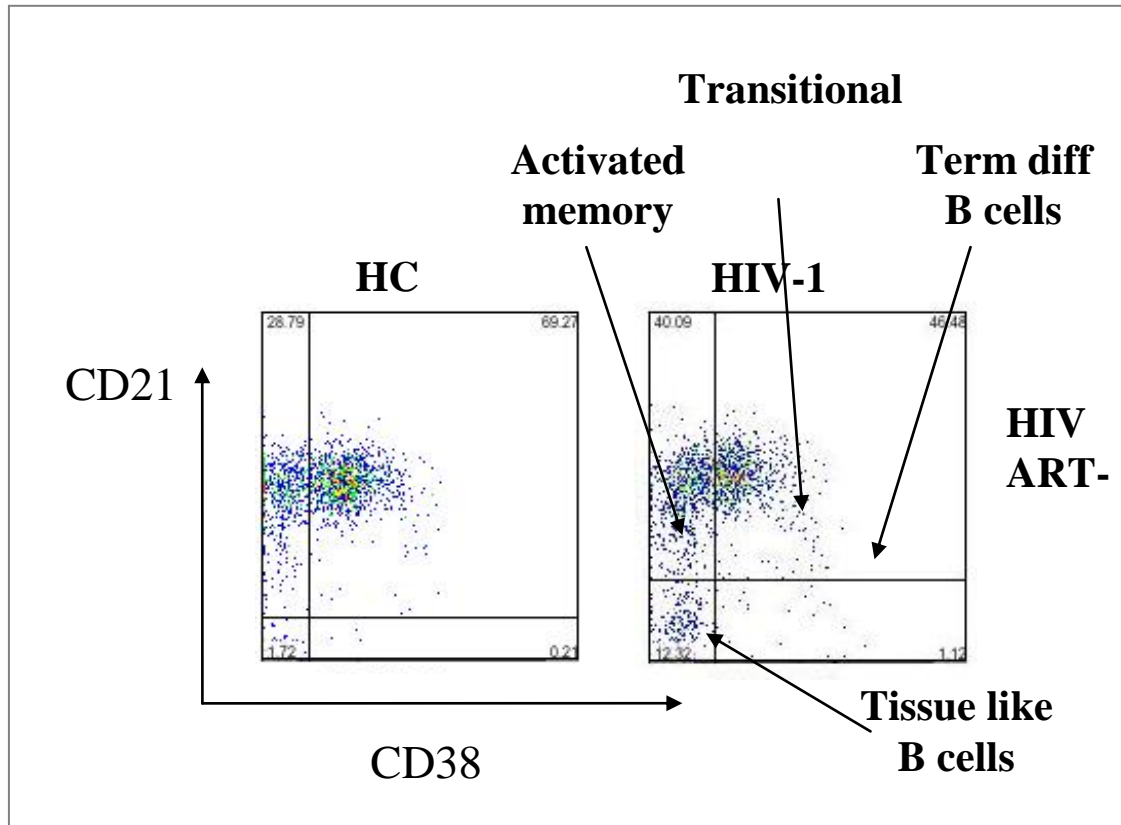


Figure 39: CD38^{lo}CD21^{lo} B cell phenotyping in a representative healthy control (HC) and an untreated HIV-1 infected individual.

Phenotyping using a CD38 and CD21 monoclonal antibody cocktail was performed as described by Warnatz *et al* (Rakhmanov, Keller et al. 2009). Warnatz *et al* used a combination of CD38 negativity and CD21 negativity to measure tissue like B cells. An initial concern was that this gating strategy might include some activated CD27⁺CD21^{low} memory B cells since these are CD21^{int/low}. Other CD21^{low} B cells such as plasmablasts and transitional B cells are CD38 bright, therefore these would be excluded by gating out CD38⁺ cells. I analysed the CD27⁺CD21^{low} activated memory cells and found these to be CD27^{int}CD21^{int} (see Fig.39). By using isotype gating, these cells were excluded from analysis.

5.4 Study One Results

5.4.1 Analysis of phenotyping strategies for the measurement of tissue like B cells

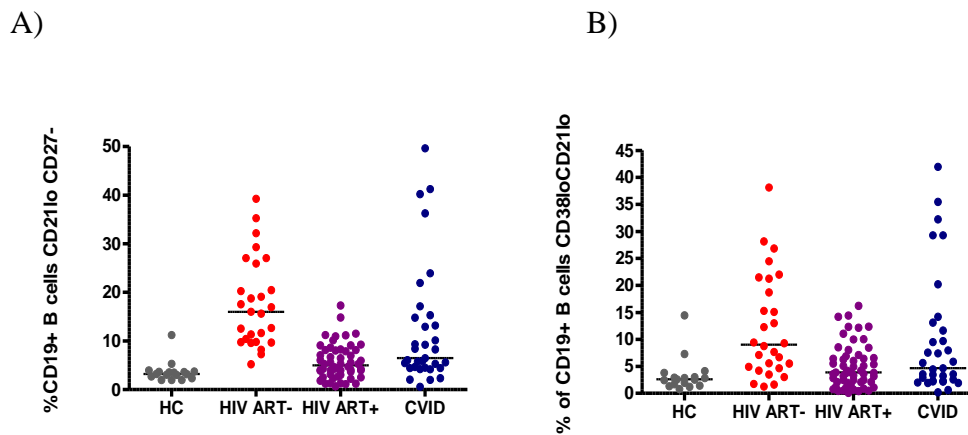


Figure 40: Scatter plots to show the relative distribution of tissue like B cells using two different phenotypes for tissue like B cells

Plots displaying data points and horizontal lines indicate median values for tissue like B cells as a percentage of CD19+ B cells, for healthy controls (HC) (n=17), untreated HIV-1 infected patients (HIV ART-) (n=29), treated HIV-1 infected patients (HIV ART+) (n=55) and CVID patients (n=28). Gates were set using an isotype matched control for CD38/CD21 tube: 40A) CD19+CD21lowCD27low B cells, B) CD19+CD38lowCD21low B cells. The Mann-Whitney U test was used to analyse non-parametric data.

CD27loCD21lo tissue like B cells are present at low numbers in healthy individuals and significantly increased in ART- ($p < 0.0001$) and ART+ HIV infection ($p < 0.0001$) and CVID (Fig. 40 A). There is a significant expansion of CD38loCD21lo tissue like B cells in both the HIV ART- and CVID patients in comparison with the healthy control group (Fig.40 B). Levels of CD38loCD21loB cells in ART treated HIV-1 infected individuals overall were not significantly different from HC due to an outlier ($p = 0.11$) but were expanded in some individuals. Therefore, this warranted further investigation in an additional study. The median percentage of CD27loCD21lo tissue like B cells was higher than CD38CD21lo tissue like B cells in the HIV ART- group, also see Fig. 40. This suggests the CD27loCD21lo subset may contain contaminating transitional B cells since these are also CD27-CD21int/low and also expanded in untreated HIV infection. CD10 positivity can be used phenotypically to exclude transitional B cells from analysis. Further phenotyping using CD10 will be performed to confirm the expansion of these populations at all stages of HIV-1 infection.

5.4.2 Analysis of the association between memory B cells and tissue like B cells

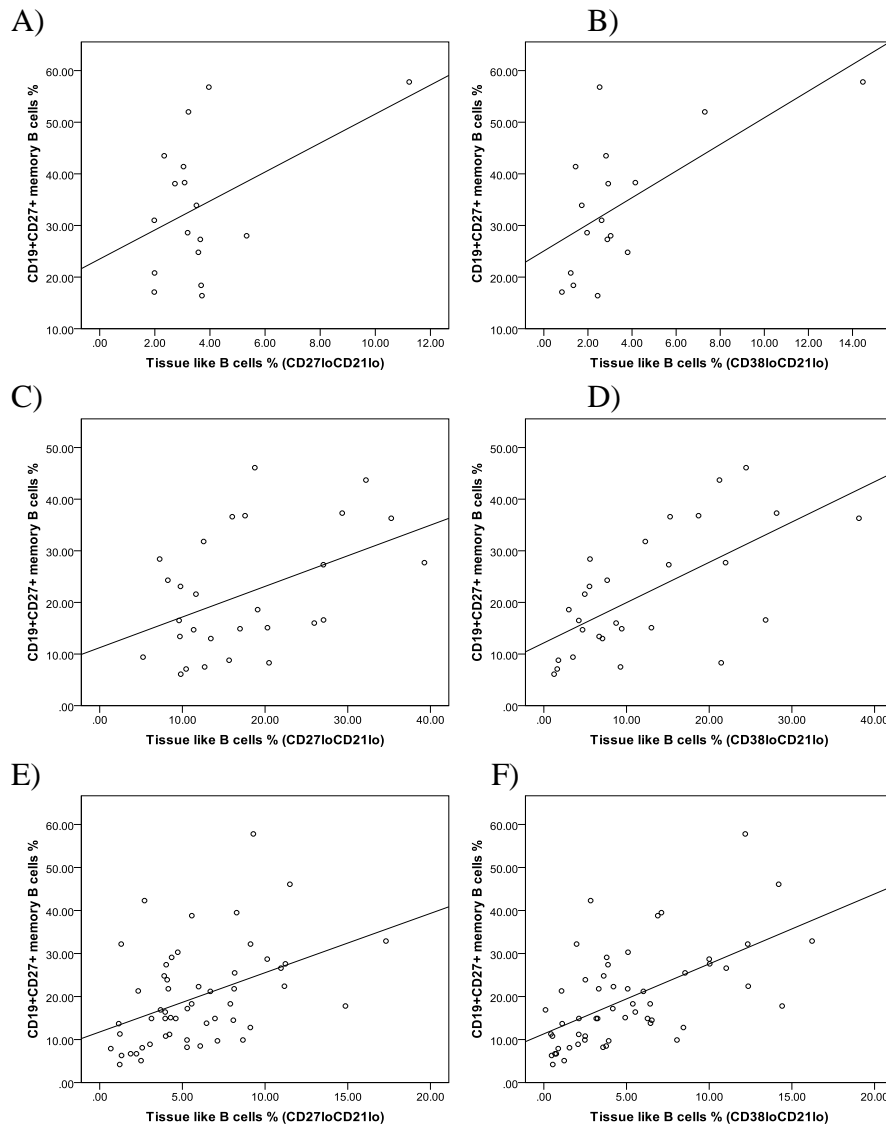


Figure 41: Scatter plots showing associations between CD27+ memory B cells and tissue like B cells using CD27loCD21lo or CD38loCD21lo phenotyping in HC, untreated HIV-1 infection and treated HIV-1 infection.

Fig. 41A) Associations between CD27+ memory B cell percentages using CD27loCD21lo tissue like B cell percentage and **41B)** CD38loCD21lo percentage in healthy controls (n=17); **Fig. 41C)** Associations between CD27+ memory B cell percentages using CD27loCD21lo tissue like B cell percentage and in **41 D)** CD38loCD21lo percentage in untreated HIV-1 infection (n=29). **41E)** Associations between CD27+ memory B cell percentages using CD27loCD21lo tissue like B cell percentage and in **41F)** CD38loCD21lo percentage in treated HIV-1 infection (n=55). All data gated as a percentage of CD19+ B cells. Data analysed using Spearman's rank correlation coefficient.

It can be seen that the percentage of CD38^{lo}CD21^{lo} tissue like B cells positively correlate with proportions of classical CD27⁺ memory B cells in the blood of healthy controls ($p=0.03$) (Fig.41B), and HIV infected individuals on and off treatment (both $p<0.001$) (Fig.41D and Fig.41F). Using CD27^{lo}CD21^{lo} gating, percentages of CD27^{lo}CD21^{lo} B cells did not correlate with total memory B cells of HC (Fig.41A) but showed significant correlations with total classical memory in untreated HIV infection ($p=0.04$) (Fig.41C) and treated HIV infection ($p<0.001$) (Fig.41E). However, from Fig.41, it is clear that the ratio of tissue like B cells to classical memory B cells is increased in HIV infection, regardless of phenotyping strategy employed.

CD38^{lo}CD21^{lo} tissue like B cells percentages were found to be negatively correlated to proportions of naïve B cells in HIV-1 infected individuals: untreated ($\rho=-0.661, p<0.0001$) treated HIV-1 infection ($\rho=-0.643, p<0.0001$) and healthy controls ($\rho=-0.403, p=0.04$). No correlation was found between tissue like B cells (CD38^{lo}CD21^{lo} or CD27^{lo}CD21^{lo}) and CD4 count or B cell count in any patient group or control. In untreated HIV infection, no correlation was found between tissue like B cell proportions and viral load or progressive disease by stratifying CD4 counts.

5.4.3 Loss of memory B cells in HIV-1 infection is associated with reduced numbers of tissue like B cells

The CD27^{low}CD21^{low} phenotyping may include contaminating transitional B cells, so further analysis of study one data was restricted to tissue like B cell percentages using CD38^{low}CD21^{low} phenotyping.

5.4.4 Untreated HIV infection:

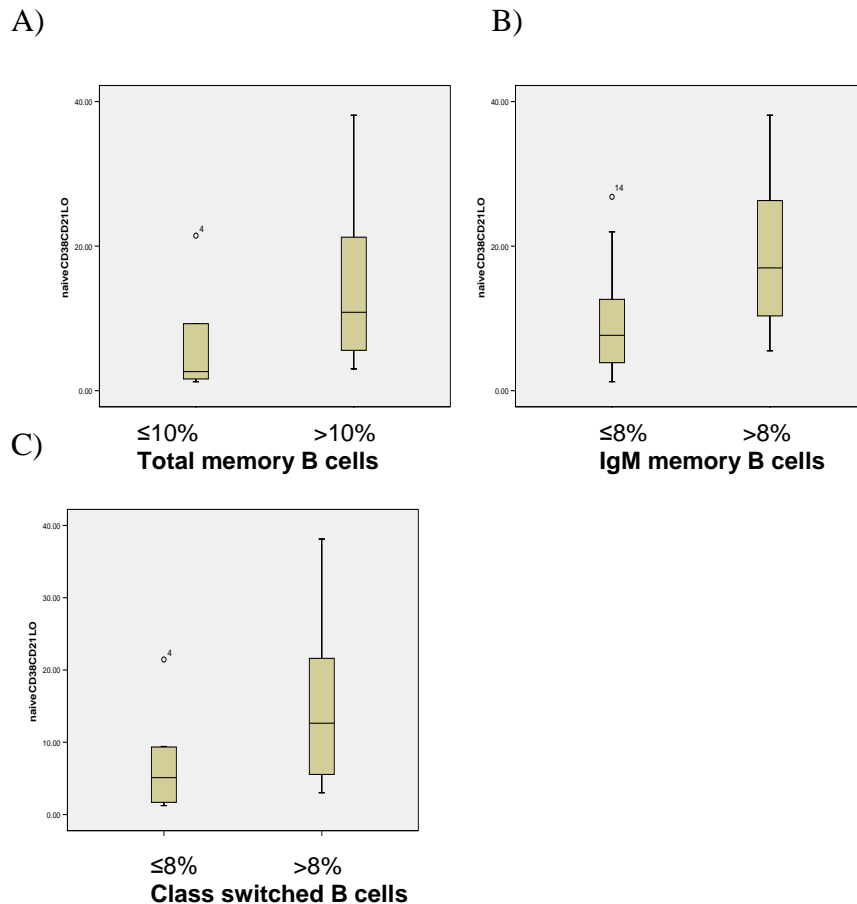


Figure 42: Influence of tissue like B cell percentages on CD27⁺ memory B cell subsets in untreated HIV infected patients

Box plots show CD38^{lo}CD21^{lo} tissue like B cells as a percentage of CD19⁺ B cells in untreated HIV infection (n=29). Patients stratified using modified cut-off values taken from the Paris CVID classification: 42A) Total classical memory B cells ($\leq 10\%$ (n=6) or $> 10\%$ (n=23)), 42B) IgM memory B cells ($\leq 8\%$ (n=19) or $> 8\%$ (n=10)) and 42C) class switched memory B cells ($\leq 8\%$ (n=7) or $> 8\%$ (n=22)). The Mann-Whitney U test was used to analyse non-parametric data.

Levels of the tissue like memory B cell subset are significantly higher in those HIV infected individuals who have higher proportions ($> 10\%$) of total classical memory B cells ($p=0.03$) (Fig.42A). Loss of the IgM memory subset ($< 8\%$) in untreated HIV infected individuals is significantly associated with reduced percentages of the tissue like memory compartment ($p=0.04$) (Fig.42B). Loss of class switched memory B cell subset in a smaller proportion of HIV infected untreated patients is also associated with a loss of the expanded tissue memory B cell compartment ($p=0.04$) (Fig.42C).

5.4.5 *Reduced numbers of both classical memory and tissue like B cells are also seen in a proportion of HIV infected individuals on ART.*

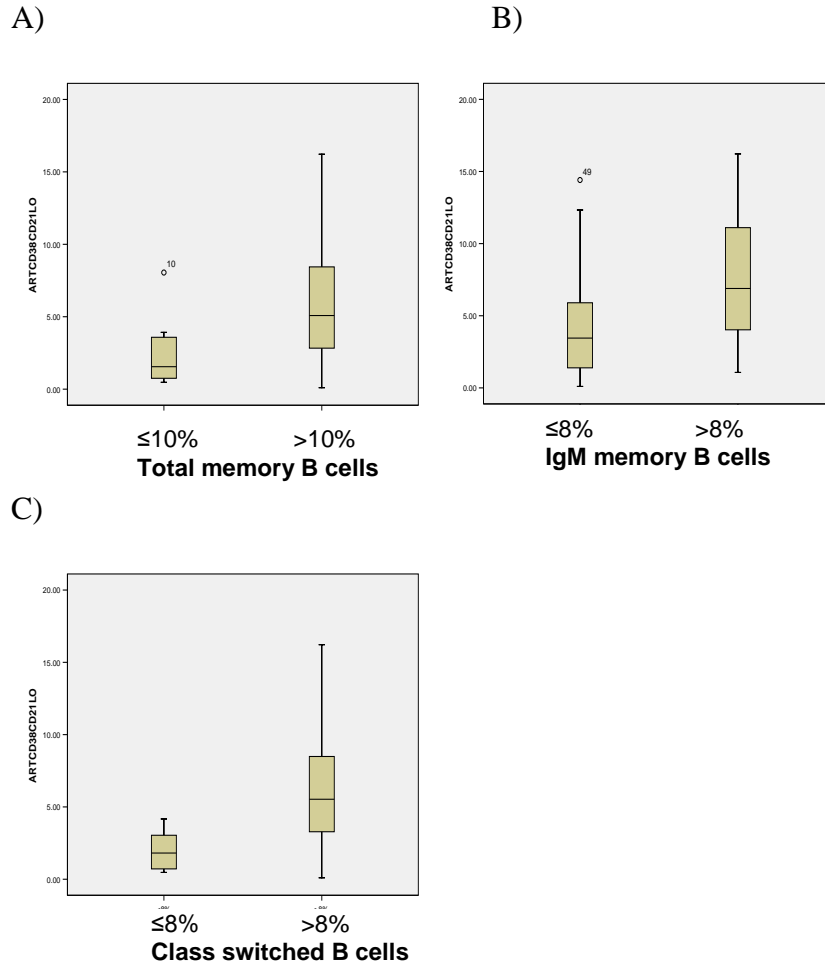


Figure 43: Influence of tissue like B cell percentages on CD27 memory B cell subsets in HIV infected patients on ART

Box plots show CD38^{lo}CD21^{lo} tissue like B cells as a percentage of CD19⁺ B cells in HIV infected patients on ART (n=55). Patients stratified using modified cut-off values taken from the Paris classification: 43A) Total classical memory B cells ($\leq 10\%$ (n=13) or $>10\%$ (n=42)), 43B) IgM memory B cells ($\leq 8\%$ (n=40) or $>8\%$ (n=15)) and 42C) class switched memory B cells ($\leq 8\%$ (n=17) or $>8\%$ (n=38)). The Mann-Whitney U test was used to analyse non-parametric data.

Expansions of tissue like B cells exist in a subset of patients on ART. Tissue like B cells are significantly higher in HIV infected individuals with higher proportions of total memory B cells (p=0.02) (Fig.43A). Loss of the IgM memory subset in 70% (p<0.01) (Fig.43B) and class switched memory B cell subset in 25% (p=0.001) of

treated HIV-1 patients was significantly associated with reduced percentages of tissue like B cells (Fig.43C). The tissue like B cells proportion has no correlation with time on ART, suggesting this is a stable subset.

5.4.6 *High levels of tissue like B cells in HIV infected patients on treatment are associated with good response to tetanus toxoid vaccination*

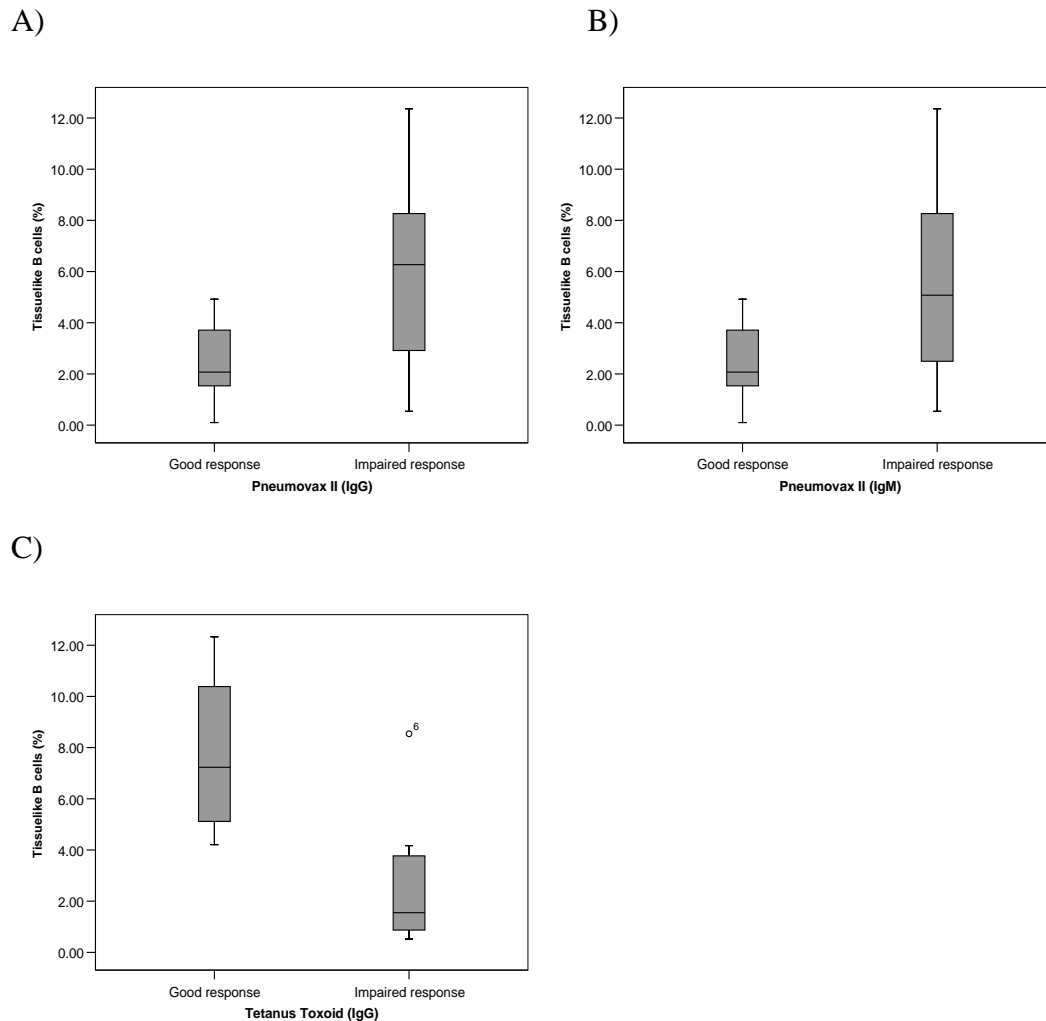


Figure 44: Influence of tissue like B cell percentages on post immunisation responses in treated HIV infected patients

Box plots display CD38^{lo}CD21^{lo} tissue like B cells as a percentage of CD19⁺ B cells and subjects stratified according to a good or impaired response to immunisation. 44A) IgG pneumococcal antibody response to Pneumovax II vaccination (good response n=7, impaired response n=11), 44B) IgM pneumococcal antibody response to Pneumovax II vaccination (good response n=7, impaired response n=12), and 44C) IgG tetanus antibody response to

vaccination with Tetanus Toxoid (good response n=4, impaired response n=9). The Mann-Whitney U test was used to analyse non-parametric data.

From Fig.44A) and Fig.44B), there was a trend for higher peripheral tissue like B cell percentages to be associated with an impaired response to Pneumovax (IgM or IgG) but this was not significant (IgM p=0.08, IgG p=0.05). At the very least, there was no positive correlation between tissue like B cells and a good response to Pneumovax, suggesting high percentages of these cells are not helpful for T cell independent immunity. In Fig.44C, a significant association was seen between a good response to tetanus toxoid vaccination and higher levels of tissue like B cells (p=0.02), suggesting high tissue like B cell percentages are associated with protective T cell dependent immunity in HIV-1 infected patients on ART. The tetanus toxoid vaccination data was based on small numbers (n=4 good responders and n=9 poor responders) but suggests promise for a larger study.

5.5 Study Two: additional markers for tissue like B cells to characterise the presence of the tissue like B cell population in untreated and treated HIV infection and CVID

In this section of the study, 14 patients naïve to HIV treatment, 11 patients on ART, 23 CVID and 11 HC were evaluated for tissue like B cells using established immunophenotyping for tissue like memory (CD27-CD10-CD21^{low} CD19⁺ B cells) (Moir, Ho et al. 2008). The additional tissue like B cell markers CD11c (trafficking molecule) and FCLR4 (inhibitory receptor) were used to further characterise tissue like B cells in healthy controls, HIV and CVID patients.

Table 11: Study characteristics of immunophenotyped patient cohort

	HC (n=11)	HIV ART- (n=14)	HIV ART+ *(n=14)	CVID (n=23)
<i>Patient Demographics</i>				
Age (yrs.)	34 (27-45)	35 (32-42)	42 (40-48)	48 (37-57)
Viral Load (copies/ml)	NA	27375 (8643-40268)	<50	NA
Time on ART (mos.)	NA	NA	88 (69-134)	NA
CD4 (cells/μl)	812 (667-971)	335 (296-467)	447 (412-553)	552 (447-895)
CD8 (cells/μl)	351 (297-487)	818 (639-1052)	732 (596-862)	409 (222-639)
CD19 (cells/μl)	205 (164-257)	114 (86-150)	222 (119-258)	159 (68-233)
<i>B cell subsets</i>				
%CD19+CD27+	31.1 (23.6-42)	32.5 (26.0-44.5)	24.0 (15.9-32.0)	8.7 (4.6-35.9)
%CD19+Csmem	13.8 (11.0-18.2)	18.7 (16.2-20.6)	12.8 (9.2-21.4)	1.1 (0.6-5.1)
%CD19+IgMmem	19.3 (12.3-23.4)	13.4 (6.6-24.9)	7.9 (6.0-11.4)	5.4 (3.8-25.3)
%CD19+Naïve B	66.7 (56.6-73.6)	63.7 (49.7-70.1)	73.8 (62.6-81.1)	90.2 (4.6-35.9)
%CD19+Tissue B*	2.3 (1.9-5.8)	15.0 (7.5-18.4)	9.0 (3.9-11.9)*	10.7 (6.0-20.2)

*Note: Three ART+ patients were not tested for tissue like B cells. Median and interquartile range Populations analysed using the Mann-Whitney U Test for non-parametric data.

The HIV-1 patients on ART were not significantly older than controls and untreated HIV-1 patients (see Table 11). The CVID patient group was not significantly older than healthy controls. CD19+ B cell counts were significantly lower in the untreated HIV-1 cohort compared to healthy controls ($p<0.002$). CD4 T cell counts were significantly lower in untreated HIV-1 infected patients ($p<0.0001$) and HIV-1 ART+ patients ($p<0.01$) compared to healthy controls. Memory B cell (CD19+CD27+) percentages were not significantly different in the HIV-1 infected patients but reduced in CVID compared with controls ($p<0.01$). IgM memory B cells were significantly reduced in the HIV ART+ group ($p<0.001$) compared to controls and decreased in the HIV ART- group but this was not significantly different from healthy controls. Class switched memory B cells were decreased in the CVID cohort compared to healthy controls ($p<0.0001$). Tissue like B cell percentages were significantly increased in

untreated HIV patients ($p < 0.001$) and CVID patients ($p < 0.02$) but not in treated HIV-1 patients ($p = 0.06$).

5.5.1 Phenotyping for tissue like B cells using CD10 exclusion, as described by

Moir et al

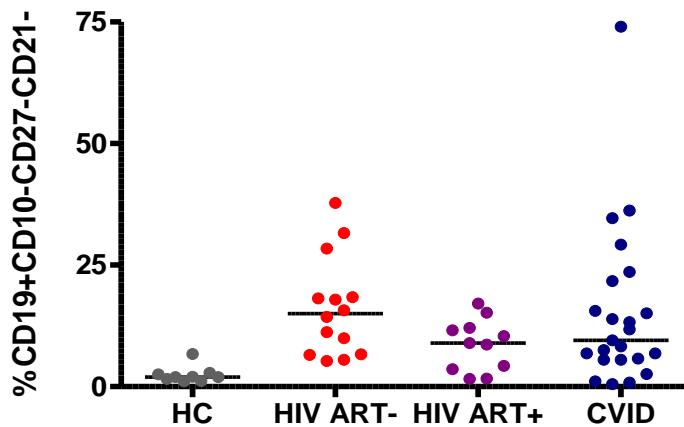


Figure 45: Tissue like B cells as a percentage of mature B cells in untreated and treated HIV infected patients, CVID and healthy controls.

Plot displaying data points and horizontal lines median values for tissue like B cells (CD21^{low}CD27-CD10-) as a percentage of CD19+CD10- B cells, for healthy controls (HC) (n=11), untreated HIV-1 infected patients (ART-) (n=14), treated HIV-1 infected patients (ART+) (n=11) and CVID patients (n=23). Gates were set using intensity of CD21 expression. Transitional B cells were excluded by gating for CD19+CD10- B cells. The Mann-Whitney U test was used to analyse non-parametric data.

Using the Moir gating strategy, CD21^{low}CD27-CD10- B cells were significantly expanded in untreated HIV patients ($p = 0.001$), consistent with previous results and CVID patients ($p = 0.014$), (see Fig.45). Few CD21^{low}CD27-CD10- tissue like B cells were present in the peripheral blood of healthy controls. In the treated HIV infected cohort (n=11), there was a trend for elevated tissue like memory B cells in comparison with the healthy control cohort but this narrowly failed to reach statistical significance, this is likely to be due to small cohort sizes in the ART+ group ($p = 0.06$).

5.5.2 Tissue like B cells and association with IgM memory B cells

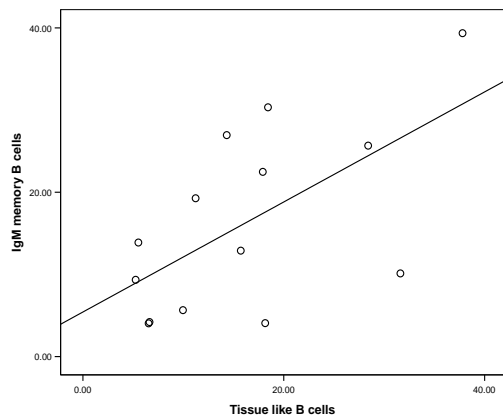


Figure 46: Scatter plot to display the correlation between IgM memory B cells and tissue like memory B cell percentages in the untreated HIV infected cohort (n=14).

