BIOLOGICAL B-CELL STIMULATORY BIOMARKERS IN HIV-ASSOCIATED NON-HODGKIN LYMPHOMA

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Dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Master of Science in Medicine in the branch of Molecular medicine & Haematology

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DECLARATION

I, **Lauren Ashton Cruywagen,** declare that this dissertation is my own work. It is being submitted for the degree of Masters of Science in Medicine in the branch of Molecular Medicine & Haematology to the University of the Witwatersrand. It has not been submitted before for any degree or examination at this or any other University.

_____

(Signature of candidate)

_____day of June 2016 in Johannesburg_____

DEDICATION:

To my mother for her endless sacrifices and motivation throughout my studying career

PRESENTATIONS ARSISING FROM THIS STUDY

- Poster presentation at PathRed National Congress, April 2015.
  Title: Cytokine expression in HIV-positive individuals with B-cell Non-Hodgkin lymphoma

- Abstract submission for Keystone International Symposia; Viruses and Human Cancers
  Title: Cytokine expression in HIV-positive individuals with B-cell Non-Hodgkin lymphoma

ABSTRACT:

Background: Human Immunodeficiency Virus (HIV) is an epidemic in South Africa with a rise in AIDS-defining malignancies, particularly Non-Hodgkin Lymphoma (NHL). B-cell stimulatory markers have been implicated in the risk and development of HIV-associated NHL. The mechanisms of the pathogenesis of HIV-associated NHL have not been fully elucidated but include: B-cell hyperactivation mediated by the over production of cytokines as a consequence of reduced immunosurveillance associated with CD4+ T-cell deficiencies.

AIM: This study aimed to investigate and quantify the expression of B-cell stimulatory biomarkers in plasma and determine their contribution to lymphomagenesis in an HIV positive South African cohort.

Methods: Plasma samples from HIV positive patients with confirmed NHL and HIV positive patients without evidence of lymphoma were assessed for ten cytokines: Interleukin (IL) IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, granulocyte-macrophage colony stimulating factor (GM-CSF), interferon gamma (IFNγ) and tumour necrosis factor alpha (TNFα), as well as two soluble factors, sCD23 and sCD30 by Luminex Technology and ELISA respectively. Peripheral blood mononuclear cells from a HIV positive patient with Burkitts lymphoma were used to establish an *in vitro* culture.

Results: Cytokines IL-6, IL-8 and IL-10 concentrations (pg/ml) were significantly elevated in HIV positive patients with NHL compared to the HIV positive controls. Soluble factors CD23 and CD30 concentrations (U/ml) were also elevated in HIV positive patients with NHL.

Conclusion: IL-6, IL-8 and IL-10 may play a key role in stimulating B-cell proliferation and lymphomagenesis. IL-6 and IL-8 are pleiotropic pro-inflammatory cytokines, and IL-10 an anti-inflammatory and/or regulatory cytokine, that act as growth factors in a paracrine or autocrine manner for HIV-associated lymphoma cells.

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ABBREVIATIONS:

BCR	B-cell receptor
Bcl6	B-cell CLL/lymphoma 6
BL	Burkitts Leukaemia/ Lymphoma
BMC	Bone marrow mononuclear cells
CHB	Chris Hani Baragwanath Hospital
CMJAH	Charlotte Maxeke Johannesburg Academic Hospital
CNS	Central nervous system
CRP	C-reactive protein
DLBCL	Diffuse Large B-cell Lymphoma
EBV	Epstein - Barr virus
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked Immunosorbent assay
FLC	Free-light chains
GIT	Gastrointestinal tract
GM-CSF	Granulocyte-macrophage colony stimulating factor
HAART	Highly active anti-retroviral therapy
HHV-8	Human herpesvirus-8
HIV	Human Immunodeficiency Virus
HL	Hodgkin Lymphoma
IFNγ	Interferon gamma
Ig	Immunoglobulin
IgH	Immunoglobulin heavy chain

IL	Interleukin
KSHV	Kaposi's sarcoma herpesvirus
MAb	Monoclonal antibody
NHL	Non-Hodgkin Lymphoma
NHLS	National Health Laboratory Services
PBMC	Peripheral blood mononuclear cells
PEL	Primary effusion Lymphoma
PI	Propidium Iodide
PL	Plasmablastic Lymphoma
QC	Quality Control
RPMI	Roswell Park Memorial Institute
sCD Antigen	Soluble CD Antigen
SDF-1α	Stromal derived factor 1 alpha
Th	T helper cells
TMB	Tetrametylbenzidine
TNFα	Tumour necrosis factor alpha
WHO	World Health Organization

CHAPTER 1.0

INTRODUCTION

1.1 Development and differentiation of B-cells

The adaptive immune response is divided into the cell-mediated (the T-cell response) and humoral responses (the antibody response mediated by B-cells) [1]. During cell-mediated immunity, cytotoxic T-cells destroy intracellular pathogens. The humoral response involves the production of antibodies by B-cells under the control of CD4+ T cells [1, 2].