In patients naïve to ART, using the published CD19+CD27-CD10-CD21- phenotype as characterised by Moir *et al* (Moir, Ho et al. 2008), a positive correlation was found between the levels of tissue like B cells (CD27-CD21-CD10) and IgM memory B cells, using Spearman's rank correlation coefficient. This confirmed data reanalysed from the functional immunity study ($p < 0.05$) (Fig.46). No correlation was found between tissue like B cells and class switched memory B cells or total memory B cells ($p = 0.06$) (graphs not shown).

5.5.3 Use of FCLR4 as a marker for peripheral tissue like B cells

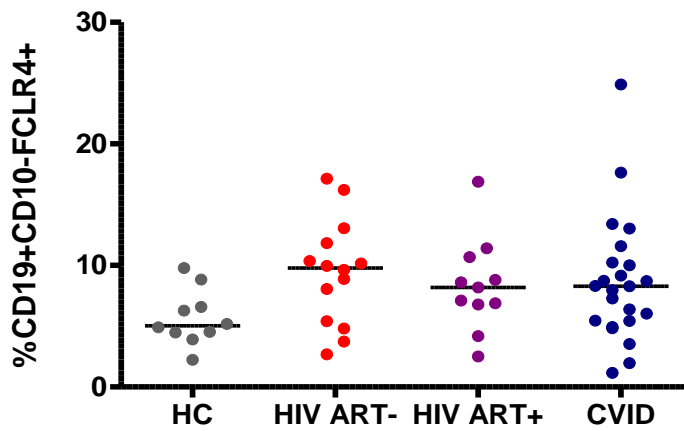


Figure 47: Percentages of mature B cells expressing FCLR4 in healthy controls and disease groups.

Plot displaying data points and horizontal lines indicate median values for FCLR4+ B cells (CD19+CD10-FCLR4+) as a percentage of CD19+CD10- B cells, for healthy controls (HC) (n=11), untreated HIV-1 infected patients (ART-) (n=14), treated HIV-1 infected patients (ART+) (n=11) and CVID patients (n=23). Transitional B cells were excluded by gating for CD19+CD10- B cells. The Mann-Whitney U test was used to analyse non-parametric data.

FCLR4 expression was present on approximately 5% of circulating mature B cells in healthy individuals. Although there was a trend for increased FCLR4 expression on all disease groups, (see Fig.47), this was only significant for the untreated HIV infected cohort (p=0.03). Since 20 to 50% of peripheral blood B cells in healthy controls are memory B cells, it can be seen that FCLR4 expression is limited to a small subset of B cells.

5.5.4 *FCLR4+* B cell populations as a percentage of the mature B cell compartment in HIV-1 infection, CVID and HC

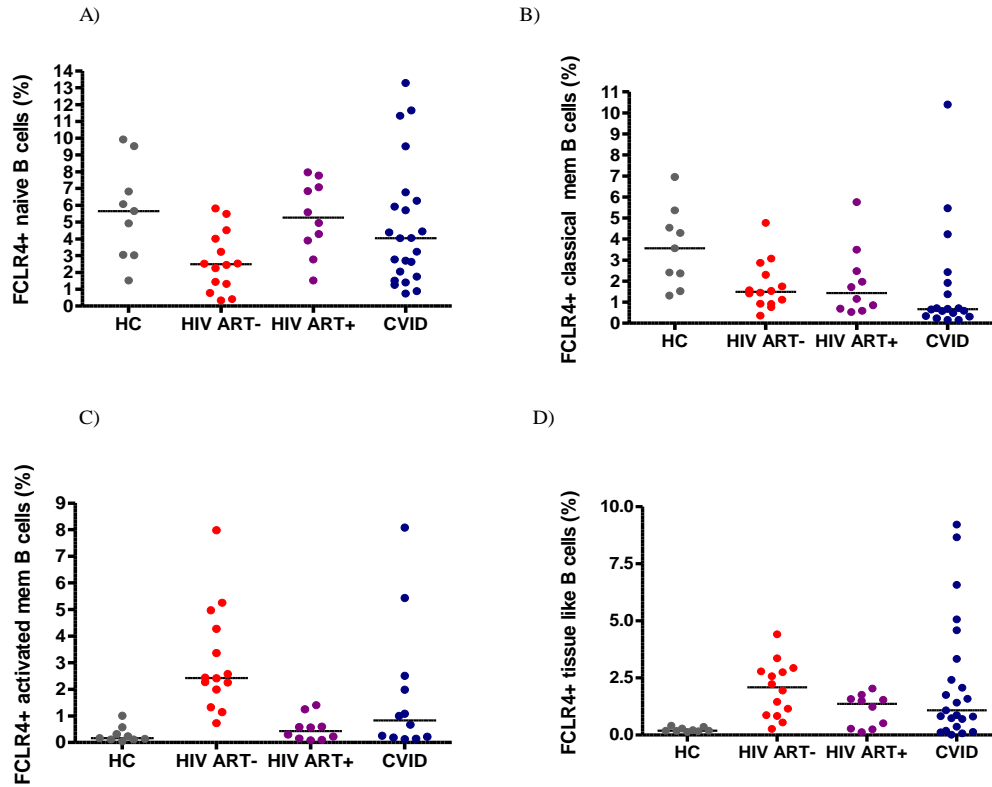


Figure 48: Percentages of FCLR4+ mature B cell subsets in the peripheral B cell compartment of healthy controls and disease groups.

Plots displaying data points and horizontal lines indicate median values for FCLR4+specific B cell subsets as a percentage of CD19+/CD10- B cells, for healthy controls (HC) (n=11), untreated HIV-1 infected patients (HIV ART-) (n=14), treated HIV-1 infected patients (HIV ART+) (n=11) and CVID patients (n=23). 48A) Naïve B cells (CD27- CD21+CD10- B cells) B) Classical memory B cells (CD21+CD10-CD27- B cells). C) Activated memory B cells (CD21-CD10-CD27+ B cells), D) Tissue like B cells (CD21-CD10-CD27- B cells). Transitional B cells were excluded by gating for CD19+CD10- B cells. The Mann-Whitney U test was used to analyse non-parametric data.

From Fig.48A, some naïve B cells of all populations stained positive for FCLR4. Intriguingly, FCLR4+ CD21+ naïve B cells are significantly reduced in patients with untreated HIV infection ($p<0.01$), which then significantly normalises on treatment, ($p<0.01$). Once again, the CVID B cell compartment showed considerable heterogeneity, in some patients, naïve B cells expressing FCLR4 represent >10% of the peripheral B cell compartment, however overall the difference in naïve FCLR4+ B cells was not significant between CVID patients and healthy controls, due to some CVID patients having little or no FCLR4+ B cells. The percentage of classical memory B cells expressing FCLR4 represent about 4% of peripheral B cells in healthy controls (Fig.48B), in comparison with 30-50% CD19+CD27+ B cells, confirming as discussed previously that not all memory B cells are positive for FCLR4. CD21+ classical FCLR4+ memory B cells are reduced in HIV infected individuals naïve to treatment ($p=0.01$), on treatment ($p=0.04$) and also reduced in patients with CVID ($p<0.01$). This may be a consequence of the overall reduction in classical memory B cell subsets in HIV infection and CVID.

Fig.49C demonstrated that FCLR4+ activated (CD21-) memory B cells are also present and significantly expanded in patients with untreated HIV infection ($p<0.001$) in comparison with healthy controls. This population is also expanded in a proportion of patients with CVID ($p=0.04$) and in some HIV infected patients on ART, however, overall this population has normalised in the treated HIV cohort. Expression of FCLR4+ by activated memory B cells was significantly reduced in HIV infected patients on treatment in comparison with untreated HIV infected patients ($p<0.001$). Up to 5% of peripheral B cells of untreated HIV infected patients are tissue like B cells expressing FCLR4 (Fig.48D), this population is also significantly expanded in HIV infected patients on treatment ($p=0.01$). This population is also expanded in patients with CVID ($p=0.01$), in some patients, tissue like FCLR4+ B cells comprise up to 10% of the B cell compartment. FCLR4+CD21-CD10-CD27- B cells represent a very small population of HC B cells (<1%), suggesting this phenotype is aberrantly expanded in HIV and a high proportion of patients with CVID.

5.5.5 Association between FCLR4+ tissue like B cells and memory B cell subsets

Table 12: FCLR4+ Tissue like B cell correlations with memory B cell subsets

<i>FCLR4+ tissue like B cell percentages (% CD19+ B cells CD27-CD10-CD21-FCLR4+)</i>	<i>Correlation with IgM memory B cell (Spearman's rho)</i>	<i>Correlation with total memory B cell</i>	<i>Correlation with CS memory B cell</i>
Healthy Controls (n=11)	-0.100	0.117	-0.050
HIV ART- (n=14)	0.741* p<0.01	0.732* p<0.01	0.196
HIV ART+ (n=11)	0.5	-0.282	0.045

From Table 12, the percentage of FCLR4+ tissue like B cells is strongly correlated to IgM memory B cell and total memory B cell percentages. This correlation was not found in HIV treated patients or HC, only significant scatter plots are shown below.

A)

B)

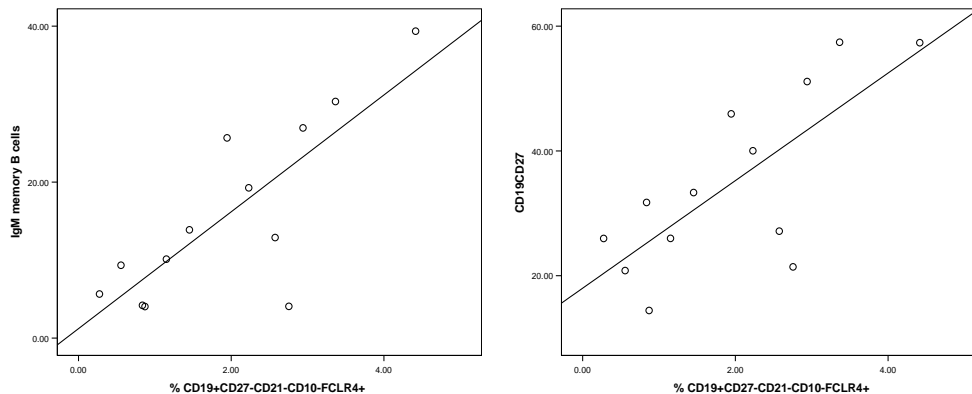


Figure 49: Scatter plots to show correlations between IgM memory B cells or classical memory B cells and tissue like memory B cells expressing FCLR4 in the untreated HIV infected cohort.

Fig.49A) IgM memory B cell percentages vs. FCLR4+ tissue like B cells and 49B) CD19+CD27+ total memory B cell percentages vs. FCLR4+ tissue like B cells in the untreated HIV infected patients (n=14). Data analysed using Spearman's rank correlation coefficient.

The correlation between IgM memory B cells and FCLR4+ tissue like memory B cells in patients with untreated HIV infection was highly significant (p=0.002) (Fig. 49A). From Fig.49B, the correlation between total memory B cells and FCLR4+ tissue like memory B cells in HIV untreated patients was also significant (p<0.007).

5.5.6 Selective expression of FCLR4 by various B cell subsets in HIV infection, CVID and in HC

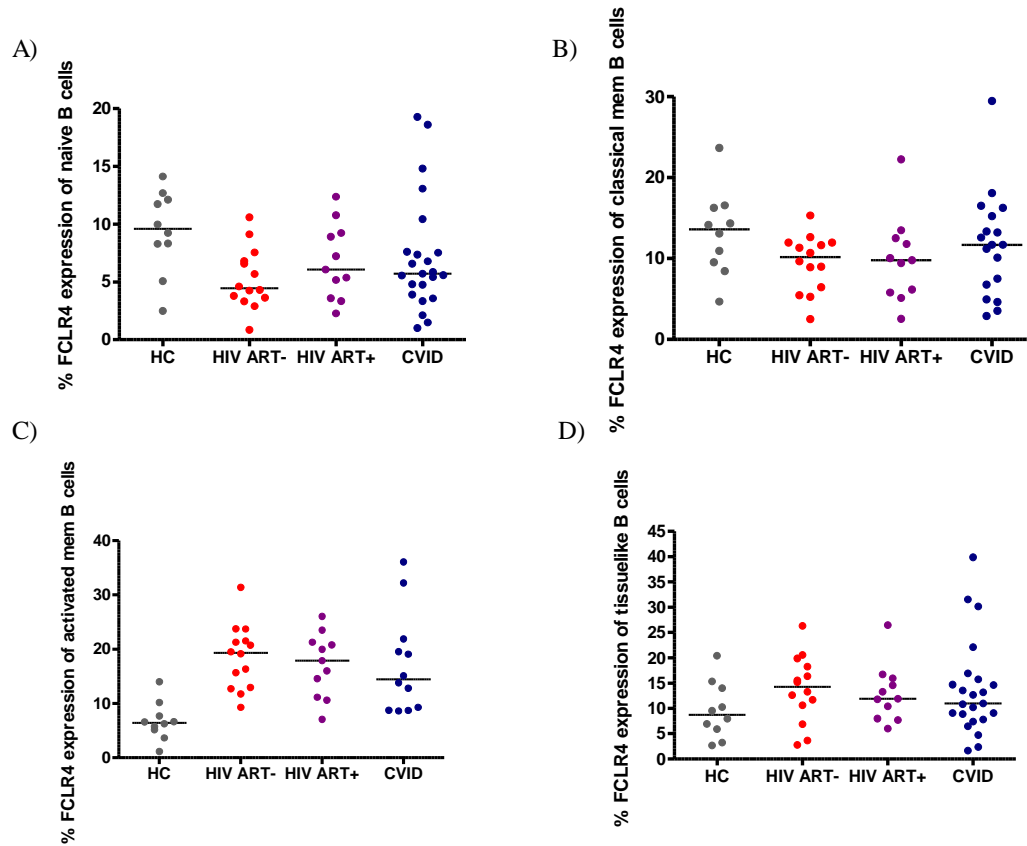


Figure 50: Percentage FCLR4 expression by specific B cell subsets in the peripheral B cell compartment of healthy controls and disease groups.

Plots displaying data points and horizontal lines indicate median values for FCLR4 expression as a percentage of each B cell subset, gated on CD19+ B cells, for healthy controls (HC) (n=11), untreated HIV-1 infected patients (HIV ART-) (n=14), treated HIV-1 infected patients (HIV ART+) (n=11) and CVID patients (n=23). 50A) Naïve B cells (CD27- CD21+CD10- B cells), 50B) Classical memory B cells (CD21+CD10-CD27- B cells), 50C) Activated memory B cells (CD21-CD10-CD27+ B cells) and 50D) Tissue like B cells (CD21-CD10-CD27- B cells). Transitional B cells were excluded by gating for CD19+CD10- B cells. The Mann-Whitney U test was used to analyse non-parametric data.

FCLR4 is believed to be a marker of tissue like B cells. Therefore the proportion of FCLR4 found within each B cell subset was then examined (see Fig. 50). FCLR4 expression by naïve B cells was significantly reduced in the untreated HIV infected patient group compared to healthy controls ($p < 0.01$) (Fig.50A). No statistical difference was found between HC and any disease group regarding percentages of classical memory B cells expressing FCLR4 (Fig.50B). FCLR4+ expression was over-represented in the activated memory B cell compartment in all three disease states compared to healthy controls, suggesting FCLR4+ is significantly upregulated on activated memory B cells in HIV infection ($p < 0.001$) and CVID ($p < 0.01$) (see Fig. 50C). FCLR4 expression was not significantly up regulated by the tissue like B cell subset in HIV infection or CVID, representing 12% of the tissue like B cell subset in HIV infected untreated patients, compared to 10% of the tissue like B cells in the HC group, see Fig.50D. FCLR4+ tissue like memory B cells therefore represent only a small subset of CD27-CD21-CD10- B cells in both HIV infection and in the healthy population. Therefore an increased percentage of FCLR4+tissue like B cells (CD10-CD21-CD27-FCLR4+) in the B cell compartment in HIV infection reported in Fig. 49D, is not a selective expansion of FCLR4+ cells but rather represents an expansion of the whole tissue like B cell subset comprising a proportion of FCLR4+ tissue like memory B cells. FCLR4+ expression of the tissue like B cell subset was considerably heterogeneous in the CVID patient group (see Fig.50D), in some patients, a third of tissue like B cells expressed FCLR4.

5.5.7 Intensity of FCLR4 expression on various B cell subsets

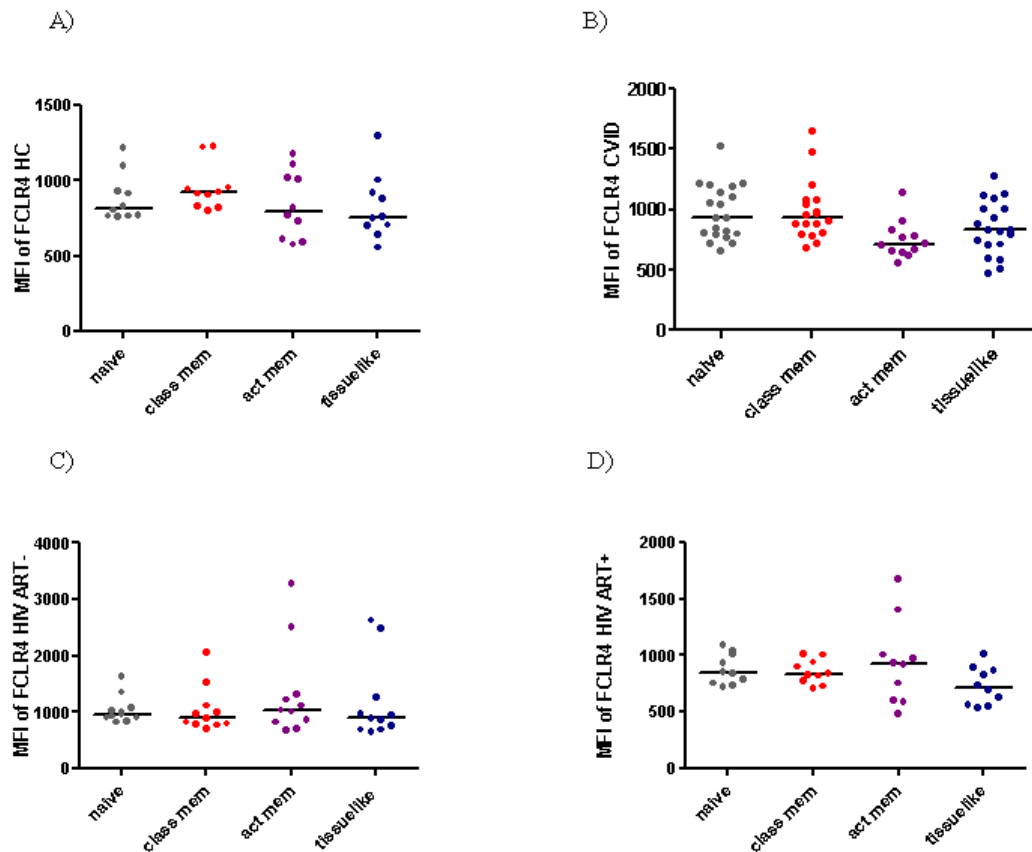


Figure 51: Median Fluorescence Intensity (MFI) of FCLR4+ expressed by B cell subsets in disease groups and healthy controls.

Plots displaying data points and horizontal lines indicate median values for FCLR4+ median fluorescent intensity of specific B cell subsets: naïve B cells, classical memory B cells, activated memory B cells and tissue like B cells. Gated on CD19+ B cells. 51A) healthy controls (HC) (n=10), B) CVID patients (n=20), C) untreated HIV-1 infected patients (HIV ART-) (n=10) and treated HIV-1 infected patients (HIV ART+) (n=10). Transitional B cells were excluded by gating for CD19+CD10- B cells. The Mann-Whitney U test was used to analyse non-parametric data.

From Fig. 51, the mean MFI of FCLR4 was marginally increased in untreated HIV infection, due to a small number of individuals displaying high levels of FCLR4 on all B cell subsets, however this was not statistically significant. This was also true for several individuals with CVID, however overall there was no significant difference between the MFI of HC and CVID. Patients with CVID had reduced FCLR4 on activated memory B cells in comparison with naïve B cells ($p=0.003$), however, few CVID patients had sufficient activated memory B cells to measure statistically and

this may have skewed the results. Overall, FCLR4 MFIs were not significantly different between B cell subsets of each cohort.

5.5.8 *The use of CD11c expression on peripheral blood B cells as an additional marker for tissue like B cells*

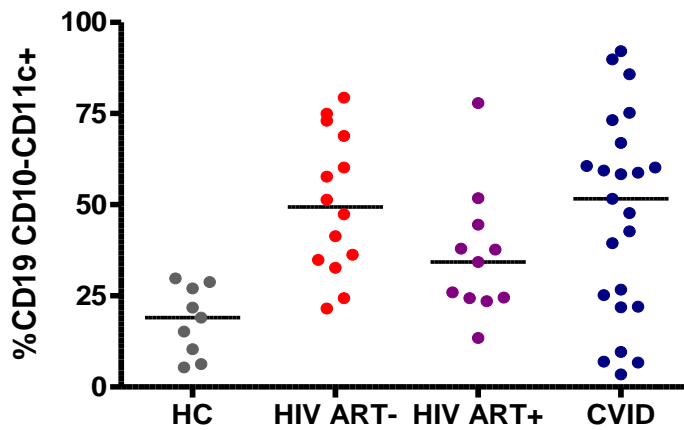


Figure 52: CD11c expression as a percentage of mature peripheral B cells

Plot displaying data points and horizontal lines indicate median values for CD11c+ B cells (CD19+CD10-CD11c+) as a percentage of CD19+CD10- B cells, for healthy controls (HC) (n=11), untreated HIV-1 infected patients (HIV ART-) (n=14), treated HIV-1 infected patients (HIV ART+) (n=11) and CVID patients (n=23). Transitional B cells were excluded by gating for CD19+CD10- B cells. The Mann-Whitney U test was used to analyse non-parametric data.

CD11c expression of B cells is significantly higher in HIV infected patients off treatment in comparison with healthy controls ($p < 0.001$) (see Fig.52). This partially normalises in the HIV ART+ cohort but is still significantly higher than levels in healthy controls ($p < 0.02$). CVID patients displayed a heterogeneous expression of CD11c, whilst overall levels were higher in this cohort of patients ($p = 0.01$), almost 100% of mature B cells expressed CD11c in some individuals with CVID, whilst in other patients the expression of CD11c was extremely low.

5.5.9 *CD11c+ B cell populations as a percentage of the mature B cell compartment in HIV-1, CVID and HCs*

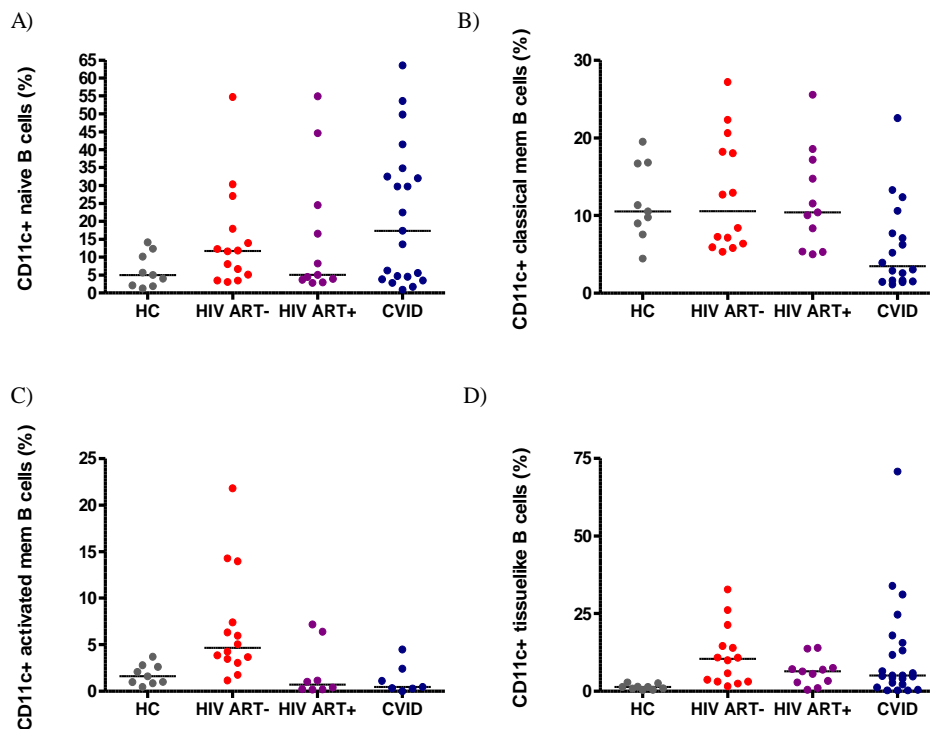


Figure 53: Percentages of CD11c+ mature B cells in the peripheral blood B cell compartment of healthy controls and disease groups.

Plots displaying data points and horizontal lines indicate median values for CD11c+specific B cell subsets as a percentage of CD19+/CD10- B cells, for healthy controls (HC) (n=11), untreated HIV-1 infected patients (HIV ART-) (n=14), treated HIV-1 infected patients (HIV ART+) (n=11) and CVID patients (n=23). 53A) Naïve B cells (CD27- CD21+CD10- B cells) B) Classical memory B cells (CD21+CD10-CD27- B cells). C) Activated memory B cells (CD21-CD10-CD27+ B cells), D) Tissue like B cells (CD21-CD10-CD27- B cells). Transitional B cells were excluded by gating for CD19+CD10- B cells. The Mann-Whitney U test was used to analyse non-parametric data.

Tissue like memory B cells expressing CD11c are not predominant in the blood of healthy controls but are significantly expanded in the blood of patients with untreated HIV infection (11% median) ($p < 0.001$), patients on ART (6%) ($p < 0.01$), and in patients with CVID (11% of B cells) ($p = 0.01$) compared to healthy controls (Fig.53D). The HIV ART- data is consistent with a previous publication (Moir, Ho et al. 2008), here this population is shown to be still elevated in HIV-1 infected patients on ART therapy. In Fig.53A, the percentages of naïve B cells expressing CD11c was

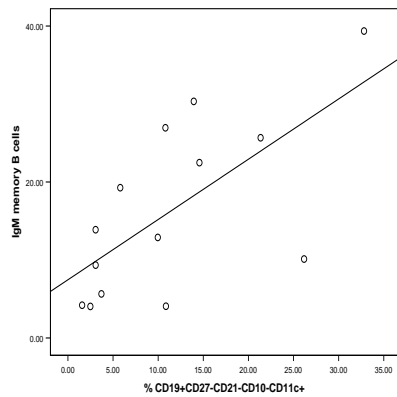
not statistically significant in HIV infected patients or CVID patients ($p=0.06$) in comparison with healthy controls. Patients with CVID had significantly fewer classical memory B cells expressing CD11c ($p<0.01$) but this is likely to be due to an overall reduction in classical memory B cells in these patients (Fig.53B). No differences in the percentages of CD11c+classical memory B cells were found between HIV infected individuals and healthy controls. Interestingly, the percentage of CD11c activated memory B cells was increased in untreated HIV infected patients ($p<0.001$) but not in the ART treated cohort (Fig.53C). The difference between percentages of CD11c activated memory B cells normalised with ART therapy, demonstrated by a significant difference between the HIV infected groups ($p=0.02$). To investigate our hypothesis that percentages of the tissue like subset are associated with loss of the IgM memory B cell subset seen in HIV infection, correlations between percentages of each subset were examined statistically.

Table 13: CD11c+ Tissue like B cell correlations with memory B cell subsets

<i>CD11c+ tissue like B cell percentages (% CD19+ B cells CD27-CD10-CD21-CD11c+)</i>	<i>Correlation with IgM memory B cell (Spearman's rho)</i>	<i>Correlation with total memory B cell</i>	<i>Correlation with CS memory B cell</i>
Healthy Controls (n=11)	-0.400	-0.250	-0.250
HIV ART- (n=14)	0.635**($p= 0.01$)	0.512	-0.042
HIV ART+ (n=11)	-0.382	-0.100	0.200

The percentage of CD11c+ tissue like B cells is strongly correlated to IgM memory B cell and total memory B cell percentages in untreated HIV infection (see Table 13). This correlation was not found in HIV treated patients or HC, significant scatter plots are shown over.

A)



B)

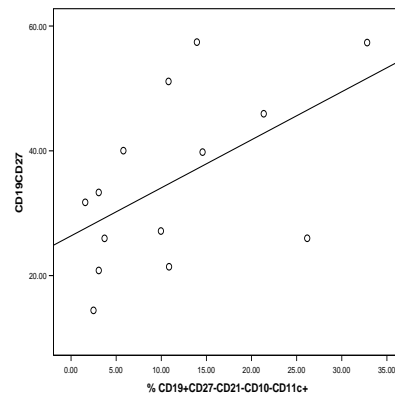


Figure 54: Scatter plots to show correlations between IgM memory B cells or classical memory B cells and tissue like B cells expressing CD11c in the untreated HIV infected cohort (n=14).

54A) IgM memory B cell percentages vs. CD11c+ tissue like B cells and 54B) CD19+ CD27+ total memory B cell percentages vs. CD11c+ tissue like B cells. Data analysed using Spearman's rank correlation coefficient.

As seen in Fig.54 above and as found in the first part of this chapter using the Warnatz gating strategy (Rakhmanov, Keller et al. 2009), loss of the IgM subset is not directly correlated with an increase of tissue like B cells in HIV infected subjects naïve to ART. In fact there is a positive correlation between tissue like B cells and IgM memory B cells ($p < 0.02$), as shown in Table 13. Therefore, decreased levels of the IgM memory B cell subset correlates with decreased levels of tissue like B cells in this group. The correlation between total memory B cells and CD11c+ tissue like memory B cells in HIV infection narrowly missed significance ($p = 0.06$).

5.5.10 Expression of CD11c on mature B cell subsets

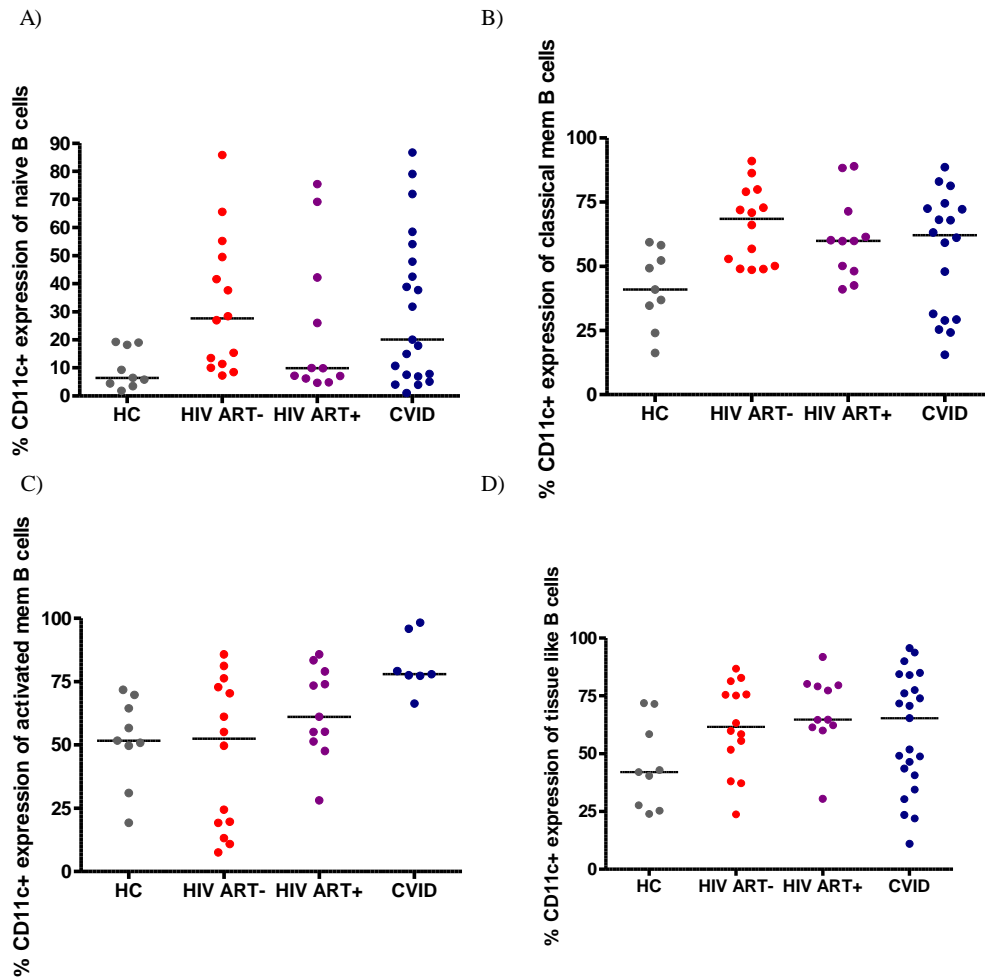


Figure 55: Percentage expression of CD11c by mature B cells in the peripheral blood B cell compartment of healthy controls and disease groups.

Plots displaying data points and horizontal lines indicate median values for CD11c expression as a percentage of each B cell subsets, gated on CD19+ B cells, for healthy controls (HC) (n=11), untreated HIV-1 infected patients (HIV ART-) (n=14), treated HIV-1 infected patients (HIV ART+) (n=11) and CVID patients (n=23) on: 55A) naïve B cells (CD27- CD21+CD10- B cells), B) Classical memory B cells (CD21+CD10-CD27- B cells). C) Activated memory B cells (CD21-CD10-CD27+ B cells). D) Tissue like B cells (CD21-CD10-CD27- B cells). Transitional B cells were excluded by gating for CD19+CD10- B cells. The Mann-Whitney U test was used to analyse non-parametric data.

From Fig.55A, it can be seen that CD11c expression is increased on naïve B cells of untreated HIV infected patients ($P < 0.001$) and CVID patients ($p = 0.046$) in

comparison with the naïve B cells of healthy controls tested. Fig.55B demonstrates CD11c expression was also significantly increased on classical memory (CD10-) B cells of HIV patients on ART+ ($p<0.001$) or off ART- ($p<0.001$) in comparison with healthy controls. The expression of CD11c on 40% of non-activated and activated memory B cells of healthy controls, suggested that CD11c is a memory marker. However, naïve B cells of all disease states showed a high expression of CD11c, suggesting on naïve B cells this could be a marker of immune activation. Activated memory B cells of CVID patients showed a significant upregulation of CD11c expression however, few patients with CVID had activated memory B cells (Fig.55C). Levels of CD11c were raised on tissue like B cells of ART+ treated HIV infected individuals ($p<0.01$) but not ART- HIV infected individuals ($p=0.06$) or CVID patients in comparison with healthy controls (Fig.55D). CD11c expression showed great heterogeneity, even in healthy controls. Therefore, at least 25% of HC tissue like B cells do not express CD11c. CD11c is not specific to tissue like memory B cells.

5.5.11 *CD11c is expressed at a higher intensity on CD21^{low} tissue like and CD21^{low} activated B cells than classical memory or naive B cells*

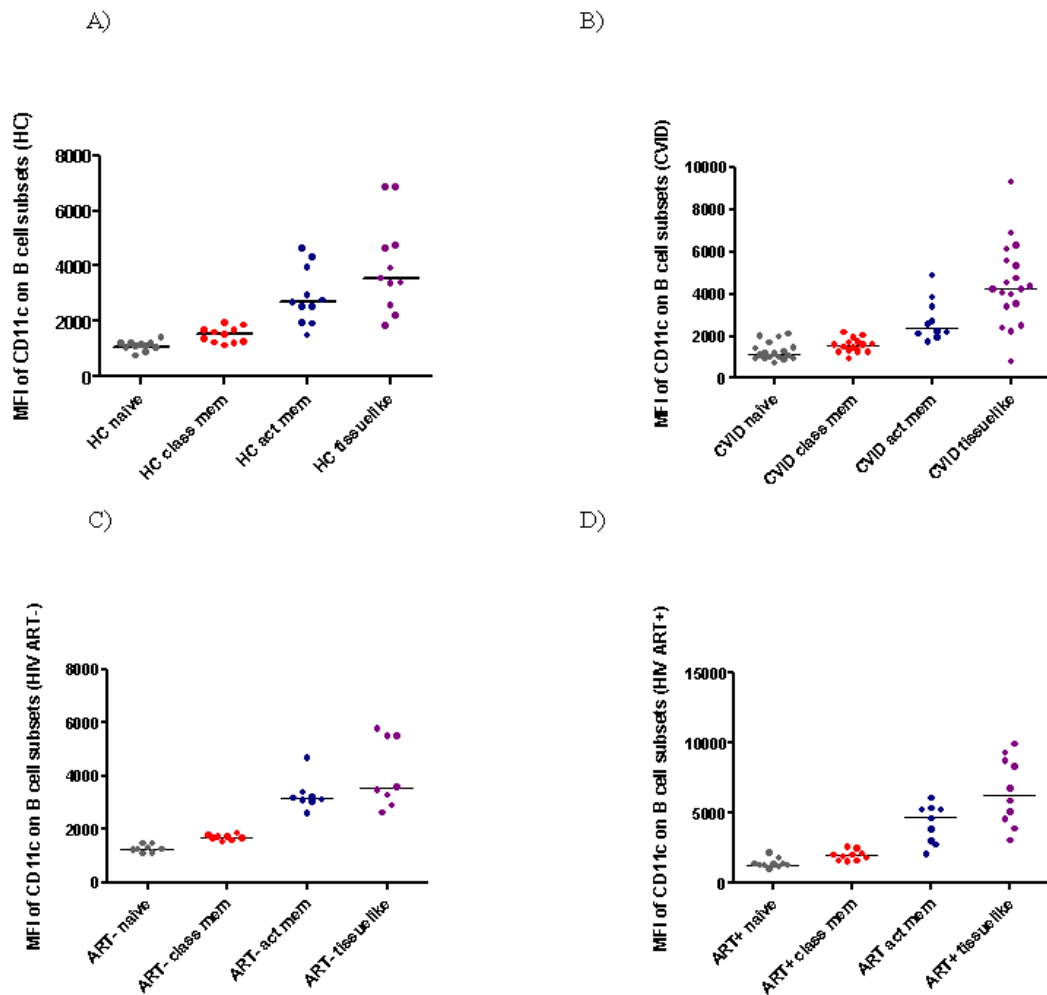


Figure 56: Mean Fluorescence Intensity (MFI) of CD11c+ expressed by B cell subsets in disease groups and healthy controls

Plots displaying data points and horizontal lines indicate median values for CD11c+ median fluorescent intensity of specific B cell subsets: naïve B cells, classical memory B cells, activated memory B cells and tissue like B cells. Gated on CD19+ B cells. 56A) healthy controls (HC) (n=10), B) CVID patients (n=20), C) untreated HIV-1 infected patients (HIV ART-) (n=10) and treated HIV-1 infected patients (HIV ART+) (n=10). Transitional B cells were excluded by gating for CD19+CD10- B cells. The Mann-Whitney U test was used to analyse non-parametric data.

The MFI of CD11c staining was investigated since tissue like B cells are reported to strongly express CD11c. This can be seen in Fig. 56, CD11c is expressed more highly on tissue like B cells and activated memory B cells than classical memory or naive B cells and therefore expression of CD11c on tissue like or activated memory B cells can be denoted as CD11c^{hi} (see Fig.56). This is true for healthy controls, CVID and untreated and treated HIV patients. Tissue like B cells from HIV infected patients on treatment showed a significant trend of higher CD11c expression than HC or untreated HIV patients (both p=0.03). Tissue like B cells from patients with CVID expressed CD11c at a higher intensity than tissue like B cells from HC tested, see Fig.56, however this was not statistically significant. Activated memory B cells expressed CD11c at a lower level than tissue like B cells in CVID but again, there was a wide range of CD11c intensity in activated memory B cells in CVID.

5.5.12 Summary Tables of Results from Study One and Study Two

Table 14: Expansions of tissue like B cells in HIV and CVID

<i>Tissue like B cell gating strategy</i>	<i>HIV ART-</i>	<i>HIV ART+</i>	<i>CVID</i>
CD21-CD27-	+++	+++	++
CD38-CD21-	+++	+/-	++
CD27-CD10-CD21-	+++ p=0.001	+/- p=0.06	++ p=0.01
CD27-CD10-CD21- CD11c	+++ p<0.001	++ p<0.01	++ p=0.01
CD27-CD10-CD21- FCLR4	+++ p<0.001	++ p<0.01	++ p<0.01

Legend: Data analysed using Mann Whitney U test.

+++ *Highly significant expansion compared to HC (p≤0.001)*

++ *Significant expansion compared to HC (p≤0.01)*

+/- *Expansion seen in a subset of patients (overall ns)*

Table 15: Associations between memory B cell subsets and tissue like B cells in the untreated HIV infected cohort

<i>Tissue like B cell markers in untreated HIV infection (%CD19+ B cells)</i>	<i>Correlation with IgM memory B cell (Spearman's rho)</i>	<i>Correlation with total memory B cell</i>	<i>Correlation with CS memory B cell</i>
CD21-CD27- (n=28)	0.251	0.394* p=0.04	0.429* p=0.02
CD38-CD21- (n=27)	0.563** p=0.002	0.628** p<0.001	0.528* p<0.01
CD27-CD10-CD21- (n=14)	0.634* p<0.05	0.516 (p=0.06)	0.086
CD27-CD10-CD21-CD11c (n=14)	0.635** p<0.02	0.512* p<0.05	-0.042
CD27-CD10-CD21-FCLR4 (n=14)	0.741** p=0.002	0.732** p=0.002	0.196

See Appendix for tabular associations between memory B cell subsets and tissue like B cells for the other patient groups.

5.6 Discussion

To summarise the results in this chapter, the presence of tissue like B cells has been confirmed in healthy controls, HIV patients on and off ART treatment and in patients with CVID. Staining of CD11c and FCLR4 identified potential subsets of tissue like B cells but were not specific to tissue like B cell populations. In the untreated HIV-1 infected cohort, levels of tissue like B cells were significantly associated with percentages of IgM memory B cells but not to any marker of T cell disease progression such as viral load or CD4 T cell count, suggesting an intrinsic B cell defect. Finally, increased levels of tissue like B cells indicated a better response to tetanus toxoid vaccination in HIV infected patients on treatment.

5.6.1 Tissue like B cell numbers are elevated in HIV infection and CVID using any combination of tissue like B cell markers.

From the Functional Immunity study, reanalysed data of CD38loCD21lo tissue like B cells determined that these cells are found in low numbers (<5%) in peripheral blood of healthy controls. A negative correlation between CD38loCD21lo tissue like B cells and proportions of IgD antigen naïve mature B cells was seen. In HIV infection, the proportions of CD38loCD21lo peripheral blood B cells is increased and is most striking in the viraemic patients at a median percentage of ~15% and at a factor of 1:2 to classical memory B cells. However, this proportion does not completely normalise after ART treatment to healthy control levels.

5.6.2 Correlations between IgM memory B cells and tissue like B cells in untreated HIV infection

Four different gating strategies in two cohorts of untreated HIV infected patients confirm the significant association between loss of IgM memory B cells and loss of tissue like memory B cells in untreated HIV infection, which has not been previously reported. The peripheral memory B cell compartment of untreated HIV infected individuals is heterogeneous, as reported by ourselves and others. We previously reported a loss of IgM memory B cells in untreated HIV infection, which was not restored by ART therapy. A number of other publications confirmed our findings (D'Orsogna, Krueger et al. 2007). However, the cohort of patients studied in this chapter were likely to be patients with primary/early HIV infection since overall IgM

memory B cells were not reduced and CD4 counts were higher overall, possibly due to patients commencing ART early in line with the current BHIVA treatment guidelines. There was still considerable heterogeneity in percentages of B cell memory: some had reduced IgM memory and others expanded IgM memory. Yet levels of tissue like B cells correlated with levels of IgM memory. It may be possible that in early HIV infection, IgM memory B cells and tissue like B cells are expanded, to be lost simultaneously during a later stage of disease. Unfortunately, due to lack of information regarding time since transmission and no functional studies, it is impossible with the small cohort studied here to confirm this. In treated HIV infected individuals, despite the CD38^{lo}CD21^{lo} analysis showing no significant difference in the median population between treated patients and healthy controls, there was still a small expanded population of CD38^{lo}CD21^{lo} B cells in a proportion of patients, contrary to the Moir study (Moir, Ho et al. 2008) (2008) but in agreement with a recent paper examining CD27⁻ IgG⁺ memory B cells (Cagigi, Du et al. 2009). This may be due to sample size, whilst we studied 55 HIV ART treated patients, the Moir study presented data only on 12 individuals.

5.6.3 The expansion of CD11c⁺ or FCLR4⁺ tissue like B cells is demonstrated in a subset of HIV infected patients on treatment

Using additional markers of tissue like B cells (CD11c, CD10 and FCLR4), this study has confirmed this population is expanded in untreated HIV infection and in addition, has demonstrated that this B cell population is still expanded in some patients with treated HIV infection, which has not been previously reported to our knowledge. An additional gating phenotype used to identify tissue like B cells (CD38^{lo}CD21^{lo} B cells) showed a trend of expanded tissue like B cells in patients on ART: large expansions were seen in some HIV infected patients on treatment. Whilst it has been demonstrated here that CD11c and FCLR4 are not specific to tissue like B cells, in combination with CD27⁻CD21⁻CD10⁻ markers, these label a cell population expanded in HIV and CVID disease and almost not seen in healthy controls. Interestingly, in the untreated HIV infected cohort, of an average total of 15% CD27⁻CD21⁻CD10⁻ tissue like B cells, 11% express CD11c and 2.5% express FCLR4 on average. Since these antigens were tested in different discrete combinations of antibodies, it is unclear whether some of these cells express both CD11c and FCLR4 or whether these are discrete subsets of tissue like B cells.

5.6.4 *The use of FCLR4 as a marker for tissue like memory B cells*

In this study, staining of B cells with anti-FCLR4 produced less positive staining of B cells than expected from the one comparable study in the literature (Moir, Ho et al. 2008). Due to differences in populations stained and gating anomalies, the data from the Ehrhardt study (2005) cannot be closely compared (Ehrhardt, Hsu et al. 2005). However, in the Moir study, gated similarly, up to 25% of B cells in HIV viraemics and 60% of the tissue like B cells expressed FCLR4 (Moir, Ho et al. 2008), whilst here, the median value for FCLR4 positivity was 10% for total CD10⁻ mature B cells and 15% of tissue like B cells expressing FCLR4 in the HIV untreated patients. This difference may be due to different monoclonal antibodies used in the two studies: Moir *et al* had obtained FCLR4 monoclonal antibody personally from the group of Ehrhardt, whilst we purchased a commercially available antibody from BioLegend (Moir, Ho et al. 2008). However, this is unlikely, because the data obtained from healthy controls was comparable between the two studies, ~5% of total mature CD10⁻ B cells were positive for FCLR4 in this study and the Moir work, suggesting differences are unlikely to be entirely due to antibodies. For the HIV treated group, we found an increase of FCLR4 expression to ~10% although this was not statistically significant. This is in discordance with the study by Moir *et al*, who did not find any increase at all in FCLR4 expression in this group (Moir, Ho et al. 2008). Due to the concordance between studies of FCLR4 in the peripheral B cells of healthy controls, the differences in FCLR4 expression may be more likely caused by differences in patient cohorts, especially time since diagnosis and disease progression. Both this study and the study by Moir *et al* examined a similar number of HIV naïve patients (~15), it is possible that with much larger cohorts of patients, any differences in patient characteristics such as disease progression and time since diagnosis would have been reduced. The Moir *et al* paper does not provide the CD4 count information of the patients used, so this cannot be compared. Viral load results are given for 40 patients (median 18,606), in the Moir study, however only 15 patients are used in the B cell phenotyping studies (Moir, Ho et al. 2008). In the untreated HIV infected cohort measured in this chapter, the previously unreported correlation between the percentage of IgM memory B cells and tissue like B cells expressing FCLR4 was very striking and stronger than the correlation between total CD27⁺CD19⁺ memory B cells and FCLR4.

5.6.5 *FCLR4 expression on activated and classical memory B cell subsets*

We also found that FCLR4 is upregulated on activated memory B cells in untreated HIV infection and CVID (both the percentages of FCLR4+ CD10-CD27+CD21- and the percentages of activated memory B cells expressing FCLR4 are increased in untreated HIV infection and CVID), however, slightly down regulated on classical memory and naïve B cells in comparison with healthy controls. In our cohort of HIV infected untreated patients, the number of peripheral FCLR4+ activated memory B cells was higher than the number of peripheral FCLR4+ tissue like memory B cells (3% vs. 2.5%). The upregulation of FCLR4 on activated memory B cells is in agreement with Moir *et al*, who also report that plasmablasts also seem to express high levels of FCLR4 (Moir, Ho et al. 2008). There is no information given as to why FCLR4 is upregulated on activated memory B cells, possibly as a negative regulator to help inhibit activation. Therefore, measurement of total FCLR4 expression is unhelpful in HIV infection since this marker is down regulated on some subsets and upregulated on others. It is more useful as an additional marker of a subset of CD27-CD21-CD10- tissue like B cells.

5.6.6 *The use of CD11c as a marker for tissue like memory B cells*

CD11c, a tissue homing marker, was highly upregulated in HIV infection and CVID on all B cell subsets, although at an increased intensity on both activated and tissue like memory B cells in all disease cohorts and healthy controls. Upregulation of CD11c on tissue like B cells has been reported in HIV infection, however, that study did not measure CD11c expression on activated memory B cells. Presence of high levels of CD11c on activated memory B cells however, meant that gating for high intensity CD11c expression is not specific enough to label for tissue like memory B cells without CD27, CD10 and CD21, since activated memory B cells also express CD21 dimly. CD11c does stain a higher percentage of CD27-CD10-CD21- cells than FCLR4 expression and using CD11c made staining for tissue like memory B cells more specific than CD27, CD21 and CD10- alone.

5.6.7 Correlations between markers of disease progression in untreated HIV infected patients

In untreated HIV infection, using any combination of markers, numbers of tissue like memory B cells are expanded. However, individuals were found to be extremely heterogeneous as to the extent of the B cell compartment affected, with up to 40% of peripheral B cells bearing tissue like B cell markers in some patients. No correlation was found with any combination of tissue like B cells markers and CD4 counts or viral load using CD27-CD21-CD10 staining. This was confirmed using data reanalysed from the Functional Immunity study (study one): proportions of CD38^{lo}CD21^{lo}/CD27^{lo}CD21^{lo} tissue like B cells were not associated with viral load (in untreated HIV infection), time on ART (treated patients), CD4 T cell counts or B cell counts in the healthy control or patient group, but to the proportions of naïve B cells (negative correlation) and memory B cells (positive correlation). Since the expansion of tissue like B cells is not linked to markers of T cell disease progression nor time, this points towards intrinsic B cell activation occurring in HIV infection at an unknown time point. No study in HIV infection measuring CD21^{low} B cells has also evaluated corresponding CD4 or B cell counts.

5.6.8 High levels of tissue like B cells may predict a good response to vaccination with protein antigens in HIV infected patients on ART

The data that suggests that the presence of a tissue like B cell (CD38^{lo}CD21^{lo}) subset is associated with a good antibody vaccination response to tetanus toxoid, yet had no bearing on pneumococcal antibody responses is intriguing. This trend is supported by a previous study, which reported that IgG antibodies to tetanus toxoid were produced in vitro by both CD27⁺ and CD27⁻ B cells, whilst pneumococcal polysaccharide antibodies were produced only by CD27⁺ B cells (Wirhth and Lanzavecchia 2005). Response to tetanus toxoid requires T cell dependent humoral immunity, whilst the response to pneumococcus encapsulated bacteria is T cell independent. HIV responses to protein components such as gp120 are expected to be predominantly T cell dependent (Chirmule, Oyaizu et al. 1992). This data may give weight to evidence that certain HIV IgG responses are enriched in the tissue like memory B cell compartment but does not support that these cells are exhausted in treated HIV infection. HIV treatment is known to reduce immune activation and viral load, possibly without these confounders, these tissue like memory cells are able to provide a functional immune

response. It may also suggest that these patients that made a good response also have high levels of switched memory B cells which are known to be associated with a good response to tetanus toxoid vaccination.

5.6.9 *Tissue like B cells in CVID*

This study has reported that CD21^{low} B cells in CVID are CD19⁺CD21⁻CD27⁻CD10⁻, a phenotype reported in an exhausted tissue like B cell population reported in HIV infection. Greater than 20% CD21^{low} B cells have long been regarded as a separate subset of CVID patients associated with splenomegaly and granulomatous disease (Warnatz, Wehr et al. 2002). Reports of an innate CD21^{low} B cell population in CVID was published whilst my data was being analysed (Rakhmanov, Keller et al. 2009; Isnardi, Ng et al.). In this current study, both published phenotypes of tissue like memory B cells were examined in patients with CVID (CD19⁺CD38⁻CD21⁻ B cells, as shown previously (Rakhmanov, Keller et al. 2009) or CD19⁺CD27⁻CD10⁻CD21⁻ B cells, demonstrating these subsets are significantly increased in patients with CVID. In this cohort of CVID patients, a proportion of the cohort had greater than 10% of tissue like B cells, with a median value of 12.5%. However, levels of tissue like B cells was extremely heterogeneous in patients with CVID and may reflect the numerous (and some undefined) underlying molecular B cell defects causing disease. This was consistent with previous results and shows the benefit of stratifying patients according to tissue like B cell percentage. Since some healthy controls had up to 7% peripheral tissue like B cells, the EURO Class strategy of stratifying patients to >10% (present tissue like B cells) or <10% tissue like B cells (absent tissue like B cells) seemed appropriate. In addition, some of these tissue like B cells stained positive for CD11c or FCLR4, consistent with the Rakhmanov paper (Rakhmanov, Keller et al. 2009). However, whilst CD11c was expressed by approximately 70% of tissue like B cells, a maximum of 10% of B cells were positive for FCLR4. CD11c was overexpressed on all B cell subsets of many of the CVID cohort, however, high MFI intensity of CD11c expression was limited to tissue like and activated memory B cells.

The tissue like B cell population expanded in CVID and RA is reported in two recent studies to be a polyclonal, somatically unmutated, IgM⁺D^{+/-} non isotype switched population with poor proliferative capacity and increased levels of apoptosis. In

addition, CD21^{low} tissue like B cells in CVID and RA have been found to express germ line autoreactive antibodies and were unresponsive to BCR/CD40 triggering (unable to calcium flux). Rakmanov *et al* termed these tissue like B cells an innate like B cell population, since these cells had many similarities with murine B1 cells, however, these cells did produce more IgM on stimulation than naïve B cells of healthy individuals (Rakhmanov, Keller *et al.* 2009). In contrast, Isnardi *et al* proposed that tissue like B cells are instead anergic B cells (Isnardi, Ng *et al.*). These papers attribute very similar functional and phenotypic features to either innate like B cells or an anergic state and it is likely they are describing the same cell. Rakmanov *et al* did suggest that the CD21^{low} tissue like B cells in CVID/RA do display some features of anergy, however, he stated that the ability of these cells to secrete IgM antibody after stimulation, high expression of IgM and CD86 argues against the classic profile of anergy as described by Campier *et al* (Cambier, Gauld *et al.* 2007). Isnardi *et al* did not measure antibody secretion after stimulation and so an antibody secretory function cannot be excluded (Isnardi, Ng *et al.* 2010). FCLR4⁺ B cells in CVID were reported to be predominantly IgG or IgA expressing, (Rakhmanov, Keller *et al.* 2009) in contrast to another study, which determined CD21^{low} B cells of CVID and RA are positive for IgM/D but that CD21^{low} B cells of healthy controls are mainly class switched (Isnardi, Ng *et al.* 2010).

5.6.10 Limitations of study

This study was most limited by the small size of the HIV infected cohorts. The HIV infected treated group was extremely small (n=11), whilst the HIV infected untreated group needed enlarging and stratifying into two groups. More patients with progressive disease need to be tested, a lack of this potential subgroup was due to difficulties in recruiting patients with progressive disease that were not on treatment. In addition, staining with FCLR4 and CD11c found these markers not specific to tissue like B cells and therefore, multiple antibody staining was required.

5.7 Conclusions

In this chapter, levels of the tissue like B cells reported to be expanded in HIV viraemic individuals were shown to be expanded overall in untreated HIV infection, some patients with HIV and a subset of patients with CVID. Memory B cells could be expanded as part of the B cell immune response against HIV infection e.g. by B cell growth factors and may reflect an early dysfunctional viral specific response to HIV infection. Although the HIV uninfected cohort had a higher level of IgM memory B cells than expected, the loss of these cells in some patients paralleled a loss of the expanded tissue like B cell subset, which has not previously been reported and may have important implications for humoral immunity in HIV infection. Levels of tissue like B cells did not correlate with T cell counts or viral load, suggesting expansion or loss was T cell independent.

In CVID, both published phenotypes of tissue like memory B cells were found to be expanded in patients with CVID. Expanded tissue like B cells are thought to be innate non-switched B cells and therefore, in some ways functionally quite different from the 'exhausted' tissue like memory B cells seen in HIV infection and at low levels in the peripheral blood of healthy controls. However, the reported functional defect/anergy of tissue like B cells in CVID may be due to the underlying genetic defect causing CVID. Certain mutations may affect maturation of the tissue like B cell subset in ways discrete from the maturation of classical memory B cells, which are often almost absent in CVID. An alternative hypothesis is that B cell growth factors meant to stimulate the development of B cell memory subsets are instead stimulating only functionally inadequate tissue like B cells.

6. Chapter VI: Further assessment of dysregulation of B cell subsets and loss of memory B cells in immune deficiency: evaluating the BAFF/APRIL chemokine axis and the role of follicular helper T cells

6.1 Introduction

Studies in Chapter III have shown there is a failure in B cell differentiation in patients with HIV-1 infection and some patients with CVID, leading to an expansion of a heterogeneous immature B cell subset called transitional B cells and a reduction in mature B cell memory cells. The maturation of peripheral B cells and their progression to mature memory B cells is regulated by two proteins called B cell activating factor (BAFF), and A Proliferating-Inducing Ligand (APRIL) (Schneider 2005; Tangye, Bryant et al. 2006). Reduced or absent protein expression within the BAFF/APRIL signalling network has been implicated in impaired humoral immune responses in patients with primary antibody deficiency (Salzer, Chapel et al. 2005; Rizzi 2007; Warnatz, Salzer et al. 2009) and to disease progression in HIV-1 infected patients (Rodriguez, Valdez et al. 2003; Moir, Malaspina et al. 2004).

6.1.1 Function of BAFF and APRIL

BAFF otherwise known as BlyS or TNFSF13B and APRIL or TNFSF13, are two closely related members of the tumour necrosis factor (TNF) family. These are homotrimeric type II transmembrane proteins necessary for the growth and survival of peripheral blood B cells. BAFF and APRIL are primarily produced by cells of myeloid origin such as macrophages, neutrophils and dendritic cells and production can be upregulated by LPS, G-CSF, type 1 interferons, or CD40L stimulation.

BAFF and APRIL have numerous roles throughout B cell development depending on whether the appropriate receptor is expressed on the B cell. BAFF and APRIL regulate the maturation of transitional B cells and their progression to mature memory B cells, as demonstrated by murine studies and humans with primary immune

deficiency (Mackay and Schneider 2009). These chemokines have important roles in B cell survival, BAFF and APRIL are able to protect mature B cells from apoptosis by modulating anti-apoptotic proteins such as Bcl2 and decreasing pro-apoptotic proteins such as Bax. BAFF and APRIL are able to induce CSR in antigen naïve B cells in a CD40 T cell independent fashion in association with other cytokines, predominantly through their receptor TACI (Castigli, Wilson et al. 2005). BAFF is also able to bind to BAFF-R to induce CSR. Whilst receptors such as BAFF-R and BCMA are receptors for Ig isotype switching to IgG or IgE, it is thought that switching to IgA is mediated entirely through TACI.

6.1.2 Murine models of BAFF and APRIL dysfunction

Mice functionally deficient in BAFF (BAFF^{-/-}) or APRIL (APRIL^{-/-}) demonstrate the important roles of these chemokines in B cell development and maturation. Functional BAFF^{-/-} transgenic mice (using soluble over expression of TACI or BCMA) have elevated transitional T1 B cells but few T2 or mature B cells in the periphery (Schneider, Takatsuka et al. 2001 {Gross, 2001 #365}). Subsequently, these animals have reduced serum immunoglobulin levels and impaired T cell-dependent and T cell-independent immune responses (Gross, Dillon et al. 2001). Autoimmunity develops as BAFF^{-/-} mice age. A small population of mature CD21^{low}CD23⁻IgM⁺IgD⁺ B cells survives in the spleen in the absence of B cell activating factor (BAFF) in BAFF^{-/-} mice, indicating some redundancy in the B cell chemokine axis (Gorelik, Cutler et al. 2004). B cells of APRIL^{-/-} mice display impaired class switching to IgA and so reduced serum IgA levels are present (Castigli, Scott et al. 2004). Bone marrow tetanus toxoid-specific plasma cells seem to have a decreased rate of survival in APRIL^{-/-} mice, however normal survival of other long-lived plasma cells is reported, indeed effector memory T cells (CD44^{hi}CD62^{low}) are observed at an increased percentage (Mackay and Schneider 2009).

6.1.3 BAFF and APRIL in human pathology

Elevated plasma levels of the B cell growth factors BAFF/APRIL and TACI have been reported recently in COVID patients, however the relevance of this finding has been unknown [24]. These chemokine levels did not correlate with circulating B cell counts, naïve or memory B cell percentages which are of variable percentages in

CVID. However, in light of recent developments in the identification of specific B cell memory subsets and tissue like B cells, this finding needs to be re-examined. One publication in untreated HIV-infection found high levels of BAFF secretion which was associated with reduced CD19⁺ CD27⁺ memory B cells [25] and another linked BAFF secretion with disease progression [26]. Plasma levels of APRIL had not been previously determined in HIV infection. Whether these chemokines are able to affect B cell homeostasis is yet to be fully defined, however, possible increased production of BAFF may be a mechanism for increased bone marrow output of transitional B cells or increased survival of activated B cells.

BAFF and APRIL promote B cell growth and survival, so unsurprisingly elevated levels of these chemokines have been found in patients with human B cell lymphomas: Non-Hodgkin's Lymphoma (NHL) (He, Chadburn et al. 2004), Multiple Myeloma (MM), Waldenstrom's Macroglobulinaemia (WM), Chronic Lymphocytic Leukaemia (CLL) (Tangye, Bryant et al. 2006). B cell lymphomas express appropriate chemokine receptors to respond to BAFF and APRIL, proliferate and are able to produce BAFF (He, Chadburn et al. 2004). HIV infection is associated with an increased risk of lymphoma 60 to 160 times greater than uninfected individuals, especially B cell NHL (Gucalp and Noy 2010) and Castleman's disease. CVID patients are also at increased prevalence of B cell lymphomas, it is possible that elevated levels of B cell growth factors may be a risk factor for the development of malignancy. Viruses are capable of inducing chemokine secretion by B cells themselves: EBV infected B cells are able to produce BAFF and APRIL [23] and induce CSR through Latent Membrane Protein-1 (LMP-1), a CD40-like EBV protein. Elevated BAFF levels are reported in patients with autoimmunity, in which autoreactive B cells have been able to survive tolerance (SLE, Sjogren's syndrome, RA) (Daridon, Burmester et al. 2009). Some non-haematopoietic cells in autoimmune states may be able to produce BAFF and APRIL e.g. synovial cells in the synovium of patients with RA (Daridon, Burmester et al. 2009).