B-cells develop in the bone marrow from pluripotent haematopoietic progenitor stem cells and migrate to secondary lymphoid organs for further maturation [3-5]. The common lymphoid progenitor or haematopoietic stem cell gives rise to a Pro-B cell. During this stage, there is germline rearrangement of the immunoglobulin heavy chain (IgH) gene of the B-cell receptor [4, 6] [7, 8]. The Pro-B cell matures to a Pre-B cell and the rearrangement of the kappa and lambda light chain genes are initiated. Should the rearrangements result in the formation of a functional B-cell receptor, the B-cell will be released from the bone marrow as a mature B cell (Figure 1.1) [3, 4]. B-cell receptors are secreted as immunoglobulins or antibodies which bind to antigens from pathogens. They have a number of functions including pathogen opsonisation, neutralisation, activation of complement and stimulation of natural killer cell killing (antibody-dependent cellular cytotoxicity) in order to illicit a specific immune response [2, 9]. [4, 10].

Mature B-cells undergo further maturation and antibody diversification by two processes; somatic hypermutation and isotype class switch recombination [11]. This usually occurs in the germinal centre of the peripheral lymphoid tissue [4] and is mediated by additional signals from CD4+ T cells (T helper cells). And/or by direct cell-cell contact or through the secretion of cytokines such as IL-4 and IL-5. During somatic hypermutation, point mutations accumulate in the V region of both the heavy and light chain genes [12-14]. Isotype switching allows cells to switch the class of antibody from IgM which is first produced to IgG, A or E. Somatic hypermutation increases the affinity of antibodies for particular antigens during the late primary or secondary immune response.

Mature activated B-cells terminally differentiate into antibody secreting plasma cells and memory B cells post germinal centre (Figure 1.1) [1, 4].

Defects in B-cell activation results in abnormal antibody production and failure to switch from the low affinity antibody IgM to IgG, A or E. The ability to negatively select self-reactive B-cells is consequently affected [15, 16] .