6.1.4 IL-7

IL-7 is a pleiotropic cytokine of the gamma chain family that plays a crucial role in CD4 T cell homeostasis, proliferation of naïve T cells, survival of T cells and B cell

development in the bone marrow of a healthy host. IL-7/IL-7R deficient individuals display severe T cell lymphopenia, indicating the importance of IL-7 in controlling T cell thymopoiesis and homeostasis. It is established that HIV-1 infection disturbs this IL-7/T cell axis. Plasma IL-7 levels are increased in HIV-1 infection (Juffroy, Bugault et al.; Napolitano, Grant et al. 2001; Malaspina, Moir et al. 2006) and yet paradoxically, this increase is associated with disease progression and decreased CD4⁺ T cell counts (Napolitano, Grant et al. 2001; Malaspina, Moir et al. 2006). Both IL-7R expression and IL-7 binding have been shown to be impaired in HIV-1 infection (Juffroy, Bugault et al.) and suggest decreased responsiveness to IL-7 is a factor. Regarding B cells, IL-7 is thought to increase the production of human precursor B cells from stem cells in cord blood and especially in adult bone marrow (Parrish, Baez et al. 2009). Whilst complete IL-7R α deficiency causes a T-B⁺ SCID (Puel, Ziegler et al. 1998), partial IL-7R α deficiency has been shown to cause severe hypogammaglobulinaemia in three cases of T-B⁺ SCID from a consanguineous family (Roifman, Zhang et al. 2000).

Increased levels of serum IL-7 have been associated with increased CD10⁺ transitional B cell numbers in the peripheral blood of patients with HIV-1 infection (Malaspina, Moir et al. 2006) and idiopathic CD4 lymphopenia (Malaspina, Moir et al. 2007) by the same group, it is thought that this cytokine may alter homing and migration of B cells to sites of B cell maturation by altering the expression of chemokine receptors. Increased IL-7 levels have also been reported in a subset of patients with CVID (Holm, Aukrust et al. 2005), these patients displayed increased numbers of CD8 T cells and increased CCR7⁻ effector memory T cells and an impaired response to IL-7 in some cases.

6.1.5 Receptors for BAFF and APRIL

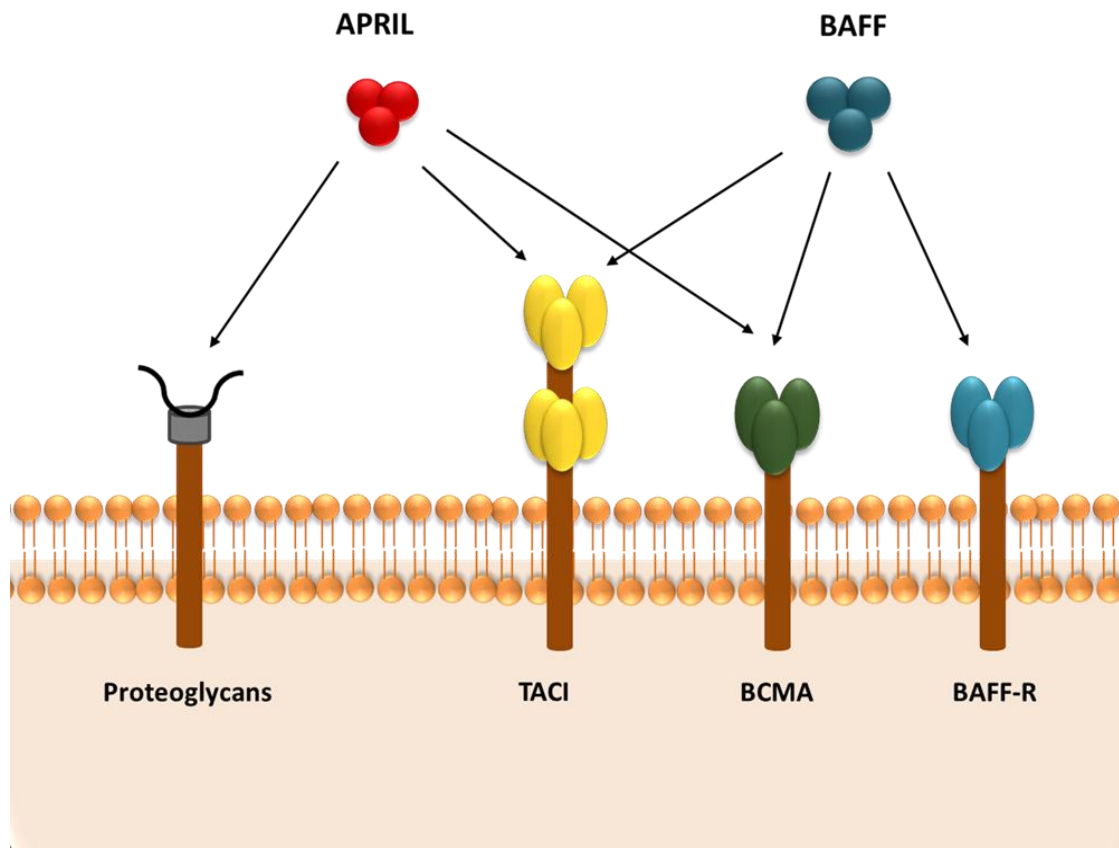


Figure 57: BAFF/APRIL and their receptors. Modified from (Lee, Ozcan et al. 2008)

BAFF and APRIL signal through a number of receptors limited to B cells: BAFF-R, B Cell Maturation Antigen (BCMA) or Transmembrane Activator and calcium modulating Cyclophilin ligand (CAML) Interactor (TACI) (see Fig. 57). BAFF has highest affinity for BAFF-R, to which APRIL does not bind, conversely BAFF is thought to bind to BCMA only weakly. APRIL additionally binds to heparin sulphate proteoglycans. BAFF and APRIL binding receptors are expressed at different times during B cell development, as can be seen in Fig. 58 below.

6.1.6 BAFF Binding Receptor expression during B Cell Development

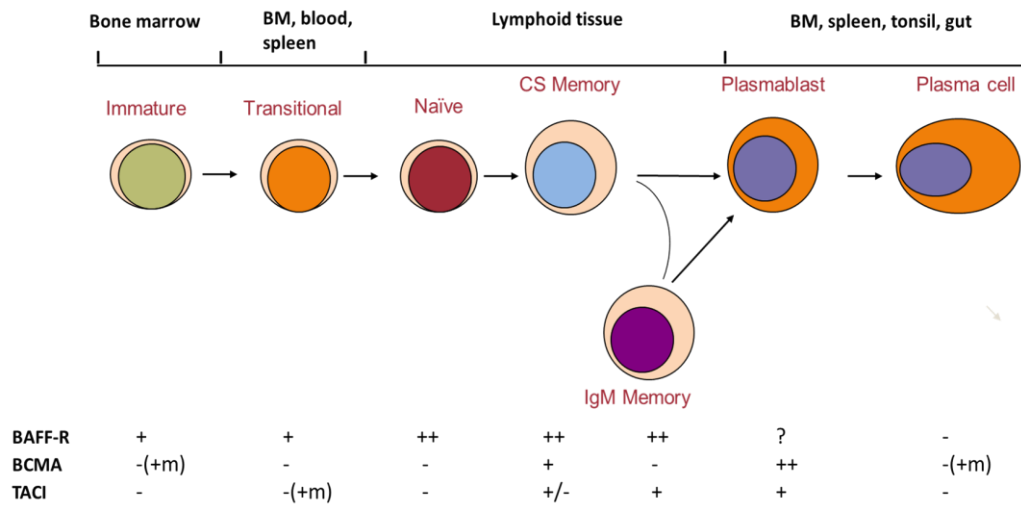


Figure 58: BAFF Binding Receptors. Modified from (Tangye, Bryant et al. 2006)

6.1.7 BAFF-R

BAFF-R, (TNFRSF13C) is generally considered to be expressed at a high level by human peripheral B cells, however, evaluations of the individual B cell subpopulations is incomplete or inconsistent (see Fig.58). Naïve B cells, transitional B cells that have left the bone marrow, human mature naïve B cells and memory B cells express BAFF-R, however the expression of BAFF-R on plasmablasts and plasma cells is unclear: BAFF-R was reported to be expressed by tonsillar plasma cells (PC) but not blood or bone marrow PC in one study. Some papers have suggested BAFF-R expression is lower on human germinal centre B cells (CD38+IgD-) (Ng, Sutherland et al. 2004; Zhang, Park et al. 2005), quantified in the latter study by a lower mean fluorescence intensity measurement (MFI). In healthy individuals, reduced BAFF-R expression may be associated with activation, as shown *in vitro* (Darce, Arendt et al. 2007) and/or associated with terminal differentiation into immunoglobulin secreting cells (ISCs) (Avery, Kalled et al. 2003). BAFF-R $-/-$ mice have a similar impaired clinical phenotype to BAFF $-/-$ mice, such as decreased lifespan of germinal centres and impaired class switch recombination (Mackay and Schneider 2009).

Homozygous BAFF-R deficiency in two siblings with late-onset CVID have recently been identified (Warnatz, Salzer et al. 2009). These patients were found to have a recessive in-frame deletion (del89–96) within the TNFRSF13C gene encoding BAFF-

R, which removed eight hydrophobic amino acids in the BAFF-R transmembrane region and prevented functional BAFF-R synthesis. BAFF-R^{-/-} B cell development was arrested at the transitional B cell stage, all further mature B cell subsets were reduced or absent, with especially reduced IgM memory B cells, B cell lymphopenia and hypogammaglobulinaemia (IgG and IgM). Normal IgA levels were present. Interestingly, these patients displayed decreased TACI expression by residual CD27⁺ B cells and impaired response to pneumococcal vaccination (Warnatz, Salzer et al. 2009). In another study, several lymphopenic CVID patients were shown to express low BAFF-R on the B cells and low BAFF binding activity (Rizzi 2007). In HIV infection, few studies have addressed BAFF-R expression by different B cell subpopulations. In one study, the percentage of CD19⁺ B cells expressing BAFF-R was shown to be reduced in HIV viraemic patients in comparison with HIV aviraemics or healthy controls (Moir, Malaspina et al. 2004) and this seemed to be associated with low expression of CD21 on CD19⁺ B cells in HIV infection. However, it is now known that the CD21^{low} population is heterogeneous, containing both CD27⁺ and CD27⁻ B cells, transitional B cells and tissue like B cells and so this finding needs further investigation.

6.1.8 TACI

TACI, also known as TNFRSF13B, is a type III transmembrane protein which plays a crucial role in T cell independent B cell isotype switching (CSR) especially to IgA (Castigli, Wilson et al. 2005). TACI also plays an important role in T cell independent immune responses e.g. to encapsulated bacteria such as pneumococcus, and has roles in B cell proliferation and plasma cell differentiation (Castigli and Geha 2007). It is controversial at present whether TACI acts as both a positive or negative regulator of B cell proliferation or survival. This chemokine receptor may have the ability to induce apoptosis (Sakurai, Hase et al. 2007). TACI is believed to be an inducible receptor in humans that is expressed on CD27⁺ memory B cells, a small subset of naive B cells (Darce, Arendt et al. 2007), tonsillar and bone marrow plasma cells. Evidence suggests that TACI is upregulated upon activation, since TACI is expressed on a proportion of CD27 negative non germinal centre B cells that showed an active phenotype (as demonstrated by upregulated CD25 and CD80) (Darce, Arendt et al. 2007).

Importantly, reduced TACI B cell expression is seen in peripheral B cells of new born mice, which is associated with defective Ig secretion after BAFF or APRIL stimulation (Kanswal, Katsenelson et al. 2008) and as such may be a crucial factor in new born susceptibility to encapsulated bacteria such as pneumococcus. In neonatal humans, which also have a susceptibility to invasive pneumococcal infection, peripheral B cells express slightly less TACI, although this was not significant (Kaur, Chowdhury et al. 2007). Splenic marginal zone B cells are thought to have elevated levels of TACI. Eight to ten per cent of patients with CVID have genetic mutations in TACI, this mutation is associated with decreased immunoglobulin production despite increased B cell numbers (Castigli and Geha 2007). TACI $-/-$ mice have impaired class switch recombination to IgA (Mackay and Schneider 2008), defective T cell independent type II antibody responses, autoimmunity, glomerulonephritis and increased rates of B cell proliferation. Mice deficient in TACI develop B cell hyperplasia and B cell lymphomas. Interestingly, increased CD4⁺ T cells in Peyer's patches of TACI $-/-$ mice have also been described.

6.1.9 BCMA

B cell Maturation antigen (BCMA) is another of the specific receptors for BAFF and APRIL and also part of the TNF super family (TNFRSF17). It is thought that in humans BCMA is expressed on plasma cells or antibody producing cells. It possibly can promote the antigen presenting function of B cells. The role of BCMA is unclear, whilst BCMA $-/-$ mice have impaired survival of long lived bone marrow plasma cells, these mice are healthy and at present, no mutations in BCMA have been reported to be associated with CVID. Systems vaccinology work has highlighted BCMA as a key immune system gene which is upregulated during successful yellow fever vaccination (Querec, Akondy et al. 2009; Pulendran, Li et al. 2010). However, few monoclonal antibodies were available for BCMA, making assessment of this receptor difficult.

6.1.10 Follicular helper T cells

Humoral vaccination responses to tetanus toxoid in HIV-1 infected patients on ART were shown to be impaired in this thesis. Tetanus toxoid is a protein antigen based vaccine, protein vaccination responses are known to be dependent on T cell help. A recently described T cell subset (T_{FH}) is proposed to be the T cell subset that gives

help to B cells during T cell dependent humoral immunity. The humoral response to protein antigens is dependent on cognate B-T cell interactions in the follicular compartments of secondary lymphoid tissues. Germinal centre founder B cells bind and process antigen presented by follicular DCs. T cell help is elicited by CD40 on GC founder B cells binding to its ligand CD40L on the surface of follicular B helper T cells (T_{FH} cell). This aids the creation of germinal centres where memory B cells with an IgD-IgM-CD27⁺ phenotype (GC/class switched memory B cells) develop and produce high affinity, class switched immunoglobulins of the IgG and IgA isotype as part of the adaptive immune response (Fig.4). T_{FH} cells have a CD4⁺ CXCR5⁺ CD45RO⁺ phenotype and express high levels of the inducible co-stimulatory molecule (ICOS) and signalling lymphocytic activation molecule (SLAM) associated protein (SAP). CXCR5 is necessary for follicular homing and is upregulated upon activation of T_{FH} cells. There is little or no data on the role or number of T_{FH} T cells in HIV infection, possibly a loss of these T cells may be associated with impaired T cell mediated immunity.

Many of the previous T_{FH} studies have used tonsillar sections in mouse or man, it is still controversial whether this subset exists in blood (Müller, Höpken et al. 2003). Some believe that the circulating subset is a 'resting' T_{FH} population (Förster, Mattis et al. 1996) or a possibly a resting T_{FH} derived memory population incapable of giving help to B cells (King, Tangye et al. 2008). Part of the controversy which reigns over the function and origin of T_{FH} in blood exists because the circulating T_{FH} express CD62L and CCR7 unlike their tonsillar counterpart. Grimbacher *et al* describe evidence that circulating CXCR5⁺ T cells must derive from a follicular precursor population by demonstrating that impaired GC formation in human ICOS deficiency correlates with decreased peripheral CXCR5⁺ T cells, whilst in ICOS knockout mice, decreased T_{FH} cells in LN and spleen correlate with a reduction of circulating CXCR5⁺ T cells in the blood, suggesting a relationship link (Bossaller, Burger et al. 2006). In agreement with this, Simpson *et al* found circulating T_{FH} numbers correlated with GC T_{FH} numbers in Sanroque mice who characteristically have high T_{FH} numbers (Simpson, Gatenby et al. 2010). Circulating T_{FH} cells may be considered a biomarker of GC T_{FH} levels in mice and man (Simpson, Gatenby et al. 2010). Five to 15% of circulating CD4⁺CD45RO⁺ T cells are CXCR5⁺ (Bossaller, Burger et al. 2006). To date, seven subsets of CXCR5⁺ cells have been identified (Vinuesa,

Tangye et al. 2005): CD57, CD25 and CD69 are differentially expressed on subsets of these cells. The requirement for CD57 is controversial, in one study in 2006, CD57 was shown to be non-essential for B cell help as measured by IgG and CXCL13 secretion (Rasheed, Rahn et al. 2006). Expression of ICOS and SAP by T_{FH} cells are critical to T_{FH} B cell help and both are required to form a sustained germinal centre, isotype switched B cell antibody response (Bossaller, Burger et al. 2006).

6.1.11 T_{FH} in primary immune deficiency

Common variable immune deficiency patients with homozygous ICOS deletions or X-linked proliferative disease (XLP) patients who have SH2D1A mutations and defective SAP protein production, lack germinal centres, specific antibodies, serological memory or memory B cells and have progressive hypogammaglobulinaemia (Grimbacher, Hutloff et al. 2003). Since these patients do not express ICOS or are deficient in SAP, a T_{FH} associated protein, they will be deficient in T_{FH} cells. The lack of T_{FH} cells contributes to a lack of GC (T cell help required) and lack of long term serological memory. ICOS deficiency is a gene mutation rarely described in CVID to date (<1%) and is seemingly restricted to Southern Germany and Austria.

6.1.12 T_{FH} and autoimmunity

Levels of T_{FH} are increased in SLE, RA and Sjogren's syndrome; in a murine mouse model of lupus nephritis, ICOS expression was increased on CD4 T cells. In agreement with this, ICOS+CD4+ T cells were found to be increased in humans in association with disease progression in SLE (Iwai, Abe et al. 2003; Hutloff, Buchner et al. 2004). ICOS blockade ameliorates SLE, RA and NOD diabetes in murine models, suggesting these T_{FH} cells may have a role in pathogenesis. Indeed, in SLE, increased titres of autoantibodies and disease severity has recently been shown to be associated with cells resembling T_{FH} T cells (Simpson, Gatenby et al. 2010), however these T_{FH} cells did not correlate with the SLE disease activity index (SLEDAI).

6.2 Aims

- 1) To measure the level of expression of B cell growth factor receptors (BAFF-R, TACI and BCMA) on mature B cell subsets (naïve B cells and IgM memory and class switched memory B cells) in HIV-1, CVID patients and healthy controls.
- 2) To evaluate plasma chemokine levels (BAFF, APRIL and IL-7) in HIV-1, CVID patients and healthy controls.
- 3) To determine whether expression of B cell chemokine receptors/levels of plasma chemokines are associated with markers of disease progression (CD4 count, viral load) or memory B cell and tissue like B cell percentages.
- 4) To measure follicular helper T cells (T_{FH}) in HC, untreated and treated HIV-1 infection and CVID.

6.3 Results

6.3.1 Patient cohort for chemokine level measurement

Untreated and treated HIV-1 infected patients were recruited from the Kobler Clinic, St Stephen's Centre, Chelsea and Westminster Hospital. CVID patients were recruited from the Respiratory Clinic at the Royal Brompton Hospital. Laboratory workers from the Immunology department were also tested to act as healthy control subjects (see Table 16). Demographic data was not available for all subjects.

Table 16: Samples tested for plasma chemokines

Cohort	Chemokine Levels
	<i>No. samples</i>
HC	22
HIV ART-	42
HIV ART+	39
CVID	32

6.3.2 Plasma levels of BAFF, APRIL and IL-7 in patients with immune deficiency and healthy controls

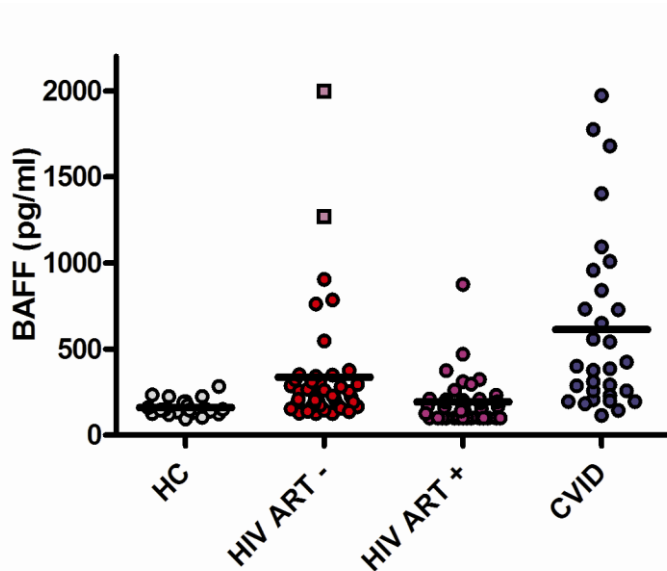


Figure 59: Plasma BAFF levels in healthy controls and disease groups.

Plot displaying data points and horizontal lines indicate median levels of plasma BAFF for healthy controls (HC) (n=22), untreated HIV-1 infected patients (HIV ART-) (n=42), treated HIV-1 infected patients (HIV ART+) (n=39) and CVID patients (n=32). Squares indicate two HIV-1 infected patients no longer receiving ART. These patients were not included in the statistical analysis. The Mann-Whitney U test was used to analyse non-parametric data.

Plasma levels of BAFF were measured in HC, a cohort of CVID patients, untreated HIV infected patients and HIV infected patients on treatment and two patients no longer receiving ART (purple squares) (see Fig.59). BAFF levels were measurable in all healthy controls in addition to disease groups. Median levels of BAFF were found to be higher overall in CVID patients and HIV untreated patients in comparison with the healthy control group (median levels 386pg/ml CVID $p<0.001$, median 221pg/ml ART-, HC group 147pg/ml, $p<0.001$) consistent with a previous publication. There was a significant difference between ART- and ART+ HIV infected individuals, confirming ART normalises BAFF levels in plasma ($p<0.001$).

Levels of BAFF in untreated HIV infected patients correlated with CD4 T cell counts, indicating increased BAFF during disease progression ($p=0.01$). (Including the two patients off treatment, this p value =0.002). Although not significant overall, high

plasma levels of BAFF were found in a proportion of HIV infected patients on ART. In a proportion of CVID patients, a strikingly elevated level of BAFF was found. In CVID, this increase in BAFF did not correlate with B cell numbers or memory B cell percentage consistent with previous findings (Knight, Radigan et al. 2007). Unlike HIV infection, there was no correlation with CD4 T cell counts in patients with CVID.

6.3.3 Levels of APRIL in immune deficient patients and healthy controls

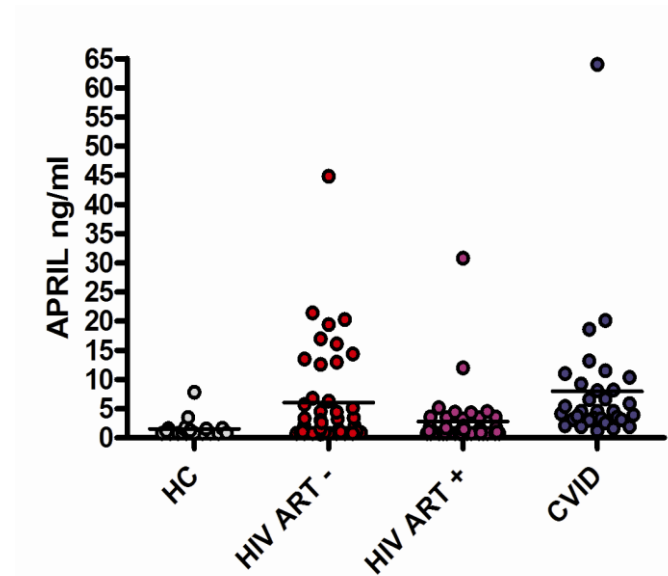


Figure 60: Plasma APRIL levels in healthy controls and disease groups.

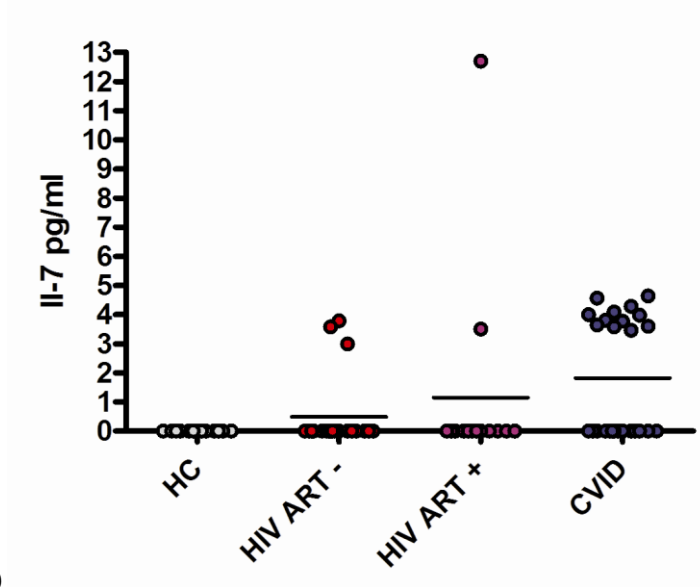
Plot displaying data points and horizontal lines indicate median levels of plasma APRIL for healthy controls (HC) (n=22), untreated HIV-1 infected patients (HIV ART-) (n=42), treated HIV-1 infected patients (HIV ART+) (n=39) and CVID patients (n=32). APRIL ELISA limit of detection was 0.8 ng/ml. All samples that were below the limit of detection were arbitrarily denoted a value of 0.8 ng/ml for consistency. The Mann-Whitney U test was used to analyse non-parametric data.

APRIL levels were strikingly elevated in almost every patient with CVID in comparison with the healthy control group (median levels 4.59 CVID ng/ml vs. 0.85 ng/ml HC, $p < 0.001$), in which, APRIL levels were almost undetectable in almost all cases (Fig.60). Levels of plasma APRIL were also elevated in individuals with untreated HIV infection (n=42), 1.79 v 0.85 $p = 0.02$. APRIL levels were normalised by ART: median plasma APRIL levels were not significantly different from the HC group (1.00ng/ml.) Further to this, there was a significant difference between

untreated patients and treated patients ($p=0.03$), suggesting ART had lowered plasma APRIL levels. There was no correlation between VL or CD4 with plasma APRIL levels in untreated HIV-1 infection.

6.3.4 Plasma IL-7 levels in patients with CVID, HIV-1 infection and healthy controls

A)



B)

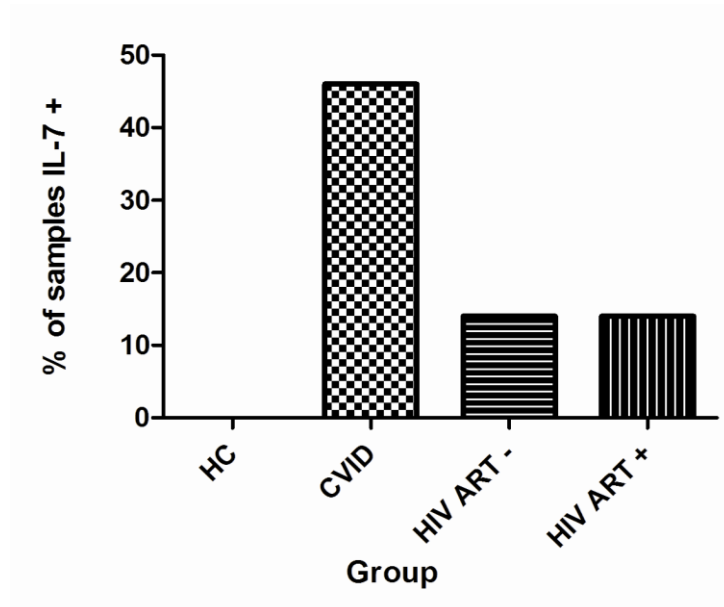


Figure 61: Plasma IL-7 levels in healthy controls and disease groups.

IL-7 measured in healthy controls (HC), untreated HIV-1 infected patients (HIV ART-), treated HIV-1 infected patients (HIV ART+) and CVID patients. Samples available for IL-7 testing: HC (n=11), CVID (n=26), HIV ART- (n=21)

and HIV ART+, (n=14). 61A) Plot displaying data points and horizontal lines indicate median levels of plasma IL-7 and 61B) graph displaying percentage of samples positive for IL-7 in all groups. The sensitivity cut off for the Diaclone IL-7 assay was 3pg/ml, all negative results were not assigned values and no statistics were performed.

IL-7 levels were not detected in many samples (Fig.61A), using this IL-7 ELISA kit. Plasma IL-7 levels were detected in a proportion of CVID patients but undetectable in all HC and most HIV ART+ patients (11/14) using this assay. Using a percentage of samples which had detectable levels of plasma IL-7 (Fig.61B), 12/26 CVID (45%) patients had measurable levels of IL-7 compared to 10% of HIV-1 infected patients and none in any of the HC. Since IL-7 was measurable in some patients with CVID, a possible correlation with CD4 T cell counts was examined but this was not significant.

6.3.5 Correlations between levels of BAFF and APRIL and B cell subsets

The finding of elevated levels of plasma BAFF and APRIL in patients with HIV or CVID was striking. This study then sought to determine whether levels of these B cell chemokines thought to play key roles in B cell growth were associated with expansion of particular B cell subsets. Extended B cell phenotyping was subsequently performed on the peripheral blood of 11 healthy controls, 23 CVIDs, 14 untreated HIV-1 infected patients and 14 HIV-1 infected patients on ART.

Table 17: Immunophenotyped patient cohort

	HC (n=11)	HIV ART- (n=14)	HIV ART+ *(n=14)	CVID (n=23)
<i>Patient</i>				
<i>Demographics</i>				
Age (yrs.)	34 (27-45)	35 (32-42)	42 (40-48)	48 (37-57)
Viral Load (copies/ml)	NA	27375 (8643- 40268)	<50	NA
Time on ART (mos.)	NA	NA	88 (69-134)	NA
CD4 (cells/ μ l)	812 (667-971)	335 (296-467)	447 (412-553)	552 (447-895)
CD8 (cells/ μ l)	351 (297-487)	818 (639-1052)	732 (596-862)	409 (222-639)
CD19 (cells/ μ l)	205 (164-257)	114 (86-150)	222 (119-258)	159 (68-233)
<i>B cell subsets</i>				
%CD19+CD27+	31.1 (23.6-42)	32.5 (26.0-44.5)	24.0 (15.9-32.0)	8.7 (4.6-35.9)
%CD19+CSmem	13.8 (11.0-18.2)	18.7 (16.2-20.6)	12.8 (9.2-21.4)	1.1 (0.6-5.1)
%CD19+IgMmem	19.3 (12.3-23.4)	13.4 (6.6-24.9)	7.9 (6.0-11.4)	5.4 (3.8-25.3)
%CD19+Naïve B	66.7 (56.6-73.6)	63.7 (49.7-70.1)	73.8 (62.6-81.1)	90.2 (4.6-35.9)
%CD19+Tissue B	2.3 (1.9-5.8)	15.0 (7.5-18.4)	9.0 (3.9-11.9)	10.7 (6.0-20.2)

**Note: three HIV ART+ patients were not measured for tissue like B cells. Data was analysed using the Mann Whitney U test for non-parametric populations.*

The HIV-1 patients on ART were not significantly older than controls and untreated HIV-1 patients (see Table 12). The CVID patient group was older than healthy controls but this was not significant. CD19+ B cell counts were significantly lower in the untreated HIV-1 cohort compared to healthy controls ($p < 0.002$). CD4 T cell counts were significantly lower in untreated HIV-1 infected patients ($p < 0.0001$) and HIV-1 patients on ART ($p < 0.01$) compared to healthy controls. Memory B cell (CD19+CD27+) percentages were not significantly different in the HIV-1 infected patients but reduced in CVID compared with controls ($p < 0.01$). IgM memory B cells were significantly reduced in the HIV ART+ group ($p < 0.001$) compared to controls and decreased in the HIV ART- group but this was not significantly different from healthy controls. Class switched memory B cells were decreased in the CVID cohort compared to healthy controls ($p < 0.0001$). Tissue like B cell percentages were significantly increased in untreated HIV patients ($p < 0.001$), and CVID patients ($p < 0.02$) but not in treated HIV-1 patients ($p = 0.06$).

6.3.6 HIV-1 untreated cohort: Associations between APRIL levels and B cell subsets

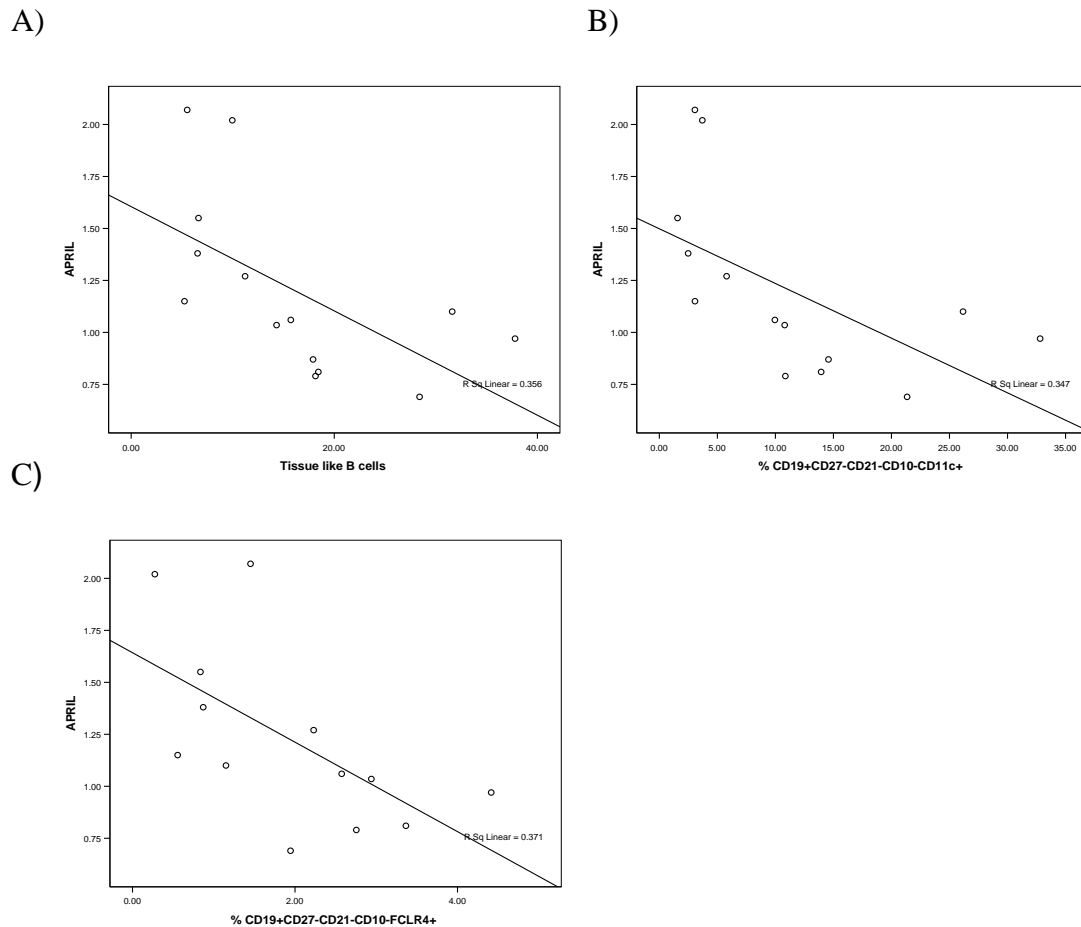


Figure 62: Scatter plots evaluating associations between APRIL levels and tissue like B cell subsets in the peripheral blood of patients with untreated HIV-1 infection.

Plots show associations between plasma APRIL and tissue like B cell subset percentages in 14 HIV-1 untreated patients in A) total tissue like B cells (CD19+CD27-CD10-CD21low) , B) CD11c+ tissue like B cells, C) FCLR4+ tissue like B cells. Data analysed using Spearman’s rank correlation coefficient.

Plasma APRIL levels were negatively correlated to percentages of tissue like B cells ($\rho = -0.754$) ($p = 0.002$), CD11c+ tissue like B cells ($\rho = -0.771$) ($p = 0.001$) or FCLR4+ tissue like B cells ($\rho = -0.692$) ($p = 0.008$), see Fig.62. APRIL levels showed a trend to be also negatively correlated with percentages of IgM memory B cells but this was not statistically significant ($p = 0.085$), probably due to the small size of the cohorts measured. APRIL levels were not associated with total B cell numbers, class switched memory B cells or total numbers of classical CD27+ memory B cells.

6.3.7 HIV-1 untreated cohort: Associations between BAFF levels and B cell subsets

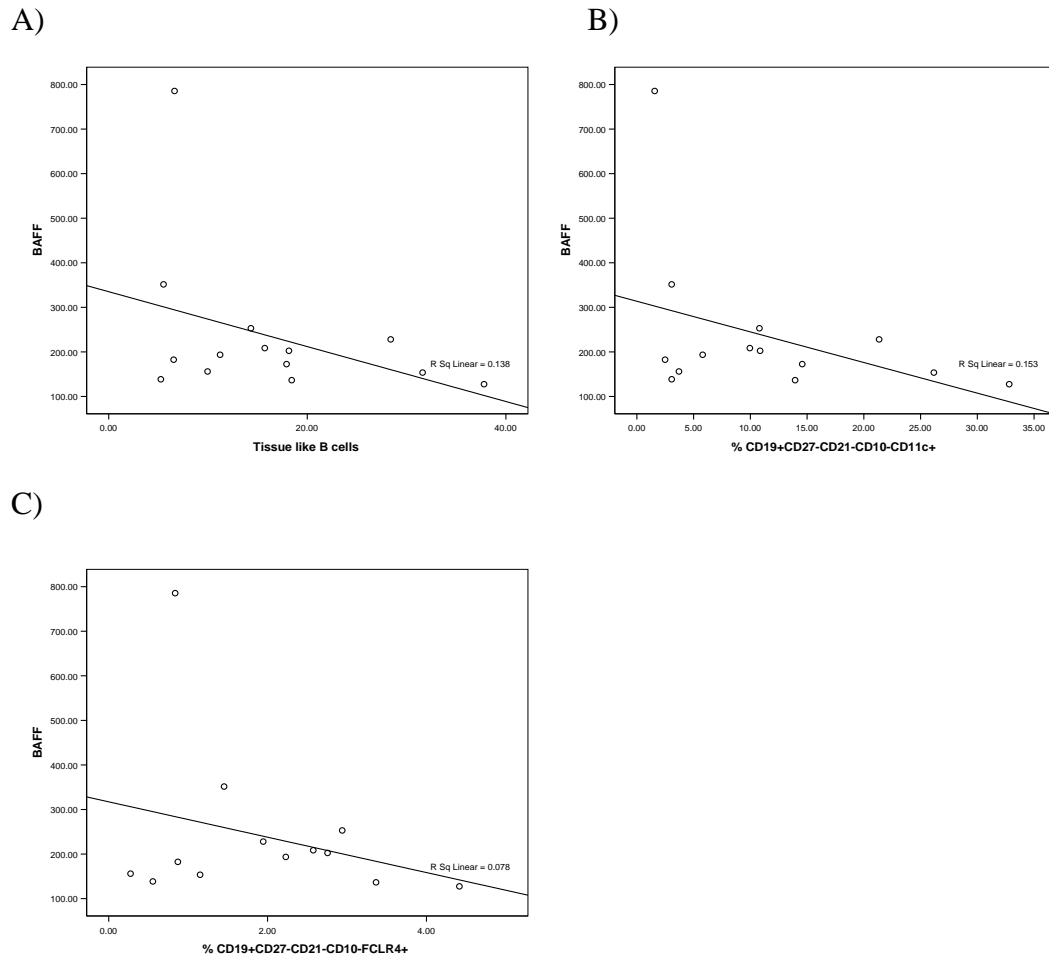


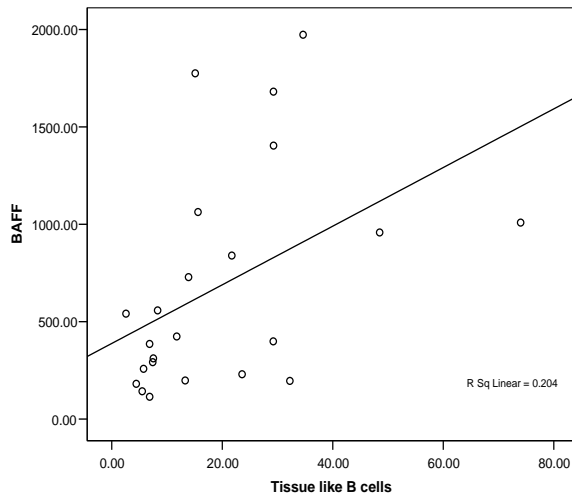
Figure 63: Scatter plots evaluating associations between plasma BAFF levels and tissue like B cell subsets in the peripheral blood of untreated HIV-1 infected patients.

Plots show associations between plasma BAFF and tissue like B cell subset percentages in 14 HIV-1 untreated patients in 63A) total tissue like B cells (CD19+CD27-CD10-CD21low), B) CD11c+ tissue like B cells and C) FCLR4+ tissue like B cells. Data analysed using Spearman's rank correlation coefficient.

In untreated HIV infection, plasma BAFF levels were not found to be significantly correlated to CD27-CD21lowCD10- tissue like B cells ($\rho = 0.215$), CD11c+ tissue like B cells ($\rho = -0.459$) ($p = 0.09$) or FCLR4+ tissue like B cells ($\rho = 0.573$) (Fig.63). In addition, BAFF levels were not associated with total B cell numbers, IgM memory B cells, class switched memory B cells, naïve B cells or total numbers of classical memory B cells (CD19+CD27+).

6.3.8 Association between BAFF and APRIL and tissue like B cell expansions in CVID

A)



B)

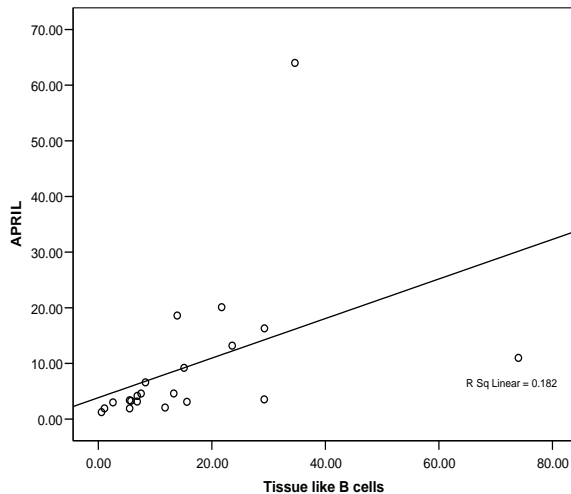


Figure 64: Scatter plots to show the correlations between plasma BAFF and APRIL levels with tissue like B cells in patients with CVID.

Plots show associations between plasma chemokine levels and tissue like B cell subset percentages (CD19+CD27-CD10-CD21low) in the peripheral blood of CVID patients. 64A) Plasma BAFF (n=22) and B) plasma APRIL (n=23). Data analysed using Spearman's rank correlation coefficient.

A significant correlation was found between BAFF or APRIL levels and innate tissue like B cells (BAFF $\rho=0.583$, $p=0.004$ and APRIL, $\rho=0.774$, $p<0.0001$) in patients with CVID (see Fig.64). Innate-tissue like B cells expressed receptors for BAFF and

APRIL (BAFF-R and TACI) establishing a potential mechanistic link between expansion of CD21^{low} B cells and elevated BAFF and APRIL concentration.

6.3.9 *High BAFF and APRIL levels are found in those CVID patients with greatly elevated tissue like B cell percentages.*

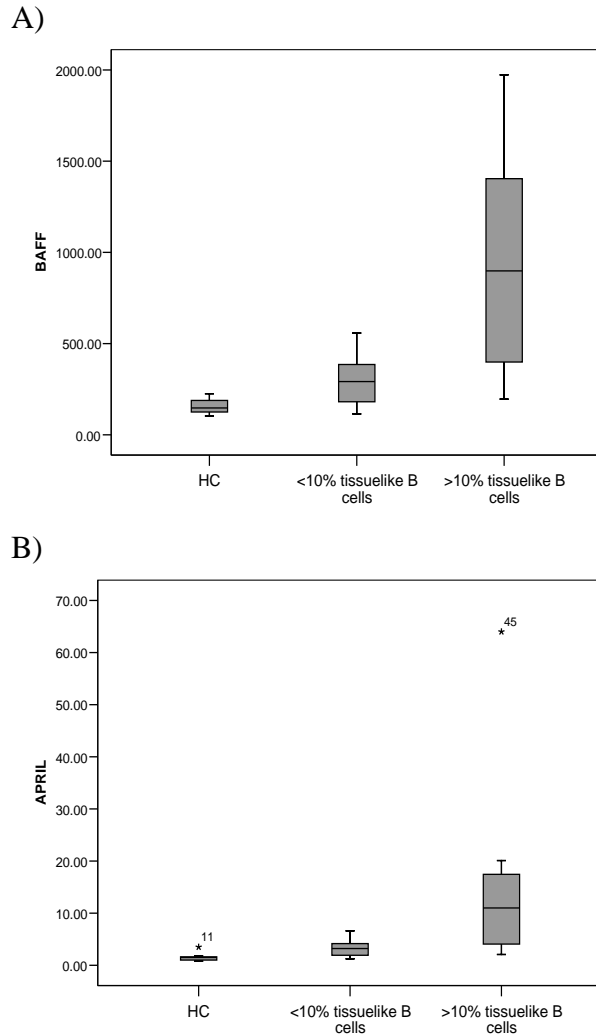


Figure 65: BAFF and APRIL levels in CVID stratified by tissue like B cell percentages

Box and whisker plots displaying median and IQR of plasma BAFF and APRIL levels in CVID patients stratified by tissue like B cell (CD27-CD10-CD21^{low}) percentages defined in EURO Class (<10 or \geq 10%) as a percentage of CD19⁺ B cells: 22A) BAFF levels (<10% (n=8) or \geq 10% (n=14)) and 22B) APRIL levels (<10% (n=9) or \geq 10% (n=14)). The Mann-Whitney U test was used to analyse non-parametric data.

From Fig.65A and Fig.65B, CVID patients with expanded tissue like B cells (>10%) had significantly higher levels of BAFF or APRIL than patients with few tissue like B cells (<10%), ($p<0.001$, $p<0.001$). 10% was the cut-off used for categorising CVID patients with expanded tissue like B cells into separate groups in the EURO Class classification (smB-CD21norm, smB-CD21lo, smB+CD21norm, smB+CD21lo).

6.3.10 Selective expansion of circulating CD21low tissue like B cells in CVID is associated with B cell lymphopenia, reduced naïve B cells and high levels of BAFF or APRIL.

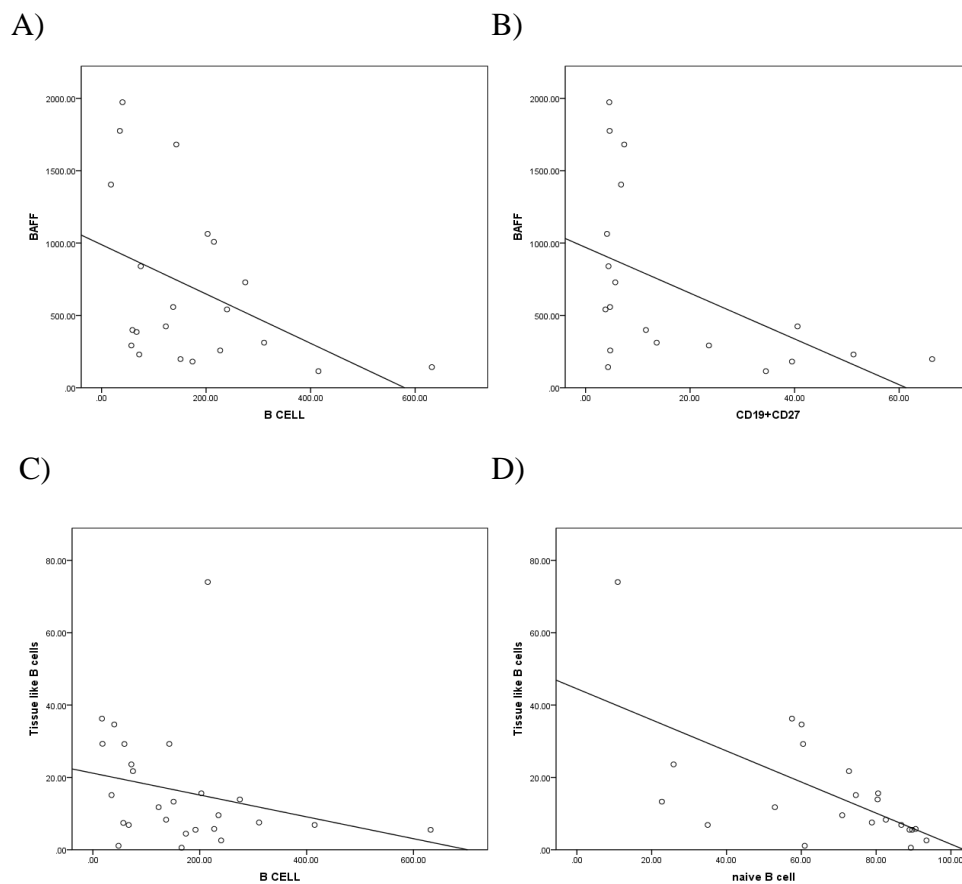


Figure 66: Selected negative B cell associations in patients with CVID

Scatter plots to show A) BAFF levels in CVID (n=22) correlate with CD19+CD27+ memory B cells, B) BAFF levels are negatively associated with total B cell counts, C) Tissue like B cell percentages are negatively associated with B cell count and D) Tissue like B cells are associated with naïve B cell percentages in CVID (n=22). Data analysed using Spearman's rank correlation coefficient.

BAFF levels in CVID patients significantly correlated negatively with B cell count ($\rho = -0.473$) ($p < 0.05$) (Fig.66A) and BAFF levels were also negatively correlated with classical memory: CD19+CD27+ ($\rho = -0.451$) ($p < 0.05$) (Fig.66B) or CD19+CD27+CD21+CD10- (non-activated classical memory, not shown) ($\rho = -0.511$) ($p < 0.05$). In Fig. 66C, tissue like B cell percentages correlate negatively with B cell count ($\rho = 0.692$) ($p = 0.014$) and naïve B cells ($\rho = 0.666$) ($p < 0.04$). APRIL levels were correlated with reduced naïve B cells ($\rho = -0.480$) ($p = 0.03$) but not classical memory ($\rho = 0.626$) or B cell count ($\rho = 0.626$) (data not shown). Therefore, a most severe immune deficiency is seen in these patients with expanded tissue like B cells and high levels of B cell chemokines.

6.3.11 Chemokine Receptors: Expression of BAFF-R on peripheral blood B cells

A)

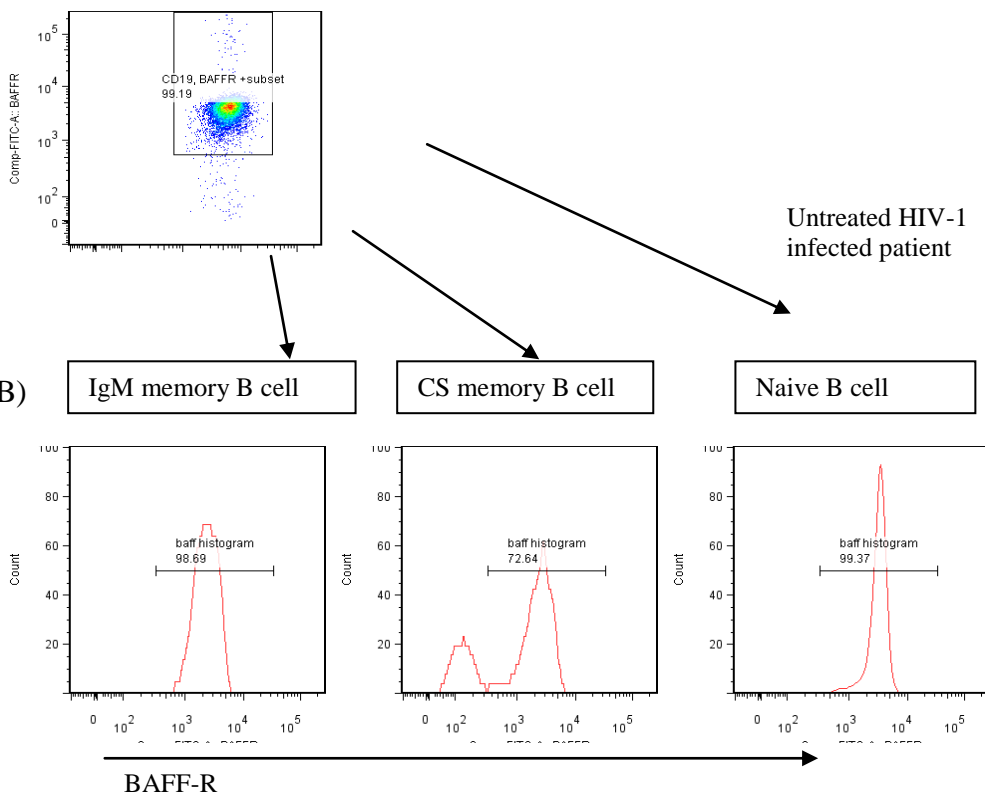


Figure 67: Expression of BAFF-R on memory B cells.

Representative histograms to show expression of BAFF-R by B cell subsets. A) BAFF-R expression by total B cells (gated on CD19+) and B) on B cell subsets. BAFF-R is reduced in HIV infection and negative on a proportion of class switched (CS) B cells. Example is representative of a HIV infected patient not on ART therapy. Histogram gated using matched isotype control.

6.3.12 BAFF-R expression on peripheral blood B cells

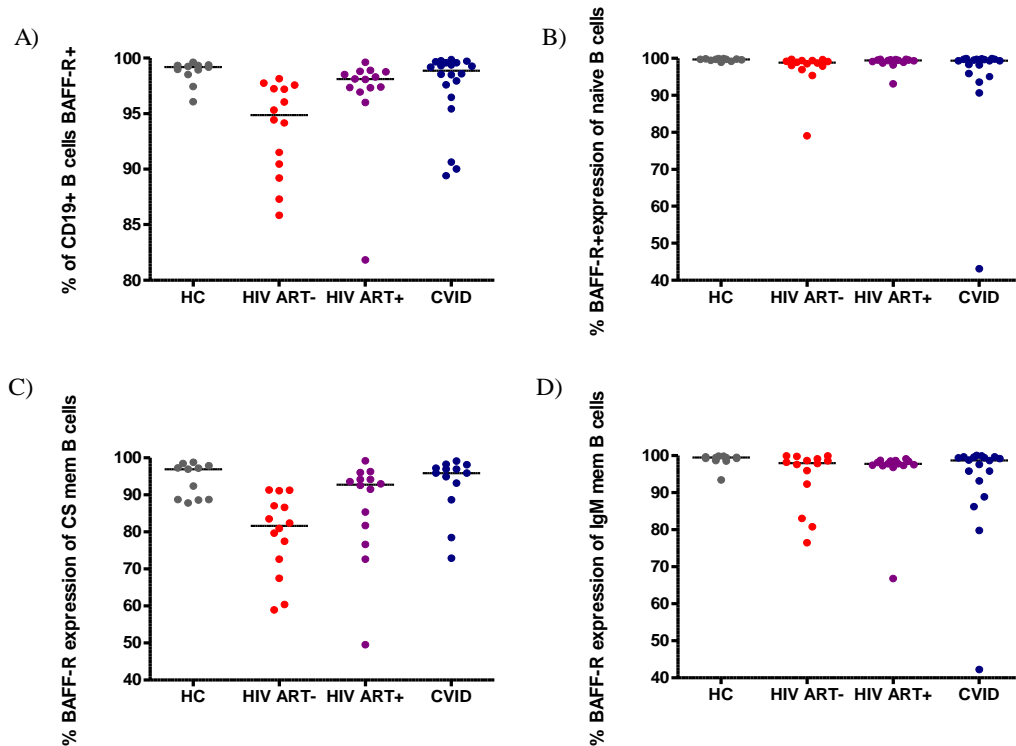


Figure 68: BAFF-R expression on mature B cell subsets in healthy controls and disease groups

Plots displaying data points and horizontal lines indicate median values for BAFF-R expression as a percentage of specific B cell subsets, gated on CD19+ B cells, for healthy controls (HC) (n=11), untreated HIV-1 infected patients (HIV ART-) (n=14), treated HIV-1 infected patients (HIV ART+) (n=14) and CVID patients (n=20). 68A) total B cells, B) naïve B cells, C) IgM memory B cells and D) class switched memory B cells. The Mann-Whitney U test was used to analyse non-parametric data.

BAFF-R is expressed at high levels on the total B cells of HC, with only 1-2% of total B cells not expressing BAFF-R, consistent with the literature (Fig.68A). Expression of BAFF-R is significantly reduced on a proportion of total B cells in untreated HIV infection in comparison with healthy controls, median percentage of CD19+ B cells BAFF-R positive: 94% v 98.6% in HC ($p < 0.001$), (see Fig.68A). This meant that a median value of 6% of B cells in untreated HIV infection were BAFF-R negative and this finding is consistent with previous studies (Moir, Malaspina et al. 2004). BAFF-R expression in HIV ART+ patients was still significantly different from that of healthy

controls (median 97%) indicating BAFF-R expression had not fully normalised in patients on ART (97%, $p=0.016$). There was no significant difference between the levels of BAFF-R on total B cells in CVID in comparison with the HC cohort ($p=0.87$), however, some patients showed reduced levels of BAFF-R.

There was a slight but significant reduction of BAFF-R in the IgD+ naïve B cells in HIV untreated patients ($p=0.002$), 96% v 99.5% and ART treated individuals in comparison with HC, 98% v 99.5% ($p=0.02$) (Fig.68B). However, this was skewed slightly by one outlier in the HIV ART- group. There was no significant difference between the levels of BAFF-R on naïve B cells in CVID in comparison with the HC cohort ($p=0.08$), however, as before, a subset of patients showed reduced levels of BAFF-R.

Next, the expression of BAFF-R was examined on memory B cell subsets to see if the loss of BAFF-R was specific to a certain memory subset. It can be seen that a proportion of class switched memory B cells of healthy controls did not express BAFF-R, (mean ~7%) a finding which has been suggested in the literature (Fig.68C). Class switched memory B cell subsets of untreated HIV patients lost the greatest amount of BAFF-R expression and this was significantly reduced in comparison with healthy controls ($p<0.0001$). Strikingly, greater than 20% of class switched memory B cells of untreated HIV infected patients did not express BAFF-R. This was not associated with CD4 count or viral load. The expression of BAFF-R on class switched memory B cells of patients on ART was on a higher proportion of B cells than untreated patients (87% of class switched memory B cells) and not significantly reduced in comparison with healthy controls ($p=0.15$). However, there did seem to be heterogeneity in BAFF-R expression, with class switched memory B cells of 4/14 patients expressing greatly reduced BAFF-R on class switched memory B cells. There was no significant difference between the levels of BAFF-R on class switched memory B cells in CVID in comparison with the HC cohort ($p=0.86$), however, some patients showed reduced levels of BAFF-R.

Although not as striking as for class switched memory B cells, interestingly, IgM memory BAFF-R expression was significantly reduced for the HIV ART+ cohort ($p<0.001$) but not for the untreated HIV ART- cohort ($p=0.06$) (Fig.68D). However,

there are small cohorts in this study: one outlier in the HIV ART+ group with extremely low BAFF-R expression may have skewed the data in that group, whilst the ART- group narrowly missed significance. This needs further investigation. In the CVID patients, the expression of BAFF-R on IgM memory B cells was heterogeneous, again there was no significant difference overall from the HC group.

6.3.13 TACI expression on peripheral blood B cells

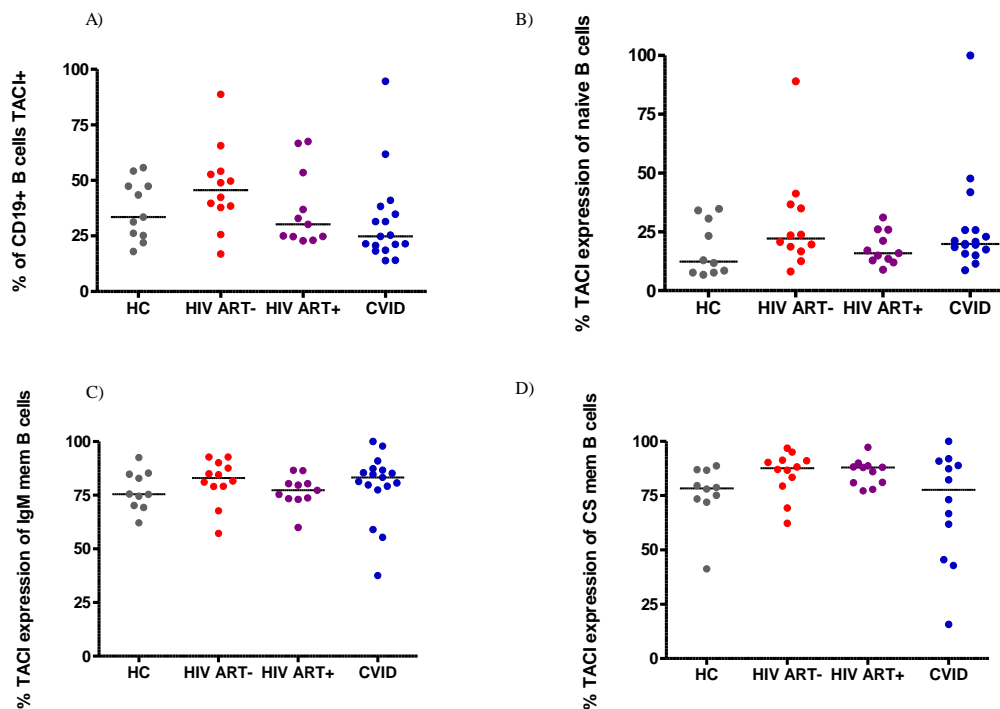


Figure 69: TACI expression by mature B cell subsets in healthy controls and disease groups

Plots displaying data points and horizontal lines indicate median values for TACI expression as a percentage of specific B cell subsets, gated on CD19+ B cells, for healthy controls (HC) (n=10*), untreated HIV-1 infected patients (HIV ART-) (n=12*), treated HIV-1 infected patients (HIV ART+) (n=11) and CVID patients (n=17*). 69A) total B cells, B) naïve B cells, C) IgM memory B cells and D) class switched memory B cells. *Note: data was omitted from D for 5 CVID patients due to less than 100 class switched memory events. Data was omitted from 1 HC, 2 ART- and 3 CVID patients due to high background staining in isotype controls. Isotype gate was set at <2% staining for all samples. The Mann-Whitney U test was used to analyse non-parametric data.

TACI was found to be expressed on 20-55% of peripheral B cells of healthy controls (Fig. 69A). The percentage of B cells expressing TACI looked to be slightly increased in untreated HIV infection, consistent with previous work (Moir, Malaspina et al. 2004) however, this was not significantly different from the HC cohort. In fact, no significant differences in overall TACI expression between healthy controls and in all disease groups were found (Fig.69A). TACI expression varied markedly according to B cell subset in healthy controls: TACI was most highly expressed by classical memory B cells, with low expression by the mature naïve B cells. Although there were no significant differences between TACI expression by the naïve B cell subsets of HIV (ART- $p=0.09$, ART+ $p=0.46$) and CVID ($p=0.18$) patient cohorts compared to the HC subset, TACI expression was elevated in the naïve B cell subset in some untreated HIV-1 patients and in some CVID patients (Fig 69B). This finding was investigated further and found to be due to CD21^{low} CD27-IgD⁺ expanded B cells expressing TACI (>70% of population, see below).

Approximately three-quarters of both IgM memory and class switched memory B cells of healthy individuals expressed TACI, consistent with the literature. TACI was found to be highly expressed on IgM memory and class switched memory B cells of healthy controls, HIV patients and individuals with CVID (Fig.69C and Fig.69D). No significant differences were seen between HIV patients, CVID and HC regarding the percentage of the IgM memory B cell subsets expressing TACI. TACI expression by class switched memory B cells was significantly increased in ART+ HIV-1 patients ($p=0.03$) but was not significantly upregulated in the ART- HIV-1 ($p=0.05$) or CVID patient group ($p=0.52$) compared to HC. This finding may be skewed somewhat by an outlier in the healthy control group. However, the lack of significance between CVID patients' TACI expression compared to healthy controls belied the fact that TACI expression of class switched B cells was markedly heterogeneous in CVID, with several patients having extremely low TACI expression on class switched memory B cells. Interestingly, two of these three CVID patients with reduced TACI expression by class switched memory B cells had normal levels of TACI expression on IgM memory B cells and naïve B cells. This needs further investigation.

6.3.14 TACI and BAFF-R expression on tissue like B cells in HIV-1 infection

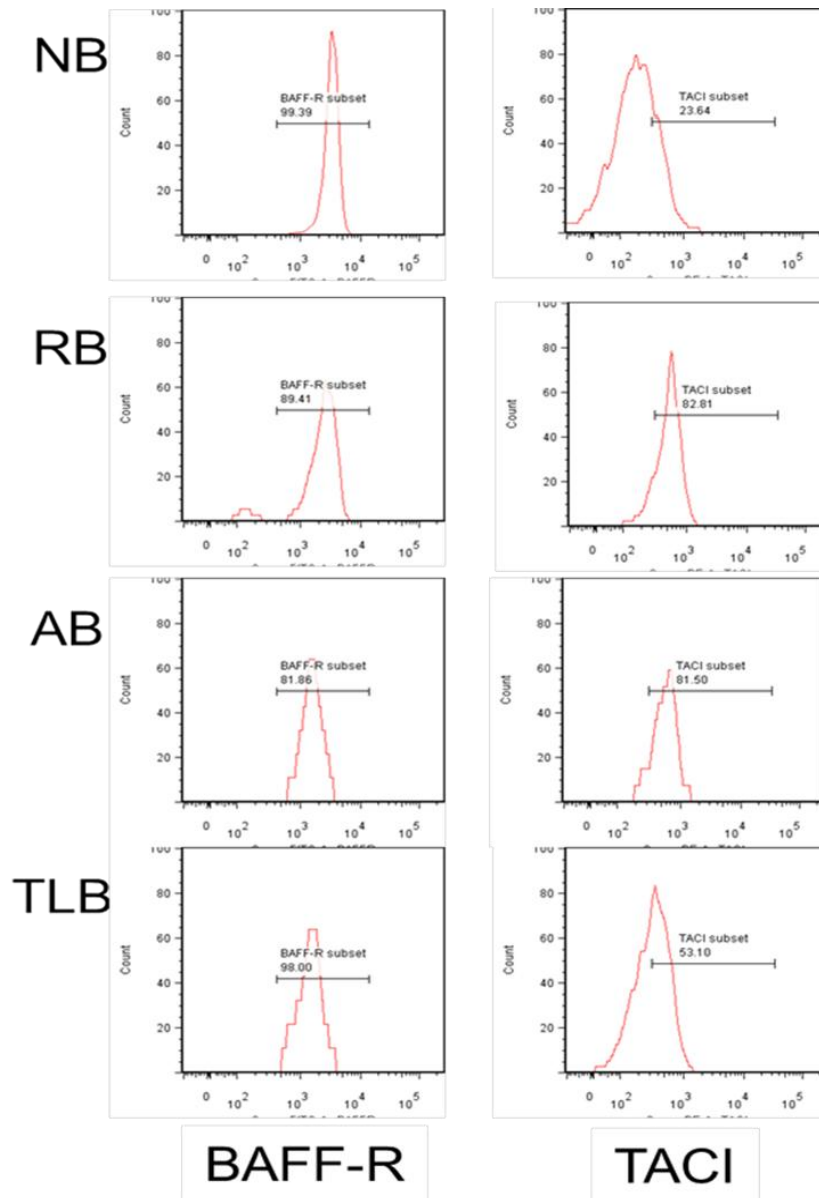


Figure 70: BAFF-R and TACI are expressed on expanded CD21low cells seen in HIV untreated patients and CVID.

Histograms to show BAFF-R and TACI expression on a representative untreated HIV infected individual on B cell subsets differentiated using CD10, CD27 and CD21. Naïve B cells (NB), resting memory B cells (RB) and the mature CD21low subsets: activated memory B cells (AB) and Tissue like B cells (TLB). At least 3 patients per group were tested and samples gated using isotype matched control.

In a subset of patients and HC, further staining was done to determine whether the CD21low subsets (tissue like and activated memory B cells) expressed BAFF-R and

TACI. A paper had speculated that in HIV-1 infection reduced BAFF-R on total B cells was due to the CD21^{low} tissue like B cells not expressing BAFF-R (Moir and Fauci 2009).

We had previously found that the percentage of total B cells that were BAFF-R negative class switched B cells was comparable to the number of total B cells that were BAFF-R negative so it was not likely that another B cell subset could have negative BAFF-R expression. Further staining with BAFF-R, CD10, CD27 and CD21 was done to determine this. Tissue like B cells express BAFF-R and TACI, a finding not previously reported (Fig.70). This finding is independent of patient group and percentage of CD21^{low} B cells: healthy controls, CVID patients and HIV infected patients (including an elite controller), all expressed TACI at a moderate to high level (50-75% of CD21^{low} cells), a similar level to that seen on classical IgM memory and switched memory B cells.

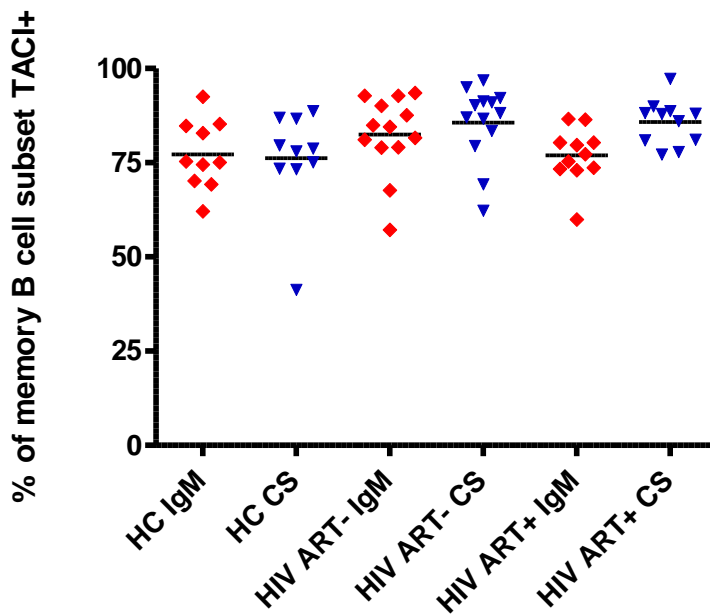


Figure 71: TACI levels on peripheral blood memory B cells.

Percentages of CD19⁺ IgM memory B cells (IgM) and class switched memory B cells (CS) expressing TACI in healthy controls (HC) (n=10), untreated HIV-1 infected patients (HIV ART-) (n=12), treated HIV-1 infected patients (HIV ART+) (n=11). The Mann-Whitney U test was used to analyse non-parametric data.

Interestingly, there seems to be a difference between the percentages of memory B cell subsets expressing TACI, especially in the HIV infected patient group on ART

(Fig.71). Levels of IgM memory B cells expressing TACI were significantly lower than the percentage of class switched B cells expressing TACI in this group ($p < 0.01$).

6.3.15 BCMA

Expression of BCMA was measured on a number of healthy controls and patients. However, it became apparent that staining using the BCMA monoclonal antibody was extremely variable and inconsistent (non-specific staining by BCMA on $>25\%$ of CD19+ B cells of a HC sample was not present when repeated) see Fig. 72.

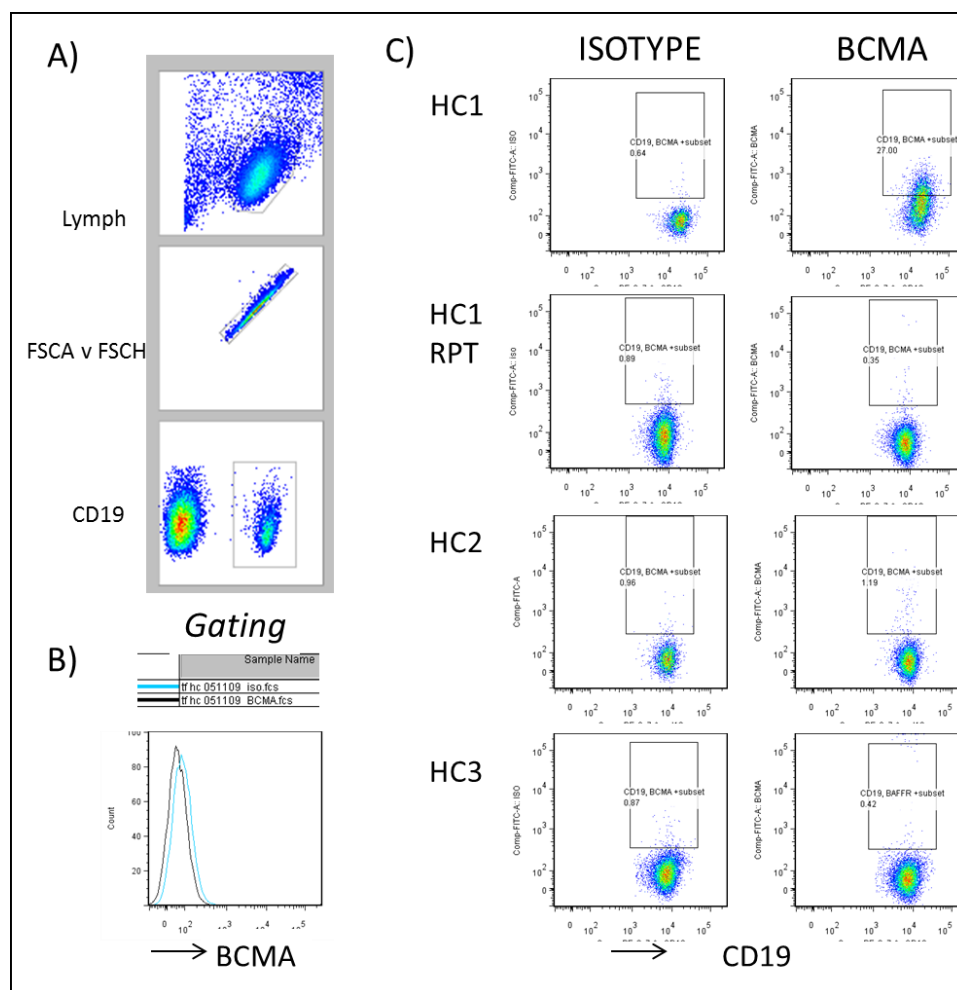


Figure 72: BCMA gating and BCMA expression by CD19+ B cells.

72A) Back-gating as shown previously. B) Antibodies to BCMA produced less staining than the matched isotype control in many cases (see Representative BCMA histogram). C) In addition, staining for BCMA with a HC sample repeated for quality control purposes (HC1), showed markedly different results. This was not an isolated occurrence. Other HC2 and HC3 samples showed minimal BCMA staining.

A second batch of anti-BCMA antibody from Alexis Biochemicals produced much less staining. At the time of the experiments, few manufacturers produced anti-BCMA monoclonal antibody and so this antibody had to be sourced from a company in the United States (Alexis Biochemicals). A publication in the literature had also reported inconsistency with staining for BCMA using available monoclonal antibodies (Benson, Dillon et al. 2008). Some studies had shown that BCMA expression could be upregulated by culture with stimulus such as CpG+IL-2, and was possibly present on activated B cells, expression by non-stimulated peripheral blood B cells is minimal and not expressed by memory B cells or naïve B cells (Darce, Arendt et al. 2007). Due to inconsistencies found in acquired flow cytometry data between BCMA batches and non-specific staining of HC samples, unfortunately, the decision was made to stop testing for BCMA by flow cytometry.

6.3.16 Measurement of Follicular helper T cells in HIV infection and CVID

As stated in Materials and Methods, the T_{FH} cells were defined as CD3+CD4+CD45RO+CXCR5+ICOS+ T cells. Initially, the T_{FH} panel included CD57, because this was reported to be a marker for T_{FH} cells (Kim, Lim et al. 2005). However, after a pilot trial examining at least ten HIV infected untreated and treated patients and healthy controls, although CD4+CD57+ T cells could be counted, especially in untreated HIV-1 infection, these CD4+CD57+ did not express any CXCR5, a standard marker of T_{FH} cells. In addition, references in the literature increasingly suggested that CD57 was not a specific marker of T_{FH} cells (Rasheed, Rahn et al. 2006). Therefore, the CD57 tube was dropped from the T_{FH} panel. See Fig. 73 below for a representative HC and untreated HIV-1 infected patient.

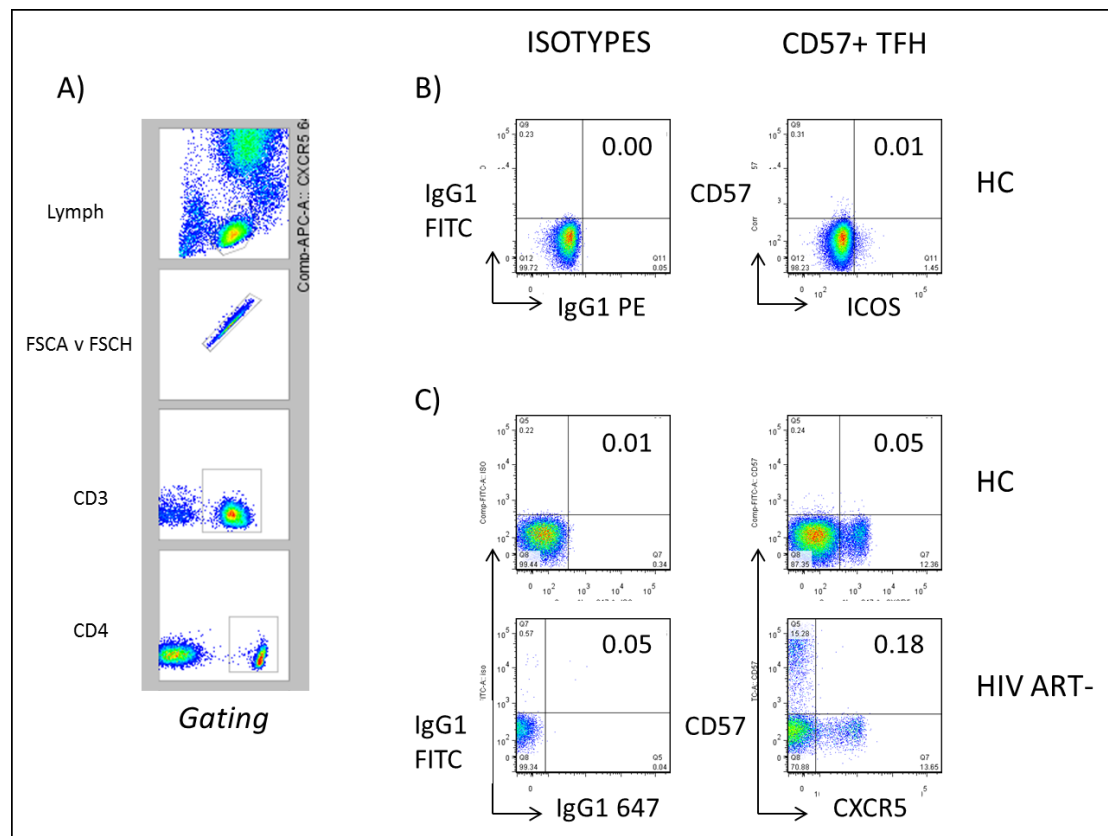


Figure 73: CD57 as a marker for T_{FH} cells.

73A) Gating strategy, B) Representative dual expression of CD57+ICOS+ levels marked in upper-right quadrant, gated using isotype-matched controls. C) Representative dual expression of CD57+CXCR5+ in a HC and HIV ART-patient, percentages in upper-right quadrant as before.

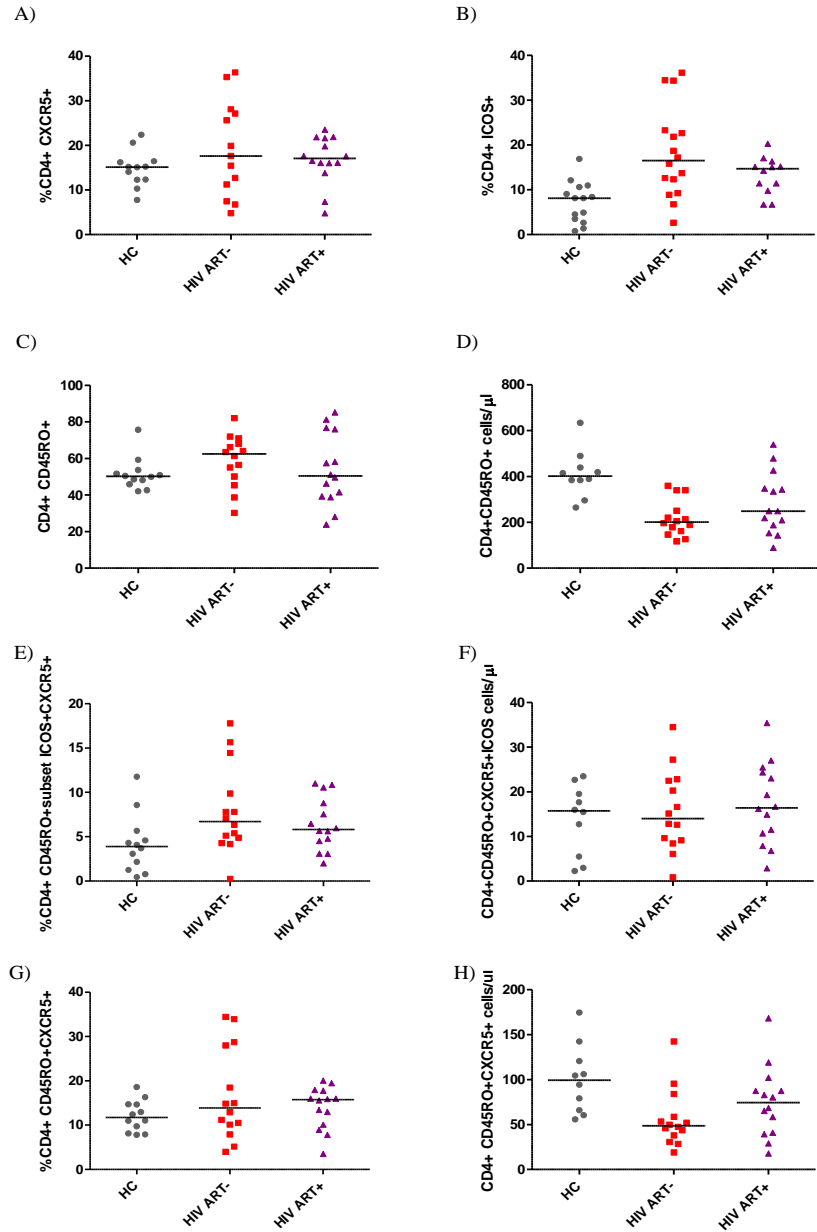


Figure 74: T_{FH} levels in untreated and treated HIV-1 infection and HC

Plots displaying data points and horizontal lines indicate median values for T_{FH} expression and associated markers as a percentage of CD4 T cell subsets or absolute counts, gated on CD3+CD4+ T cells, for healthy controls (HC) (n=11), untreated HIV-1 infected patients (HIV ART-) (n=14) and treated HIV-1 infected patients (HIV ART+) (n=14). 74A) CXCR5+ expression by CD4 T cells, B) ICOS expression by CD4 T cells, C) CD45RO expression by CD4 T cells, D) CD4+CD45RO+ absolute count, E) ICOS+CXCR5 expression by CD4+CD45RO+ T cells, F) CD4+CD45RO+ ICOS+CXCR5+ absolute count, G) CD45RO+CXCR5+ expression by CD4 T cells and H) CD4+CD45RO+CXCR5+

absolute counts. The Mann-Whitney U test was used to analyse non-parametric data.

Consistent with one paper in the literature, 5-15% of CD4+CD45RO+ T cells in the peripheral blood of healthy controls were ICOS+ and CXCR5+, previously defined as T_{FH} cells (Bossaller, Burger et al. 2006) (Fig.74E). Absolute numbers of CD4+CD45RO+ T cells are depleted in HIV-1 infection in both the untreated ($p<0.001$) and treated ($p<0.03$) groups compared to healthy controls (see Fig.74D), despite slightly raised percentages of CD4+CD45RO+ cells (non-significant, see Fig.74C). Although there was heterogeneity in the percentage of T_{FH} T cells in HIV-1 infection (see Fig.74G), the absolute CD4+CD45RO+CXCR5+ T_{FH} population was depleted in untreated infection ($p<0.005$), with a trend towards depletion in some patients of the treated group ($p=0.12$) (Fig.74H).

Overall expression of CXCR5 by CD4 T cells was not significantly different in HIV-1 infection from healthy controls (Fig.74A). Percentages of CXCR5+ICOS+ expression by CD4+CD45RO+ T cells were raised in untreated and treated HIV-1 infected groups ($p<0.03$, $p<0.05$ respectively) (Fig.74E), whilst absolute counts of these cells in HIV-1 infection were no different from healthy controls (Fig.74F), likely due to high levels of ICOS expression skewing data. ICOS was significantly upregulated on all CD4 T cells in both HIV-1 infected groups compared to HC, untreated ($p<0.001$) and treated groups ($p<0.005$) (Fig.74B) and is known to be upregulated on activated T cells (Yong, Salzer et al. 2009). Therefore, use of this marker was not helpful for labelling T_{FH} cells in HIV-1 infection, CD4+CD45RO+CXCR5+ labelling was used in preference.

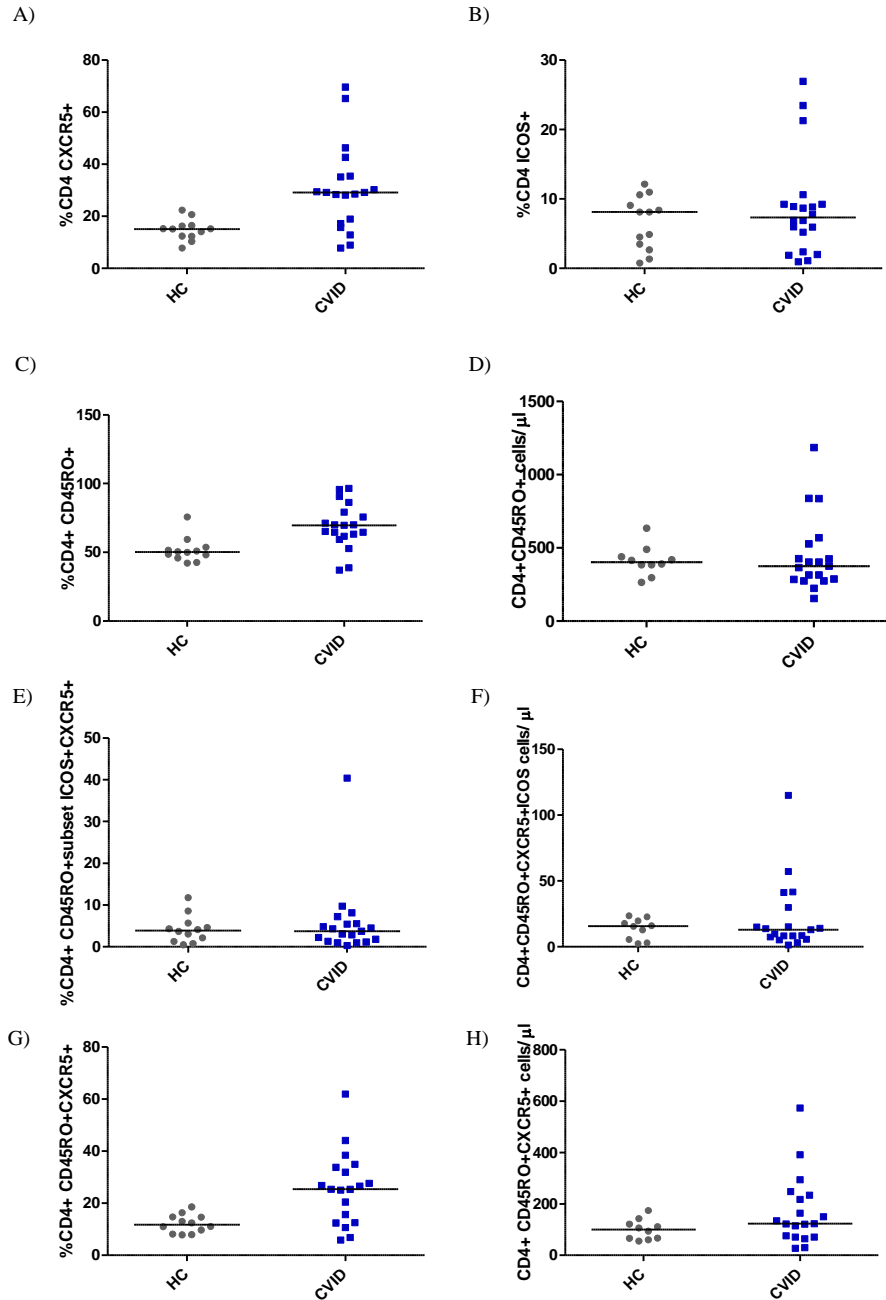


Figure 75: T_{FH} cells in CVID and HC

Plots displaying data points and horizontal lines indicate median values for T_{FH} expression and associated markers as a percentage of CD4 T cell subsets or absolute counts, gated on CD3+CD4 T cells, for healthy controls (HC) (n=11) and CVID patients (n=19). 75A) CXCR5⁺ expression by CD4 T cells, B) ICOS expression by CD4 T cells, C) CD45RO expression by CD4 T cells, D) CD4+CD45RO⁺ absolute count, E) ICOS+CXCR5 expression by CD4+CD45RO⁺ T cells, F) CD4+CD45RO⁺ ICOS+CXCR5⁺ absolute count, G) CD45RO+CXCR5⁺ expression by CD4 T cells and H) CD4+CD45RO+CXCR5+

absolute counts. The Mann-Whitney U test was used to analyse non-parametric data.

The percentage of CD4 T cells expressing CXCR5 or CD45RO were significantly higher in patients with CVID (both $p < 0.003$) compared to healthy controls (Fig.75A and Fig.75C), although individual levels of these cell markers were extremely variable. However, when absolute counts were determined, the levels of CXCR5 (data not shown) or CD4+CD45RO+ T cells were no different from healthy control levels (Fig.75D). The expression of ICOS on CD4 T cells was not statistically different from HC (Fig.75B), although a high level of ICOS expression on CD4 T cells was demonstrated in peripheral blood of a subset of patients. Interestingly, significantly increased percentages of circulating of T_{FH} cells were seen in CVID patients as measured using CD4+CD45RO+CXCR5+ ($p < 0.01$) (see Fig. 75G), however, absolute counts of CD4+CD45RO+CXCR5+ (Fig. 75H) were not significantly different from healthy controls ($p = 0.10$).

6.4 Discussion

In summary, consistent with the hypothesis of this thesis, considerable B cell dysfunction was seen in the BAFF/APRIL chemokine axis. High levels of BAFF and APRIL were found in the peripheral blood of patients with untreated HIV-1 infection, however, these were normalised by anti-retroviral therapy. BAFF was associated with CD4 counts in untreated HIV-1 infection, indicating increased levels during disease progression whilst APRIL levels were negatively associated with levels of tissue like B cells in HIV-1 infection. In CVID, elevated levels of BAFF and APRIL were associated with elevated levels of tissue like B cells and reduced naïve B cells. In turn tissue like B cells and BAFF levels were associated with B cell lymphopenia. The receptors for BAFF, BAFF-R was reduced in HIV infection, especially class switched memory B cells of untreated HIV infection. TACI receptor was shown to be present on tissue like B cells and memory B cells. Unfortunately, due to technical difficulties in staining B cells for BCMA, expression of BCMA was not able to be analysed on memory B cell subsets. Elevated percentages of T_{FH} cells were found in the peripheral blood of patients with CVID and absolute T_{FH} counts were reduced in untreated HIV infection.

6.4.1 High levels of BAFF and APRIL chemokine levels in HIV-1 infection

BAFF levels were shown to be elevated in HIV untreated patients compared to healthy controls. This was consistent with previous work (Stohl, Cheema et al. 2002; Rodriguez, Valdez et al. 2003). Levels of BAFF in untreated HIV infected patients correlated with CD4 T cell counts, indicating increased BAFF during disease progression, again this was consistent with previous work (Stohl, Cheema et al. 2002; Rodriguez, Valdez et al. 2003). Although not significant overall, some patients with HIV-1 infection on treatment had high levels of plasma BAFF.

For the first time, to our knowledge, this study has measured APRIL levels in HIV infection, showing that APRIL levels were significantly higher in the untreated HIV patients but this had almost normalised with ART therapy. However, a proportion of HIV infected patients had very high levels of plasma APRIL: APRIL levels were not related to CD4 count. This current study found that ART therapy, which is associated with reduction of HIV related B cell immune activation, reduction of HIV viral load

and restoration of CD4+ T cell counts, restored BAFF and APRIL chemokine levels to normal, implicating presence of the virus or the immune response to the virus as a factor in up-regulating BAFF and APRIL levels.

6.4.2 APRIL levels are associated with decreased levels of tissue like B cells in untreated HIV-1 infection

Interestingly, in our small cohort of untreated HIV infected individuals, levels of plasma APRIL negatively correlated with tissue like B cell percentages. APRIL levels were normalised by ART therapy in most cases. This finding needs further investigation.

6.4.3 High levels of BAFF and APRIL are seen in CVID and associated with expansions of tissue like B cells

Elevated levels of BAFF and APRIL were found in this cohort of CVID patients, consistent with a recent publication (Knight, Radigan et al. 2007) and investigations into patients with selective IgA deficiency (Fabris, De Vita et al. 2009). APRIL levels were strikingly elevated in almost every patient with CVID in comparison with the healthy control group and at very high levels in a proportion of individuals with CVID. In a proportion of CVID patients, a strikingly elevated level of BAFF was found. Consistent with a previous report, this increase in BAFF did not correlate with B cell number or memory B cell percentage, however, this previous work did not evaluate tissue like B cells (Knight, Radigan et al. 2007). Unlike HIV infection, there was no correlation between BAFF levels and CD4 T cell counts of CVID patients.

In this chapter, for the first time, a significant association was found between levels of the B cell growth factors BAFF or APRIL levels and levels of tissue like B cells in CVID. Stratifying CVID patients into greater or less than 10% of tissue like B cells demonstrated that those patients with high levels of circulating plasma BAFF or APRIL were those individuals with >10% tissue like B cells, classified as 1a in the Freiburg scheme or smB-CD21lo and smB+CD21lo in EURO Class. A previous study has shown that the Freiburg 1a CVID patients are associated clinically with splenomegaly and granulomatous disease (Wehr, Kivioja et al. 2008).

Further analysis showed that tissue like B cell numbers were strongly associated with B cell counts in CVID, specifically increased tissue like B cell percentages were associated with both B cell lymphopenia and loss of mature naïve B cells. CVID patients have functional/percentage defects in the classical memory B cell compartment. Patients with expanded CD21^{low} subsets thus have few memory B cells, few naïve B cells, B cell lymphopenia but small surviving expansions of innate, (possibly anergic or auto reactive B cells) and high levels of B cell growth factors. Elevated levels of BAFF and APRIL in these patients may contribute to clinical features of CVID e.g. autoimmune complications and/or splenomegaly is associated with expanded CD21^{low} subsets in CVID. Elevated levels of BAFF may be due to an attempt to rebalance B cell homeostasis and repopulation in these CVID patients: B cell lymphopenia after depletion therapy is associated with increased BAFF levels, suggesting BAFF is a regulator of B cell homeostasis and repopulation (Pers, Devauchelle et al. 2007; Krumbholz, Faber et al. 2008) .

6.4.4 Little data determined for IL-7 ELISA may be due to patient cohort or due to a lack of sensitivity of the Diaclone IL-7 kit.

Plasma IL-7 levels were not detected in healthy controls, most untreated HIV-1 infected patients and most patients on ART. Detectable levels were found for 46% of CVID patients. Low IL-7 levels were seen in the 14 patients in the immunophenotyped untreated HIV-1 cohort, this is perhaps explained by a lack of advanced disease since a relatively high median CD4 count was recorded for this cohort. Levels of IL-7 in the sera of patients with HIV-1 infection were lower using this Diaclone assay than reported for the R&D kit in one paper (median 18 pg/ml) (Fry, Connick et al. 2001), but not in another paper using the R&D IL-7 kit (2.12pg/ml) (Napolitano, Grant et al. 2001). In previous work, levels of plasma IL-7 were found to be related to disease progression (CD4 count or viral load) (Fry, Connick et al. 2001; Napolitano, Grant et al. 2001; Malaspina, Moir et al. 2006). The Diaclone IL-7 kit has a minimum limit of detection of 3pg/ml and may not be sensitive enough for to measure IL-7 levels in healthy subjects and HIV-1 infected patients with early disease.

Whilst increased levels of serum IL-7 have been associated with increased transitional B cell numbers in the peripheral blood of patients with HIV infection [60], the authors were not able to distinguish the effects of decreased CD4 counts and increased VL from the correlation between IL-7 and transitional B cell numbers. It is possible that the IL-7 was increased in a homeostatic response to CD4 depletion or related to viral load. Recently, IL-7 levels in HIV-1 vertically infected children were not found to be correlated with transitional B cell percentages and were lower than in uninfected individuals (Cagigi, Palma et al. 2010). The role of IL-7 in human B cell development is under debate, since much of the available published evidence was provided from murine studies (LeBien and Tedder 2008). It would have been useful to determine if levels of plasma IL-7 were associated with transitional B cell percentages, lymphopenia, CD4 or BAFF. Unfortunately, due to the lack of sensitivity of the ELISA, not enough samples had detectable IL-7 to determine any associations.

6.4.5 Expression of B cell chemokine receptors on peripheral B cell subsets in HC, patients with HIV-1 infection and CVID

To our knowledge, this is the first study to examine the expression of the B cell chemokine receptors BAFF-R and TACI on specific memory B cell subsets: IgM memory, class switched memory and tissue like memory. Unfortunately, due to technical difficulties in staining B cells for BCMA, expression of BCMA was not able to be analysed on memory B cell subsets.

6.4.6 TACI is present on classical memory B cells and tissue like B cells in HC and disease and not decreased in HIV-1 infection

This has shown TACI to be upregulated to a similar high percentage on each memory B cell subset in healthy controls, HIV-1 infected patients and patients with CVID and indicates that TACI is not selectively decreased on memory B cells in HIV infection as expected. There was even a trend for TACI to be upregulated overall and on IgD+ CD27- B cell subset in patients with HIV-1 infection. This upregulation in HIV infection was due in part to the presence of TACI on CD21^{low} tissue like B cells. The novel description of tissue like memory B cells expressing TACI at an intermediate level suggests a mechanism for this subset to be stimulated by BAFF and APRIL. TACI levels correlated with tissue like B cell numbers in untreated HIV-1 infected patients (p=0.04). This is likely to be due to TACI expression on the high percentage

of CD21^{low} tissue like B cells seen in these patients. TACI expression of class switched B cells in CVID was markedly heterogeneous, with some patients having normal levels of TACI expression, whilst other patients demonstrated extremely low TACI expression on class switched B cells, which needs further investigation. Signalling through TACI may decrease the size of the B cell pool. There is evidence that TACI could directly transmit inhibitory or apoptotic signals to B cells (Sakurai, Hase et al. 2007).

6.4.7 *Decreased BAFF-R expression on class switched memory B cells in untreated HIV-1 infection*

Loss of BAFF-R on B cells in untreated HIV infection was consistent with a previous publication and found here to be predominantly lost on the class switched memory B cell subset, which has implications for T cell dependent immunity. Loss of BAFF-R means that BAFF signalling will be reduced, which may affect CSR or survival signals in both T cell dependent and T cell independent humoral immune responses and may affect B cell counts. BAFF-R down regulation may be a physiological homeostatic mechanism to excess BAFF or may reflect early terminal differentiation. Further work should be done to evaluate loss of BAFF-R on class switched memory B cells and responses to a T cell dependent antigen such as tetanus toxoid vaccination. In untreated HIV-1 infected individuals, BAFF-R expression was not associated with CD4 counts or viral load, therefore loss of BAFF-R is not linked to disease progression and probably occurs at an early stage of infection. This study has shown for the first time that mature CD21^{low} B cells in HIV infection express BAFF-R at high levels.

Decreased expression of the BAFF receptor on B cells may cause decreased responsiveness of B cells to the B cell survival factor BAFF, which may then cause increased or early apoptosis of BAFF-R negative cells. *Moir et al* proposed that this was as a result of decreased expression of the BAFF receptor on CD21^{low} B cells (Moir and Fauci 2009). CD21^{low} expression is not specific to a particular B cell subset. This previous study did not look at mature B cells, activated memory B cells or tissue like B cells and only ascertained that immature transitional B cells, which are CD21^{low}, had decreased surface BAFF-R expression and decreased responsiveness to BAFF in vitro. BAFF can also signal through TACI receptor, which is unlikely to be

present on immature transitional B cells. This current work has extended this work into mature B cell memory subsets and the newly identified CD21^{low} tissue like B cell subset and shown that class switched memory B cells (CD21^{low} or CD21^{high}) are the predominant mature B cell population to lose BAFF-R expression in HIV-1 infection.

6.4.8 *Overexpression of BAFF and APRIL in murine models*

BAFF and APRIL are important for B cell homeostasis and normal B cell growth. High levels of BAFF and APRIL have been found in patients with dysregulated B cell compartments: autoimmune disease (SLE), B cell malignancy, immune deficiency (CVID) and in HIV. Animal models overexpressing BAFF or APRIL provide valuable information regarding the pathogenic effect of excess B cell chemokines. Transgenic mice over expressing BAFF (BAFF-Tg) display splenomegaly, high levels of lymphocyte proliferation disorders, T cell independent autoimmunity and a previously undefined expanded mature B cell subset [123, 124]. Although this cannot be confirmed without further immunophenotyping of BAFF-Tg mice, this expanded B cell subset may correspond to the expanded tissue like B cells seen in human disease and immune deficiency. Apart from the hypergammaglobulinaemia, CVID patients show many similarities with BAFF-Tg mice. In a study of MHC II deficient mice, overexpression of BAFF increased production of IgM, IgG and IgG autoantibodies (Stohl, Jacob et al. 2010). High levels of BAFF reported in autoimmune diseases are thought to override peripheral tolerance of auto reactive B cell clones, allowing these to survive deletion/anergy. April-Tg differ from BAFF-Tg mice since APRIL-Tg mice do not develop autoimmunity nor have altered B cell homeostasis. However, 40% of ageing APRIL-Tg mice develop B1 B cell neoplasia and have additional T cell abnormalities [235]. APRIL-Tg mice display increased proliferation and survival of CD4⁺ and CD8⁺ T cells, with increased production of IL-2 by CD8⁺ T cells, increased survival of super antigen-reactive T cells associated with up regulated BCL-2 and decreased percentages of T cells in peripheral lymph nodes [235].

6.4.9 *Mechanisms of BAFF and APRIL upregulation*

Increased production of BAFF and/or APRIL described in this thesis may be linked to chronically activated plasmacytoid DCs and other immune cells secreting increased

IFN- α and upregulating production of BAFF and APRIL in other immune cells. IFN- α and other pro-inflammatory cytokines such as TNF, IL-6, IL-10 and CD40-L have been described at increased levels in the peripheral blood of untreated HIV-1 infected individuals and are thought to trigger B cell hyperactivation seen in HIV-1 infection (Moir and Fauci 2009). IFN stimulated genes (ISGs) are associated with response to infection and upregulated in HIV-1 infection.

High levels of IFN- α may be a biomarker of immune activation and chronic immune stimulation of plasmacytoid DCs in pathogenic HIV infection or simian immunodeficiency virus (SIV). This chronic immune stimulation may drive disease progression to AIDS. Using SIV, where infections of rhesus macaques (RM) are characterized by immune activation and progressive CD4⁺ T cell depletion, RM produce much higher levels of IFN- α by plasmacytoid DCs in response to SIV and TLR7/9 ligands *ex vivo* in comparison with sooty mangabeys, the natural reservoir hosts for SIV that do not progress to AIDS. Sooty mangabeys do not exhibit dysfunctional immune activation and present normal CD4⁺ T cell populations, despite high levels of SIV replication (Mandl, Barry et al. 2008). Consistent with this, in non-pathogenic SIV infection of African green monkeys, increases in IFN- α and decreases in pDC were transient and only observed in acute infection (Diop, Ploquin et al. 2008). Microarray studies support the importance of IFN levels in HIV-1 pathogenesis (Giri, Nebozhyn et al. 2006). HIV-1 infected macrophages or HIV-1 gp120 stimulated macrophages upregulate IFN/IFN associated genes (Giri, Nebozhyn et al. 2006) and promote a proinflammatory environment of chronically activated macrophages. Microbial products such as LPS released in the gut during HIV associated microbial translocation may also induce the upregulation of IFN- α and TNF production.

6.4.10 Possible HIV-1 mediated modulation of BAFF function and BAFF up-regulation in HIV-1 infection

Viral HIV-1 proteins may act with BAFF to modulate the immune system by acting in synergy or by directly inducing BAFF secretion by haematopoietic cells. *In vitro*, BAFF is known to enhance optimal IgG and IgA production through CpG DNA stimulation during T cell independent class switching in healthy individuals (He, Qiao

et al. 2006). Viral dsRNA and BAFF can synergise to trigger T cell independent class switching (Xu, Santini et al. 2008). Poly (I:C), a viral RNA stimulant added with BAFF *in vitro*, induced proliferation of IgD+CD38-, GC founder/activated and GC B tonsil cells but not IgD-CD38- B cells (Xu, Santini et al. 2008). These IgD+CD38- tonsil B cells expressed TLR-3, a viral dsRNA receptor. An IgD+ B cell fraction upregulated AID and IgA/IgG production after stimulation by BAFF/IL-10/Poly (I:C). As discussed previously, the IgD+CD38-fraction may include IgD+ tissue like B cells and IgM memory B cells. Further investigations confirmed IgM memory B cells strongly express TLR-3 (Xu, Santini et al. 2008). BAFF secretion was up regulated by myeloid dendritic cells in response to IFN- α and/ or viral DNA. Therefore microbial/viral ligands can further upregulate the production of BAFF and APRIL by DCs and viral products have the ability to trigger class switching. BAFF and APRIL are known in HIV-1 infection to be able to induce CD40 independent IgG and IgA class switching (Litinskiy, Nardelli et al. 2002) and BAFF additionally in synergy with ferritin (Xu 2009).

6.4.11 Compensatory mechanisms secondary to B cell lymphopenia

BAFF blockers and B cell depletion therapy has extended current knowledge of BAFF function in lymphopenic conditions, B cell homeostasis and reconstitution of the immune system. B cell depletion therapy is associated with increased BAFF levels, suggesting BAFF is a regulator of B cell homeostasis and repopulation (Pers, Devauchelle et al. 2007; Krumbholz, Faber et al. 2008). Inhibition of BAFF by therapeutic agents in mice has demonstrated that precursor or naïve B cells are subsequently depleted, whilst memory B cells or antibody clones are predominantly not affected, suggesting pre-existing memory B cells survive independently of BAFF (Scholz, Crowley et al. 2008). BAFF and APRIL have multiple, partially resolved roles in the development of the immune system and homeostasis. Although out of the scope of this thesis, increased BAFF may also have a role in T cells function.

6.4.12 Follicular helper T cells

This study has examined the CD4+CD45RO+CXCR5+ICOS+ T_{FH} population of patients with untreated and treated HIV-1 infection for the first time, to our knowledge. The absolute number of the CD4+CD45RO+CXCR5+ subset is decreased

in untreated HIV-1 infected patients but this is not a selective depletion and may reflect the overall loss of CD4 T cells in HIV infection. The results found here contrast with one previous study investigating untreated HIV-1 infection, reported CD4+CD45RO+BLR1 (CXCR5) percentages to be lost during progressive disease (Förster, Mattis et al. 1996). This previous work preceded the advent of HIV treatment and so comprised many patients with severely reduced CD4 counts (<200 cells/ml), which may explain these differences, since only one patient in the current work fitted this criteria. The T_{FH} subset may have an important role in T cell dependent functional immunity and depletion of this subset in HIV-1 infection may have a role in the impairment of humoral vaccination responses in HIV-1 infected patients and loss of T cell dependent serum antibody levels. Although ICOS is an additional marker for T_{FH} cells, ICOS is upregulated on activated T cells (Yong, Salzer et al. 2009) and measurement was not useful for T_{FH} determination in HIV-1 infection, where a high level of preactivation of peripheral CD4 cells was seen. Some believe that abnormal expression of ICOS by naïve T cells promotes abnormal expansion of T_{FH} cells, autoantibody production and enlarged germinal centres (Vinuesa, Tangye et al. 2005), suggesting that over expression of ICOS is involved in tolerance breakdown and induction of autoimmunity, which is seen at increased rates in HIV-1 and other conditions with increased percentages of T_{FH} such as in SLE.

CVID patients have impaired T cell mediated immunity yet increased percentages of peripheral blood T_{FH} T cells (CD4+CD45RO+CXCR5). CXCR5 may be upregulated upon activation, as suggested in one early paper studying HIV (Förster, Mattis et al. 1996), however this has not been examined elsewhere. In patients with CVID, there was an extremely variable level of CD4+CD45RO+CXCR5+ T cells expressing ICOS, which may reflect the heterogeneity of T cell activation in patients with this disease. Some variability of this may be due to ICOS deficiency which has been reported in a small proportion of patients with CVID (Bossaller, Burger et al. 2006). Since ICOS is expressed by T_{FH} cells, patients with ICOS deficiency have a reduction of CD4+CD45RO+ICOS+CXCR5+ T cells by definition (Bossaller, Burger et al. 2006). The percentages of CD4+CD45RO+CXCR5+ in ICOS deficient patients were reported to be less than 5% (Bossaller, Burger et al. 2006). Evaluating our cohort of patients, we found no patients had <5% of peripheral CD4+CD45RO+CXCR5+ T cells, suggesting ICOS deficiency is unlikely in our CVID patients. In many patients

with CVID, most of the CD4 T cells were of a CD45RO phenotype and there were a higher percentage of CD4 T cells expressing CD45RO and CXCR5. This may reflect few CD45RA naïve T cells in the peripheral blood, which has been previously reported in CVID and is associated with a more severe disease and early death (Giovannetti, Pierdominici et al. 2007). However, interestingly enough, when absolute counts for CD4+CD45RO+ T cells were examined, there was no difference between memory T cells in CVID compared to controls in our cohort.

6.5 Conclusions

6.5.1 Patients with HIV-1 infection Summary

Consistent with the hypothesis of this thesis, considerable B cell dysfunction was seen in the BAFF/APRIL chemokine axis. High levels of B cell growth chemokines and dysregulated expression of B cell chemokine receptors may be one of the mechanisms playing a role in the loss of the memory B cell subsets seen in HIV infection.

6.5.2 Untreated HIV-1 cohort

The untreated HIV infected patients in this cohort were heterogeneous regarding memory B cell percentage. Consistent with results in Chapter III and in other publications, a loss of IgM memory B cell subsets was observed in some untreated HIV infected patients in comparison with healthy controls. However, whilst 4/14 patients had a very low memory B cell percentage, some of the untreated cohort had normal or increased total memory B cell subsets, which was reflected in a normal median total memory B cell count. This is in contrast with results seen in Chapter III and in other studies, where a significant loss of total memory B cell counts in HIV infection was observed. Since performing the study in Chapters III and IV, the BHIVA guidelines have changed recommendations for starting ART therapy. As a result, patients now start ART therapy when CD4 counts drop to 350 cells/ml, rather than 200 cells/ml. As was shown in Chapter III, IgM memory B cells correlate with CD4 T cell counts and so are lost during disease progression. Therefore, the patients in the untreated cohort were likely to have higher CD4 T cell counts and higher memory B cell levels. Commencing ART early may prevent some loss of peripheral memory B cells in some patients. Although it has been reported that memory B cells are lost soon from the gut during SIV infection of Rhesus macaques (Titanji, Velu et

al. 2010), this does not seem to be the case in HIV-1 infection (Levesque, Moody et al. 2009).

In summary, despite relatively higher peripheral CD4 T cell counts in this untreated HIV infected patient cohort, high levels of dysfunction was still seen in the B cell BAFF/APRIL chemokine axis. Loss of BAFF-R, especially on class switched memory B cells, high levels of plasma BAFF and APRIL and upregulated TACI on memory B cells, including the CD21^{low} expanded subset, were found and all may play a role in B cell dysregulation, impaired B cell maturation and B cell activation seen in HIV infection. BAFF-R expression is not associated with CD4 counts or viral load, therefore loss of BAFF-R is not linked to progression and probably occurs at an early stage of infection. Regarding APRIL, APRIL levels were not related to CD4 count, but negatively correlated to tissue like B cell levels and normalised by ART therapy in most cases.

6.5.3 HIV-1 infected patients on ART

In this cohort of HIV-1 infected patients on ART, reduced IgM memory B cells were found, consistent with the results in Chapter III. Interestingly, the patients in this cohort had a decreased median IgM memory B cell level lower than HIV-1 infected patients in the untreated cohort. This may reflect a cohort of treated patients that started ART therapy later, at an advanced stage of disease or it may reflect loss of memory B cells over time, despite ART treatment. From the results in Chapter III, we showed that time on ART did not restore IgM memory B cell levels. Further work will be done to determine whether the latter is the case.

ART treatment normalised BAFF and APRIL levels in most HIV-1 infected patients on ART therapy. This was despite very low IgM memory B cell counts in this cohort of patients, suggesting the loss of memory B cells occurred during primary HIV-1 infection and were not restored by ART therapy. It is possible that high levels of BAFF and APRIL are secreted to regulate B cell homeostasis, since subjects with B cell immune deficiency seem to have high levels of these chemokines (Knight, Radigan et al. 2007; Fabris, De Vita et al. 2009). How ART therapy normalises BAFF and APRIL levels is not clear. In addition, BAFF levels are known to be correlated with CD4 T cell counts in untreated HIV infection, suggesting disease progression/or

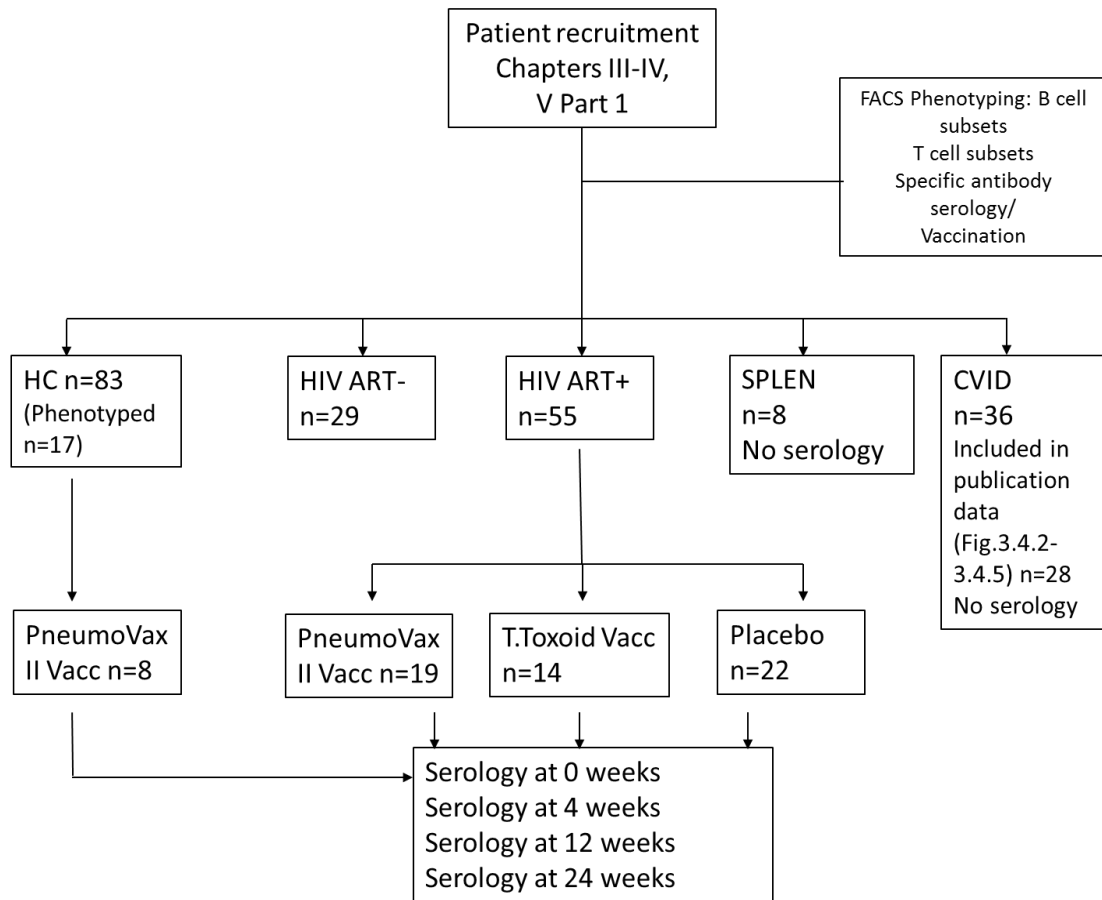
T cell count is important for regulating BAFF levels. In addition, although total BAFF-R levels were reduced in the treated HIV-1 cohort compared to healthy controls, HIV-1 patients on ART did not have a significant loss of BAFF-R on class switched memory peripheral B cells, in contrast to untreated HIV infection. This normalisation of class switched B cell terminal differentiation may reflect normalisation of BAFF and APRIL levels during ART treatment. It may be possible that in untreated HIV-1 infection, BAFF-R is down regulated on class switched memory in a negative feedback loop in response to elevated levels of B cell chemokines such as BAFF and APRIL. However, BAFF-R was significantly reduced on IgM memory B cells and naïve B cells of HIV-1 infected patients on ART. The relevance of this finding is unclear, except to consider this group had overall reduced IgM memory B cell percentages.

6.5.4 Summary for CVID cohort

Reports of an innate CD21^{low} B cell population in CVID were published whilst my data was being analysed (Rakhmanov, Keller et al. 2009; Isnardi, Ng et al.). We have extended these findings by showing that these CD21^{low} B cells express high levels of TACI (a receptor for BAFF and APRIL expressed predominantly on classical memory B cells) and are associated with B cell lymphopenia, loss of mature B cells and high levels of BAFF and APRIL. CVID patients have a high risk of autoimmune disease and lymphocyte dysregulation including a high risk of B cell lymphoma, many characteristics associated with high levels of B cell growth factors. The underlying intrinsic memory B cell defect in patients with CVID may be the mechanism causing the inhibition of hypergammaglobulinaemia in CVID, despite excess BAFF and APRIL. BAFF and APRIL may both stimulating cells through TACI which is expressed mainly on CD27⁺ memory B cells and activated memory B cells. In this aim, it was determined whether tissue like B cells expressed TACI receptor in CVID. This study demonstrated that TACI is present on the tissue like B cell subset in CVID patients, providing a mechanism for BAFF and APRIL to stimulate the growth of this tissue like B cell compartment. Consistent with findings here, a recent publication has also found TACI to be present on CD21^{low} B cells in healthy controls (Isnardi, Ng et al.).

7. Chapter VII: General Discussion

A)



B)

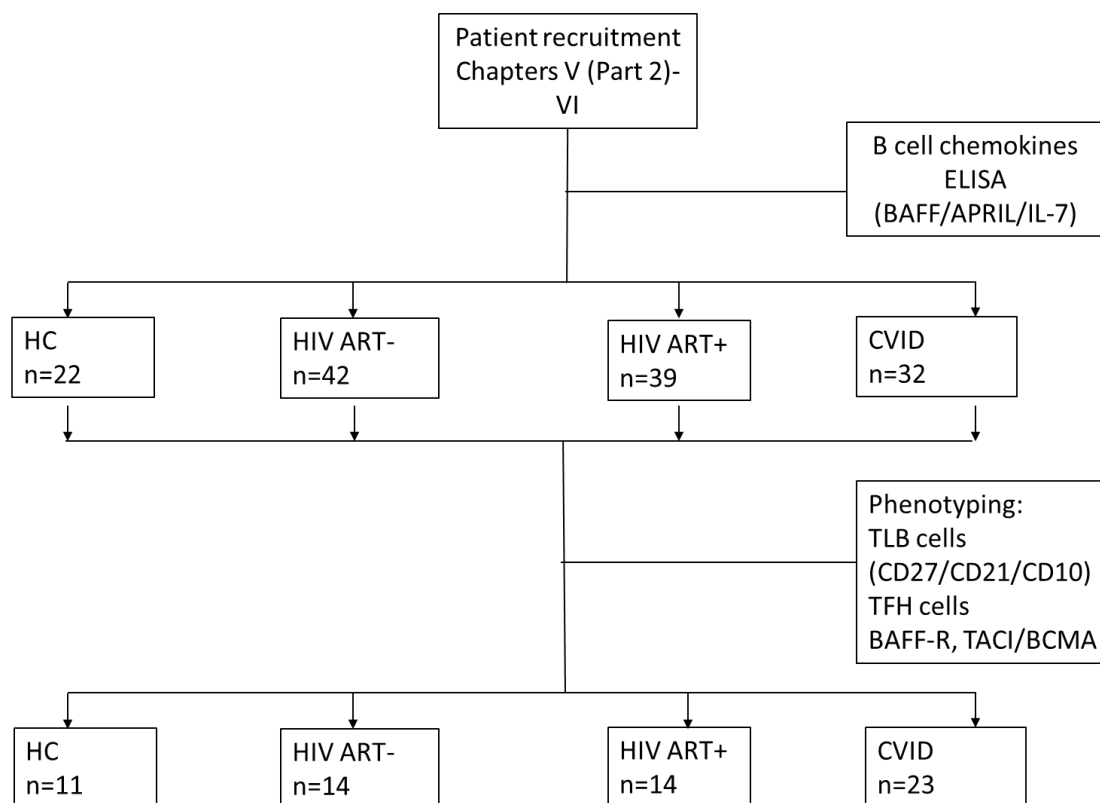


Figure 76: Summary of experimental cohorts described in this thesis: A) Experimental cohorts tested in Chapters III-V Part One and B) Experimental cohorts tested in Chapters V Part Two-VI

See Fig.76 for a summary diagram of patient cohorts studied in this thesis. Assessment of B cell subsets in healthy controls, HIV-1 infection, CVID and individuals who had undergone splenectomy showed that total memory B cell percentages were reduced in patients with CVID, HIV-1 and splenectomy. As expected, IgM memory B cell percentages were reduced in splenectomised patients and patients with CVID. In addition, IgM memory B cell percentages were significantly reduced in HIV-1 infection and not restored by ART. Overall percentages of class switched B cells were significantly reduced in patients with CVID or splenectomy. Using a B cell classification scheme to define humoral defects in CVID, 25% of HIV-1 infected individuals had marked reductions in class switched memory B cell percentages, regardless of treatment status. Transitional B cell populations were expanded in all patient groups (untreated and treated HIV-1 infection, CVID and individuals with splenectomy) compared to healthy controls. Individuals with CVID and HIV-1 infected individuals therefore displayed numerous

B cell abnormalities and in some HIV-1 patients, B cell memory defects in both subsets were similarly severe as CVID patients with the most impaired B cell memory phenotype. Reduced IgM memory B cells in 70% of untreated HIV-1 infected patients suggested functional hyposplenism similar to that seen in splenectomised individuals.

Pneumococcal IgG and tetanus IgG antibodies were statistically lower in patients with untreated and treated HIV-1 infection. There was a trend towards a reduction in IgM pneumococcal levels in both HIV groups but this was not statistically significant. IgM memory B cell percentages correlated with pneumococcal IgM levels, whilst class switched memory B cell percentages correlated with tetanus IgG levels. Vaccination in most HIV-1 infected individuals to tetanus toxoid or pneumococcus was shown to be impaired. Low pre-existing pneumococcal IgM antibodies and impaired vaccination responses were associated with a loss of IgM memory B cell subset, suggesting susceptibility to IPD. Likewise, low levels of pre-existing tetanus toxoid antibody correlated with an impaired response to vaccination. High levels of T cell immune activation were found in patients with HIV-1 infection but this was not associated with vaccination responses.

Tissue like B cells are increased in HIV patients off ART treatment and in patients with CVID compared to healthy controls. Differential staining using CD11c or FCLR4 identified potential novel subsets of tissue like B cells. However, these markers were not specific to tissue like B cell populations and had to be stained in combination with established tissue like B cell markers. In the untreated HIV-1 infected cohort, levels of tissue like B cells were significantly associated with percentages of IgM memory B cells but not to any marker of T cell disease progression such as viral load or CD4 T cell count, suggesting an intrinsic B cell defect common to both B cell subsets. This was not true for ART treated patients, suggesting a role for HIV replication. Interestingly, increased percentages of tissue like B cells indicated a better vaccine response to tetanus toxoid vaccination in HIV infected patients on treatment.

High levels of BAFF and APRIL were found in the peripheral blood of patients with untreated HIV-1 infection, however, these were normalised by ART. In untreated HIV-1 infection, BAFF was associated with disease progression whilst APRIL levels

were negatively associated with levels of tissue like B cells. In CVID, elevated levels of BAFF and APRIL were associated with elevated levels of tissue like B cells and reduced naïve B cells. In turn, tissue like B cells and BAFF levels were associated with B cell lymphopenia in CVID. The receptor with the highest affinity for BAFF, BAFF-R, was reduced on B cells in HIV-1 infection, especially on class switched B cells: a significant proportion of class switched memory B cells in untreated HIV-1 infection were negative for this receptor. The receptor TACI was shown to be present on tissue like B cells in addition to classical CD27+ memory B cells. Unfortunately, expression of BCMA was not able to be determined. Elevated peripheral blood percentages of T_{FH} cells were found in CVID, whilst in untreated HIV-1 infection, absolute T_{FH} counts were reduced.

7.1.1 Possible Mechanisms for loss of IgM memory B cells in HIV-1 infection and CVID reported in this thesis

Many deleterious mechanisms have been hypothesized to contribute to a loss of B cell memory in HIV-1 infection, such as: increased rates of cell turnover, increased apoptosis of memory B cells, decreased B cell survival factors or HIV-1 mediated bystander killing (Moir and Fauci 2009) but the exact mechanisms of T cell or B cell loss in HIV-1 infection remain undefined. IgM memory B cells require the spleen for maturation, so it can be hypothesised that levels of IgM memory B cells may be a marker of HIV-1 mediated splenic disturbance or permanent destruction of splenic architecture. HIV-1 mediated damage of splenic tissues could impair IgM memory B cell maturation, trigger apoptosis or induce CD8+ mediated B cell killing of resident IgM memory B cells, resulting in fewer circulating IgM memory B cells. It is known that CD8 T cells are increased in the spleen of HIV-1 infected individuals (Wilkins, Davis et al. 2003).

This hypothesis is supported by evidence of selective atrophy of the human splenic marginal zone in HIV-1 infected individuals (Wilkins, Davis et al. 2003). This splenic damage is thought to occur early after infection. The spleen is the predominant site for maturation of this subset so an inhospitable spleen would impair IgM memory B cell numbers. Also, the spleen is a major reservoir for memory B cells (Mamani-Matsuda,

Cosma et al. 2008). Splenic damage may increase in severity during untreated chronic infection and may be indicative of B cell progressive disease. Counting of pitted red blood cells, a sign of splenic disturbance, of HIV-1 infected individuals would help confirm this hypothesis. In several studies of untreated HIV-1 infected individuals (Titanji, Chiodi et al. 2005; Titanji, De Milito et al. 2006; D'Orsogna, Krueger et al. 2007; Hart, Steel et al. 2007; Moir, Malaspina et al. 2008), IgM memory B cell reductions are variable. A possible explanation is that viral induced splenic damage/IgM memory B cell loss is an event independent of viral replication (VL) or CD4 counts but the likelihood of this damage occurring increases the longer a patient is off ART.

Although HIV-1 does not infect B cells, HIV-1 infection is localised to secondary lymphoid tissues (Fauci 1993; Haase, Henry et al. 1996) and it is thought HIV-1 may use the spleen and secondary LNs as a viral reservoir, for example, infected FDCs are known to reside in GCs (Shen and Siliciano 2008). HIV-1 particles Nef and Env accumulate in lymphoid follicles (Moir and Fauci 2009). Lymph node architecture in HIV-1 infection is disturbed, possibly caused by fibrosis (van Grevenynghe, Halwani et al. 2008); this may perturb GC T-B cell interactions and additionally impair T cell dependent humoral immunity. Anti-retroviral therapy is thought to be only able to partially restore LN architecture (van Grevenynghe, Halwani et al. 2008) and HIV-1 structural proteins are still present in LN despite ART (Popovic, Tenner-Racz et al. 2005).

Viral particles interacting with B cells in the secondary lymphoid tissues such as LN or spleen may further disturb B cell homeostasis and function. For instance, HIV associated isotype switching (CSR) of IgM+ B cells may contribute to reduced circulating IgM memory B cells: HIV gp120 bound to tonsil B cells of an DC-SIGN+ IgM memory B cell phenotype *in vitro*, has been linked to B cell activation and induction of CSR by a BAFF-dependent mechanism (He, Qiao et al. 2006) whilst Env is also associated with increased AICD, the enzyme needed for CSR. In another study, gp120 binding to VH3+ memory B cells subsequently led to a loss of VH3 specific memory B cells (Berberian, Goodglick et al. 1993; Cagigi, Nilsson et al. 2010) suggesting a direct mechanism for viral-mediated depletion. HIV-1 infected macrophages may transfer Nef through inter-cellular conduits to inhibit B cell class

switching in follicular but not extra follicular areas of lymphoid tissues. In the extra follicular areas, upregulation of BAFF by macrophages associated with increased CSR and nonspecific IgG and IgA hypergammaglobulinaemia characteristic of HIV-1 infection (Xu 2009). Finally, Tat derived from infected T cells is thought to induce B cell apoptosis by CD95 (Fas) up regulation (Huang, Li et al. 1997).

7.1.2 Controversies regarding the role of IgM memory B cells in protection against encapsulated bacteria

Recent work on splenectomised individuals called into question the proposed non-redundant role of IgM memory B cells in protection against encapsulated bacteria (Wasserstrom, Bussel et al. 2008). Functional hyposplenism is associated with reduced IgM memory B cells in splenectomised individuals and hyposplenic conditions such as HIV-1, IBD and Coeliac disease (Carsetti, Rosado et al. 2005; Di Sabatino, Rosado et al. 2005; Carsetti, Pantosti et al. 2006; Di Sabatino, Rosado et al. 2007; Hart, Steel et al. 2007; Di Sabatino, Rosado et al. 2008). Data in this thesis supports the importance of IgM memory B cells in the immune response against encapsulated bacteria. In HIV-1 infection, which is known to increase the risk of invasive pneumococcal disease, IgM memory B cells were found to be reduced and associated with impaired responses to pneumococcal vaccination in HIV-1 treated individuals. In CVID patients with very few class switched memory B cells, the presence of IgM memory B cells was protective against the development of bronchiectasis, a known complication of respiratory infections due to encapsulated bacteria.

There is a belief held by some researchers that class switched B cells are also reduced in conditions associated with reduced IgM memory B cell compartments and that impairment of immunity is primarily caused by a lack of class switched B cells (Tangye and Good 2007). 25% of HIV-1 infected individuals in Chapter III of this thesis did display reduced class switched B cells, however 70% of untreated patients had reduced IgM memory B cells. The CVID data also did not support this. Most CVID patients had reduced class switched B cells but only a proportion of them had bronchiectasis. In contrast with some previous studies but not others, we found reductions in class switched B cell percentages were not associated with

bronchiectasis or bronchiectasis and airway limitation, whilst low IgM memory B cell percentages increased the risk of bronchiectasis in CVID patients with severely reduced class switched B cells.

It is unclear whether protection against blood-borne encapsulated bacteria is mediated entirely by IgM memory B cells or that IgM memory B cells are a marker for a poorly functioning spleen, which is required to clear blood-borne pathogens. Most of the populations characterised to have reduced IgM memory B cells also have hyposplenism. CVID is slightly different, although some CVID patients have granulomatous disease resembling sarcoid and splenomegaly, so also have a disturbed spleen. It is likely that a poorly functioning spleen affects all memory B cell subsets to some extent since the spleen is a major reservoir for memory B cells, however, an impaired spleen seems to affect circulating IgM memory B cells more, implying redundancy for class switched B cells to mature and function elsewhere, such as in the secondary lymph nodes.

The peripheral IgM memory B cell subset is assumed to be the circulating equivalent of MZB cells in the splenic marginal zone which respond to TI antigens (Weill, Weller et al. 2009). Naïve B cells, IgM memory and class switched B cells are similarly found in the spleen. The splenic IgM memory B cell subset is a similar phenotype to that seen in the blood (IgM^{high}IgD^{low}CD23⁻CD21⁺CD1c⁺, mainly CD5⁻) and constitutes most of the B cells in the splenic marginal zone (Weill, Weller et al. 2009). However, recently CD5⁺ IgM memory B cells have been identified, which could be the counterpart of the B1 subset in mice. These CD20⁺CD27⁺CD43⁺ cells, found in small numbers in cord blood and adult peripheral blood (3-11% of CD20⁺ B cells) spontaneously secrete natural IgM, are able to stimulate T cells, and have a skewed B cell repertoire (Griffin, Holodick et al. 2011), suggesting these are the antigen independent pre-diversified subset described by others (Kruetzmann, Rosado et al. 2003; Tangye and Good 2007; Tangye and Tarlinton 2009; Weill, Weller et al. 2009). This finding suggests heterogeneity exists in the IgM memory B cell subset and demonstrates much is still to be discovered about human B cell subsets and the use of CD27 as a memory B cell marker.

IgM memory B cells may not be solely involved in T cell independent humoral responses outside the GC. In experimental mice models, both IgM memory B cells and class switched B cells produced IgG in response to T cell dependent antigens (Moens, Wuyts et al. 2008; Good-Jacobson and Shlomchik 2010) suggesting IgM memory B cells are also important in T cell dependent immune responses and *vice versa* (Moens, Wuyts et al. 2008). Again, in a murine model, IgM memory B cells rapidly mobilised into GCs and were able to switch into IgG secreting cells, whilst IgG+ class switched B cells differentiated into plasma cells or replenished the memory B cell pool. Seifert *et al*, (Seifert and Kuppers 2009) demonstrated that GC derived memory B cell clones could give rise to both IgM and IgG daughter populations. The authors suggest that whilst a population of IgM memory B cells are pre-diversified early in life, later IgM memory B cells may be GC derived, possibly leaving the GC reaction earlier than class switched B cells. Taken together and with the caveat that murine memory B cells may not be anatomically nor physiologically representative of the human immune system (Weill, Weller et al. 2009), these findings indicate diversity or heterogeneity in the memory B cell pool exists, possibly to create an immune response to a diverse range of pathogens (Good-Jacobson and Shlomchik 2010) .

7.1.3 Identification of tissue like B cells

The findings presented in this thesis have provided additional information on B cell maturation and B cell biology in health and disease states. Novel B cell subsets have been described in the literature during the course of this thesis, most recently tissue like B cells expanded in viraemic HIV-1 infection and CVID and reported to be exhausted, anergic or innate B cells (Moir, Ho et al. 2008; Rakhmanov, Keller et al. 2009; Isnardi, Ng et al. 2010). This current work has shown that the tissue like B cell subset is present at low numbers in the blood of healthy individuals, still present in some patients during treated HIV-1 infection and that this subset expresses TACI and BAFF-R, chemokine receptors for B cell growth and survival factors. In addition, subsets of CD11c or FCLR4 tissue like B cells could be determined. Whether the expression of CD11c, a homing molecule, or FCLR4, an inhibitory marker, alter homing or affect function of these cells remains to be fully investigated.

Interestingly, this thesis found that IgM memory B cells and tissue like B cells are positively correlated in the peripheral blood compartment of untreated HIV-1 infected individuals. Little is known about the exact relationship between B cell subsets such as IgM memory B cells, class switched B cells and tissue like B cells in the blood, gut or tissues and this should be evaluated in future work, as agreed by others (Tangye and Tarlinton 2009). IgM memory and tissue like B cells are both thought to be enriched or mature in the tissues. Destruction of splenic architecture or LN follicles in HIV-1 infection could affect the numbers of both these B cell populations. Further work should confirm if this loss of peripheral blood IgM memory and tissue like B cells is paralleled in secondary lymphoid tissue *in vivo*. To my knowledge, measurement of tissue like B cells in the gut of HIV-1 infected individuals has not taken place.

7.1.4 Dysregulation of BAFF/APRIL axis

The BAFF/APRIL chemokine axis was studied to see if levels of B cell growth factors were important for reduced memory B cell percentages in HIV-1 infection as implicated in patients with primary immune deficiency associated with mutations in this chemokine axis. Considerable dysregulation of the BAFF/APRIL axis was reported in this thesis in patients with HIV-1 or CVID. It is unclear whether high levels of BAFF or APRIL are a primary or secondary event due to loss of memory B cell subsets, B cell lymphopenia, disturbed LN structure or induced by pro-inflammatory cytokines secreted by dysregulated DCs (see Chapter VI discussion).

7.1.5 Implications of these findings regarding HIV-1 infection

There is no cure at present to eradicate HIV infection, nor an effective vaccine to prevent transmission of disease to uninfected individuals. Despite the advent of treatment, HIV-1 infected individuals on ART still have a shorter life span than normal, even with full T cell immune reconstitution (Volberding and Deeks 2010). Data presented in this PhD adds to the current knowledge regarding the biology of B cell pathogenesis in HIV infection and the immune response to HIV-1 infection. Findings presented in this thesis demonstrated multiple B cell defects in HIV-1 infection in both B cell maturational pathways and loss of the B cell memory compartment; these defects were demonstrated to be functionally associated with

impaired responses to vaccination with important bacterial antigens. Defects in humoral immunity have implications for HIV-1 vaccination design and the induction of neutralising antibodies against HIV-1 and may be another mechanism for HIV-1 to evade the host immune response.

Impaired responses to common vaccination suggests HIV-1 infected individuals on ART continue to be susceptible to invasive bacterial disease such as that caused by pneumococcus, to which they have already have a heightened risk of infection. This means new vaccines are needed to induce protection in these individuals but whether this is achievable on the background of poor humoral immunity is debatable. However, conjugated pneumococcal vaccines have been successful in inducing immunity in young infants, a population also unable to respond to Pneumovax. Recently, a 7-valent conjugated pneumococcal vaccine has been used to vaccinate HIV-1 infected individuals in Malawi with a previous history of pneumococcal disease (French, Gordon et al. 2010). Treatment status was not assessed but efficacy of one year's duration was noted even in patients with a CD4 count of <200 cells/ μ l. Due to cohort and ethnicity differences, this study needs to be reproduced in a developed setting.

The findings in this thesis support previous evidence that current formulations of ART have their limitations, especially in reconstituting the peripheral B cell compartment. This suggests a residual immune defect which may make HIV-1 patients susceptible to IPD even on ART. It is important to be able to prevent loss of specific memory B cells and specific antibody responses in patients with HIV-1 infection. Additionally, clinicians should measure anti-pneumococcal and tetanus levels in patients with HIV-1 infection and despite the possibility that a high proportion of patients will not respond to vaccination, vaccinate patients if levels are low. Early initiation of ART may be useful in preventing some B cell dysfunction, however, this work has shown loss of IgM memory B cells is not restored by ART. Therefore, even if ART was administered early to all new patients, a pre-existing cohort of patients with impaired humoral immunity must be carefully managed. There is limited data regarding the effect of ART on GALT memory B cells in HIV-1 infection. In a single study, GALT memory B cells were not reduced during early HIV-1 infection (<43 days) (Levesque, Moody et al. 2009). Nevertheless, this data offers an opportunity to preserve GALT

memory B cell subsets using early ART treatment as suggested in many investigations of peripheral blood in HIV-1 infected adults and in vertically infected children (Cagigi, Palma et al. 2010).

7.1.6 Markers of disease progression and the use of classification systems

Evaluation of specific memory B cells may be useful biomarkers of disease progression or vaccination responses and low percentages may indicate early initiation of ART. Use of percentages and cut off levels in HIV-1 infection as used in classification schemes for CVID or using reference ranges may be more clinically important than using median levels to assess B cell composition in patient groups. Measuring IgM memory B cells and class switched memory B cells may predict which HIV-1 patients may be susceptible to invasive infection with encapsulated bacteria and these patients could be targeted for prophylactic antibiotic treatment. Since current ART does not restore memory B cell levels, a one-off measurement could be performed once successful ART is confirmed. Follow up longitudinal clinical data of our HIV-1 infected cohort would further add weight to these *in vitro* findings. The altered BAFF/APRIL chemokine axis may be a biomarker for disease progression and a risk factor for developing autoimmunity or B cell malignancies in HIV-1 infected patients. Plasma BAFF and APRIL levels could be measured routinely in untreated HIV infection or HIV treated infection, since high levels of BAFF or APRIL were found in some HIV patients on ART and may be a risk factor for development of disease complications.

7.1.7 Implications of findings for individuals with CVID

Hopefully future research work will be able to find better treatments for or a cure for CVID. Findings in Chapter III indicated the importance of IgM memory B cell percentages in protection against respiratory complications and bronchiectasis in CVID. IgM memory B cells should be incorporated into future classification schemes. Despite this heterogeneity, this thesis has shown that expansions of tissue like B cells seen in some patients and associated with splenomegaly in other work, are associated with high levels of BAFF or APRIL and B cell lymphopenia. The CD21^{low} B cells expressed receptors for BAFF and APRIL (TACI and BAFF-R). Expansions of tissue like B cells in the peripheral blood may be associated with parallel expansions of

these cells in the spleen, causing the characteristic splenomegaly reported in these patients. Expanded tissue like B cells may indicate a yet to be defined CVID aetiology and so classification schemes such as EURO Class may be useful for identifying these patients. Mice engineered to secrete increased levels of BAFF displayed splenomegaly and an undefined activated CD21^{low} subset in the spleen, therefore plasma levels of BAFF could potentially be also used as a biomarker for splenomegaly. Finally, plasma levels of BAFF and APRIL may contribute to the increased risk of development of NHL and other malignancies in CVID.

7.1.8 Common patterns between HIV-1 infection and CVID

This current work and other studies of diseases involving B cell dysregulation have highlighted several novel B cell populations expanded in disease that represent only small percentages of B cells in healthy controls. Work in this thesis compared an example of primary (CVID) and secondary immune deficiency (HIV-1), demonstrating extensive B cell dysregulation is common to both types of immune deficiency (see Table 18).

Table 18: Similarities between B cell abnormalities in HIV infection and CVID

<i>B cell abnormality</i>	<i>CVID</i>	<i>HIV ART-</i>	<i>HIV ART+</i>
Loss of specific Igs and response to vaccination	Yes	Yes	Yes
Loss of IgM memory or class switched B cells	Yes/Variable	Yes/Variable	Yes/Variable
Expansions of transitional B cells	Yes (subset)	Yes	No
Loss of BAFF-R on CS memory	No	Yes (all)	Variable
Expansions of CD21 ^{low} B cells	Yes	Yes (all)	Yes (proportion)
CD21 ^{low} cells express BAFF-R	Yes	Yes	?
CD21 ^{low} cells express TACI	Yes	Yes	?
High levels of APRIL	Yes	Yes	No (subset)
High levels of BAFF	Yes (subset)	Yes (subset)	No (subset)
Increased TFH level (%)	Variable	No	Yes

HIV-1 infection is an acquired immune deficiency whilst CVID is predominantly a genetic immune deficiency. Insights into monogenic PID disorders may aid knowledge of HIV pathogenesis. This thesis found many similarities between the two

diseases, including wide heterogeneity in the spectrum of disease severity and the extent of B cell memory loss. In both diseases, B cell dysregulation may be progressive.

7.1.9 Standardisation of B cell immunophenotyping

The studies in this thesis have used the differential staining of CD27 and IgD used in research and clinical laboratories world-wide for classical B cell memory immunophenotyping plus the differential staining of CD27 and CD21 resting memory/activated memory B cell phenotyping used by the NIH group of Moir *et al.* Both methods use CD19 as a pan B cell marker and CD27 as a memory marker. By using both phenotyping schemes, this thesis tied together parallel research findings which could not be previously compared: for example, the previously reported reduced BAFF-R expression on CD21^{low} B cells in HIV infection by Moir *et al.*, is better defined in this thesis to be reduced on class switched memory B cells. Conversely, a proportion of the expanded CD27^{low} CD21^{low} tissue like B cells reported by Moir *et al.* in HIV-1 infection express IgD and so were grouped into the naïve (IgD⁺CD27⁻) or IgD⁻ quadrant population (IgD⁻CD27⁻) of a differential IgD v CD27 dot plot and so were not previously reported to be reduced in HIV-1 infection. Earlier studies used differential staining of IgD and CD38, which is not sufficiently discriminatory for B cell memory and limits the findings of these papers. Further advances in multi-parameter flow cytometry will provide better discrimination of B cell subsets and hopefully allow a better understanding of B cell biology and aid standardisation of B cell phenotyping.

7.2 Study Limitations

Measurement of peripheral blood samples is a convenient method of sampling the immune system and is less invasive. A possible limitation of this study in HIV-1 infection is the measurement of peripheral blood and not samples from the lymphoid tissues such as spleen, LN or gastro-intestinal tract which are the main reservoirs of HIV-1 infection and also are believed to be the key reservoirs or lymphoid sites of maturation for IgM memory B cells, class switched B cells and tissue like B cells. Damage to the gut in HIV-1 infection precedes major immunological abnormalities in the peripheral blood compartment and therefore it may be more relevant to investigate

lymphoid sites. It is possible that damage to the lymphoid compartment could initially cause lymphoid B cells home to the peripheral blood in larger numbers before slowly decreasing in number as the lymphoid compartment becomes less hospitable to maturation of new naïve B cells. Secondly, peripheral blood sampling at one time point is a cross sectional study of the immune system in HIV-1 infection. This is most relevant to the measurement of samples from untreated HIV-1 infected patients, in whom it may be impossible to entirely delineate time since infection and disease stage. Damage to the B cell lymphoid compartment may not be linked to markers of T cell disease progression. There are few longitudinal studies performed to assess the B cell memory compartment and this needs further investigation.

There are many confounding variables to studying patients with CVID such as replacement Ig treatment, monogenic or polygenic causes of disease, time since development of CVID and varying complications of disease. Again, this current work is a cross sectional measurement of the peripheral B cell compartment in CVID. Respiratory complications of CVID are confounded by factors such as asthma, cigarette smoking, inhalers, diagnostic delay and replacement immunoglobulin treatment.

Flow cytometry is a powerful methodology which allows simultaneous measurement of various antigens on the surface of lymphocytes, however, similar to every assay, it has its limitations. Traditional flow cytometry does not observe and measure cell behaviour or the interactions between one cell and another. It also cannot provide morphological details about a cell and CD markers are often not specific to one cell type. Flow is a high cost assay and typically has a low throughput rate, which means rare event analysis can be extremely time-consuming. There are improvements in the field if available on site: imaging flow cytometers can now visualize and quantitate the location of fluorescence within each cell and so can provide important information about localization of specific cell markers/ give clues to possible interactions between various cells. Compensation in flow cytometry can now be automated but still requires specialist technical knowledge and training in order to maximize resolution of positive dimly stained cells from negative background staining.

Functional immunology is an important tool which can provide some in vitro ‘proof of principle’ for associations observed using statistical analysis of flow cytometric data, as described in this thesis. Immune response assays typically use exposure to specific stimuli or inhibitory molecules in culture and compare these responses to negative control cultures. Proliferation of PBMCs in culture can be measured using various assays (tritiated thymidine uptake etc.) whilst cellular secretions such as cytokine production can be measured using flow cytometry or ELISpot assays for single cytokine detection. Expanded stimulated cells can be harvested and subsequently immunophenotyped by flow cytometry to provide further information. In addition, functional assays measuring inhibition of viral/cell growth by adding specific compounds/cytotoxic cell types are increasingly being used to help determine efficacy of vaccines.

7.3 Future work

It would be useful to measure tissue like B cell populations in the gastro-intestinal tract of HIV infected individuals and parallel this with peripheral blood populations, which has not been determined previously. The aim would be to correlate this with other markers of B cell dysfunction e.g. B cell memory subsets and markers of B cell activation, to see if the expansion/loss of tissue like B cells in untreated HIV infection is paralleled in the gut. It would also perhaps be preferable to measure HIV specific memory B cells or antigen specific memory B cells e.g. by ELISpot to see whether this reflects the dysfunctions in the lymphoid compartment (Bussmann, Reiche et al. 2010).

The parallel relationship between IgM memory B cells and tissue like B cells reported in this thesis in HIV-1 infection is interesting but requires a longitudinal study during HIV-1 infection to characterise it further. Both cell types express BAFF-R and TACI and so both may be preferentially expanded from naïve B cells by BAFF or APRIL as part of the dysfunctional immune response against HIV-1 infection. It would be useful to culture sorted B cell subsets with BAFF or APRIL to determine which populations are more responsive, using tritiated thymidine or CFSE as a measure of proliferation. Findings could be confirmed using the addition of a BAFF/APRIL stimulation blocker such as Atacicept (a monoclonal antibody which blocks TACI binding). This

experiment could also be replicated using B cell subsets from CVID patients to confirm whether the reported association between high levels of BAFF or APRIL and expansions of tissue like B cells in CVID can be demonstrated *in vitro*.

Alternatively, loss of IgM memory and tissue like B cells in HIV-1 infection may result from these cells isotype switching to terminally differentiated populations. Both IgM memory B cells and most tissue like B cells express IgM and receptors for APRIL and BAFF, factors able to promote T-cell independent isotype switching (Litinskiy, Nardelli et al. 2002). Class switched memory B cells were elevated in the gut of patients with primary HIV infection (Levesque, Moody et al. 2009), due to limitations in current knowledge of B cell subsets, these cells could be differentiated from IgM memory cells. Addition of BAFF or APRIL to co-cultures of HIV viral particles (gp120, Tat or Nef) with sorted populations of IgM memory B cells or tissue like B cells could be performed. Immunophenotyping and Ig secretion from the supernatants may help determine whether cells have isotype switched.

Expansions of tissue like B cells may trigger apoptosis or loss of the whole IgM memory compartment. Higher levels of apoptosis may be a feature of both IgM memory B cells and tissue like B cells or alternatively both may be targeted for destruction by HIV-1. Apoptotic rates of the tissue like B cell subset has not been determined and needs investigation using cell culture and immunophenotyping by flow cytometry.

Surprisingly, this thesis reported higher percentages of peripheral T_{FH} cells in primary (CVID) and secondary immune deficiency (HIV-1), although in untreated HIV-1 infection, absolute numbers of T_{FH} were reduced. It would be useful to correlate T_{FH} numbers with antibody responses to tetanus toxoid and conjugated pneumococcal vaccines in HIV-1 infection to help determine if these T_{FH} cells are functional.

It would be useful to undertake a more detailed evaluation of pneumococcal vaccination serology. IgM antibodies for the four commonest serotypes (4,9V,23F,14) associated with invasive pneumococcal infection in HIV-1 adults will be determined by ELISA at baseline and post pneumococcal vaccination. IgG levels of the 10

commonest US pneumococcal serotypes (4,9V,23F,14,6B,19F,9N,6B,19F,18C,19A, 6A) associated with IPD will be assessed using fluorescent bead technology.

The role of BAFF/APRIL in the pathogenesis of HIV/CVID associated malignancies needs further investigation. These are known growth factors for lymphoid malignancies and continuing high levels may predispose patients to future development of neoplasia. Plasma BAFF and APRIL could be studied in HIV-1/CVID patients with various forms of malignancy. Longitudinal measurement would determine whether measurement would be useful in predicting the development of B cell malignancies.

7.4 Conclusions

In studies of our work, we have found a loss of IgM memory B cells in HIV infection that was not restored by ART therapy, this loss correlated with a poor response to Pneumovax vaccination in HIV infected patients on treatment and so may be a risk factor for invasive pneumococcal disease seen in HIV infection. We also reported high levels of plasma BAFF and APRIL in HIV-1 infected patients which was normalised by ART therapy. In a later study, we reported levels of peripheral IgM memory B cells in untreated HIV infected patients correlated with levels of tissue like B cells newly reported to be expanded in untreated HIV infection. There are a number of possible mechanisms which may explain these findings, however poorly understood. Loss of IgM memory B cells is a risk factor for invasive pneumococcal disease which remains a major cause of morbidity and mortality in HIV-1 infection. Elucidating the mechanisms behind this will be of substantial benefit to HIV-1 infected patients, considering BAFF and APRIL blocking agents are in trial as therapy for a number of autoimmune diseases and BAFF and APRIL levels are implicated as growth factors for B cell lymphomas, which occur at a higher rate in those with HIV-1 infection or CVID. CVID is a heterogeneous syndrome displaying failure of B cell differentiation, antibody deficiency and recurrent infections, yet the molecular basis of most CVID is unknown. Studies here have added to current knowledge of disease. Total memory B cell percentages are protective against the development of bronchiectasis in CVID whilst expansions of tissue like B cells in a subgroup of patients with CVID are associated with high levels of BAFF and APRIL and B cell lymphopenia.

In conclusion, studying B cells in both health and disease provides greater insights of the mechanisms underlying B cell immunology, response to infection and disease. This holds promise for future vaccines, better diagnostic tests and effective treatments for patients with immune deficiency.

8. Chapter VIII: Publications

Journal Articles

Hart, M. et al., *Loss of discrete memory B cell subsets is associated with impaired immunization responses in HIV-1 infection and may be a risk factor for invasive pneumococcal disease.* J Immunol, 2007. **178**(12): p. 8212-20.

Abstracts

Increases in CD21 (low) B cells are significantly associated with levels of circulating TNF family chemokines BAFF and APRIL in CVID patients.

Hart M, Page E, Ford T, Greathead L, Wilson R, Loebinger M, Henderson D, Kelleher P. IMMUNOLOGY. 131: 96-96. Dec 2010. BSI, Liverpool, 2010

Elevated levels of APRIL and BAFF in HIV-1 Infection are normalised by antiretroviral therapy.

Melanie Hart, Sally Clark, Alan Steel, Graham Moyle, Mark Nelson, Don Henderson, Francis Gotch, Brian Gazzard and Peter Kelleher. 16th Conference on Retroviruses and Opportunistic Infections, Montreal, Canada, 2009

Loss of memory B cells is associated with lung disease in CVID patients.

Melanie Hart, Sima Ateshpanjeh, Sally A Clark, Mitzi Nesbit, Don C Henderson, Robert Wilson, Peter Kelleher, UK PIN Meeting, Leeds, 2007

Marginal zone B cells are selectively depleted in HIV-1 infection and are not restored by anti-retroviral therapy.

Hart M, Clark L, Steel A, Henderson DC, Gazzard B, Wilson R, Gotch F, Kelleher P IMMUNOLOGY **116**:102-102 Dec 2005

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10. Chapter X: Appendix

Table 19: Associations between memory B cell subsets in the treated HIV infected cohort

<i>Tissue like B cell markers in treated HIV infection (%CD19+ B cells)</i>	<i>Correlation with IgM memory B cell (Spearman's Rho)</i>	<i>Correlation with total memory B cell</i>	<i>Correlation with CS memory B cell</i>
CD21-CD27- (n=54)	0.306* (p=0.02)	0.491** (p<0.001)	0.430** (p=0.002)
CD38-CD21- (n=54)	0.372** (p=0.005)	0.609** (p<0.001)	0.564** p<0.001
CD27-CD10-CD21- (n=11)	-0.491	-0.264	0.055
CD27-CD10-CD21-CD11c (n=11)	-0.382	-0.100	0.200
CD27-CD10-CD21-FCLR4 (n=11)	-0.5	-0.282	0.045

Table 20: Associations between memory B cell subsets and tissue like B cells in healthy individuals

<i>Tissue like B cell markers (%CD19+ B cells)</i>	<i>Correlation with IgM memory B cell (Spearman's Rho)</i>	<i>Correlation with total memory B cell</i>	<i>Correlation with CS memory B cell</i>
CD21-CD27- (n=17)	-0.173	0.109	0.275
CD38-CD21- (n=17)	0.363	0.529* p<0.03	0.311
CD27-CD10-CD21- (n=17)	-0.382	-0.200	0.018
CD27-CD10-CD21-CD11c (n=17)	-0.400	-0.250	-0.250
CD27-CD10-CD21-FCLR4 (n=17)	-0.100	-0.117	-0.050

Table 21: Associations between memory B cell subsets and tissue like B cells in the CVID cohort

<i>Tissue like B cell markers (%CD19+ B cells) CVID</i>	<i>Correlation with IgM memory B cell (Spearman's Rho)</i>	<i>Correlation with total memory B cell</i>	<i>Correlation with CS memory B cell</i>
CD21-CD27- (n=32)	-0.373* (p<0.04)	0.350* (p=0.05)	0.578** (p=0.001)
CD38-CD21- (n=32)	-0.114	-0.81	-0.353* (p<0.05)
CD27-CD10-CD21- (n=22)	0.143	0.03	-0.141
CD27-CD10-CD21- CD11c (n=22)	0.182	0.185	-0.035
CD27-CD10-CD21- FCLR4 (n=22)	0.132	0.149	0.06