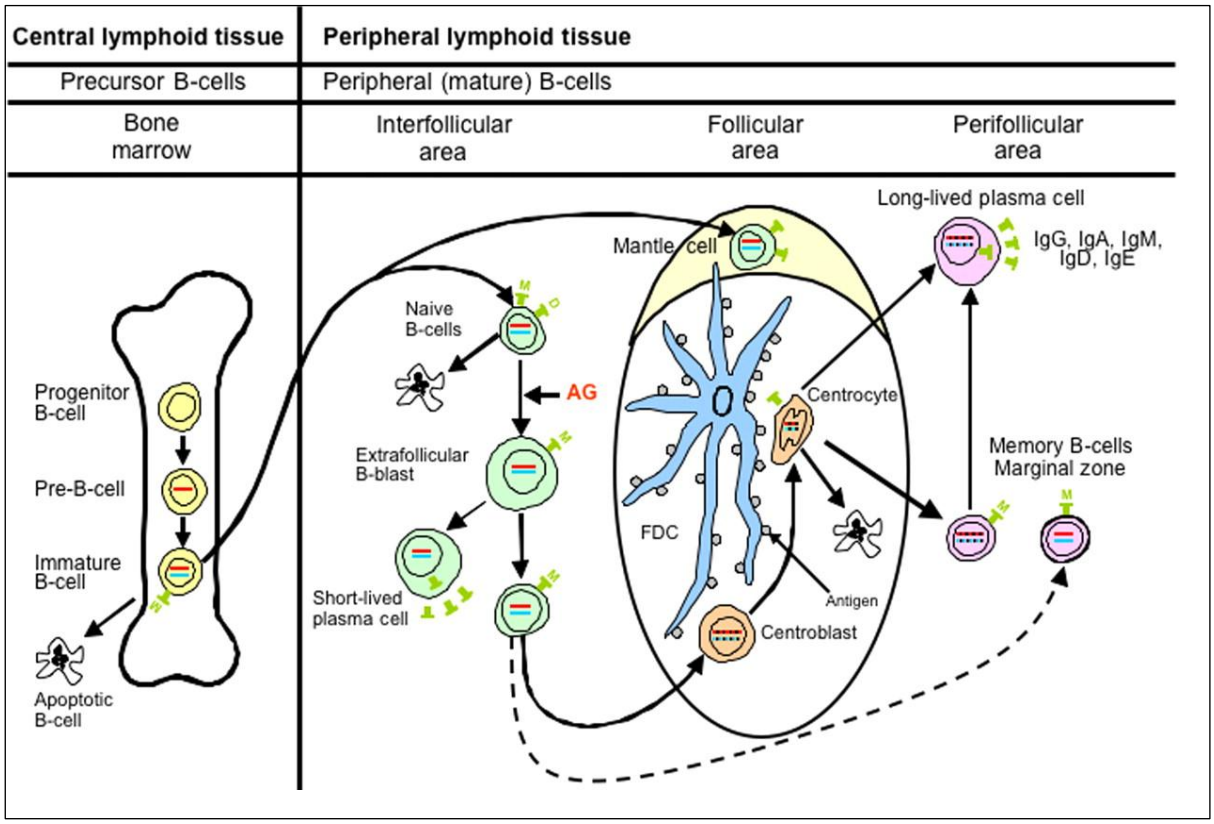

Figure 1.1: Schematic representation of the development and differentiation of B-cells.

Immature B-cells develop in the bone marrow from progenitor B-cells. Naïve mature B-cells migrate to the germinal centre in the peripheral lymphoid tissue where they differentiate into antibody secreting plasma cells and memory B cells [Taken from Jaffe *et al*, 2008. WHO Classification of Tumours of haematological and lymphoid tissues].

1.2. The development of haematological lymphoid neoplasms

Lymphomas are the most common haematopoietic malignancies of lymphoid origin [17, 18]. They frequently present as tumours in lymphoid tissues, including lymph nodes, mucosa-associated lymphoid tissue, spleen and bone marrow but can proliferate and disseminate to other soft tissues and organs [18]. B-cell lymphomas can be classified according to the stage of B-cell maturation or differentiation in which they arise (Table 1.1) [1, 19]. B-cell lymphomas are classified as either Hodgkin (HL) or Non-Hodgkin lymphomas (NHL) and according to their clinical growth behaviour as low-, intermediate or high-grade (aggressive) lymphomas [17, 19-21].

Table 1.1: Classification of lymphomas corresponding to the different stages of B-cell development and differentiation [Adapted from Weigert *et al*, 2012 and Jaffe *et al*, 2008]

Stage of Normal B-cell development/ Cell Type	Corresponding malignancy
• Pre/Pro B-cell	Precursor B-cell leukaemia/ lymphoma
• Naïve mature B-cell/ Pre Germinal centre B-cell	Mantle cell lymphoma
• Germinal centre neoplasms ➢ Centroblast	Hodgkin's lymphoma Diffuse Large B-cell lymphoma Burkitts lymphoma
➢ Centrocyte	Follicular lymphoma
• Post germinal centre	Plasmacytoma DLBCL Plasmablastic DLBCL Marginal zone lymphoma

1.3. Incidence of HIV Associated Non-Hodgkin Lymphoma in Sub-Saharan Africa

Infection rates of human immunodeficiency virus (HIV) in South Africa are high with an estimated disease burden of 6.4 million infected individuals and 2.4 million deaths as indicated by the UNAIDS 2014 HIV statistics in South Africa report [22]. High grade B-cell NHL is an AIDS-defining condition [23-25] with a reported increased risk of development in HIV positive individuals compared with the general HIV negative population [26-28]. Conversely, according to the GLOBOCAN 5 year estimated incidence and prevalence report, there is a lower prevalence of NHL in Africa (5.9%) compared with developed countries (Figure 1.2) [29]. Studies conducted between 2007 and 2009 in the Gauteng province of South Africa, however, noted an increase in the prevalence of aggressive high grade B-cell lymphomas (from 64% in 2004-2006 to 82%). Of the patients diagnosed with high grade lymphoma, 90% also tested positive for HIV infection. These high grade lymphomas included; Diffuse large B-cell lymphoma (DLBCL), DLBCL plasmablastic variant, Burkitt leukaemia/lymphoma (BL), HL and plasma cell dyscrasias [30, 31]. A 16% increase (from 24 to 40% in the last decade) in the incidence of NHL was reported in a study conducted at Chris Hani Baragwanath (CHB) hospital between the period 2003-2012 [28]. Data from the Western Cape Province in South Africa also reported increased numbers of HIV-related lymphoma (from 5-37%) between the periods of 2002-2009 [32]. DLBCL followed by BL was the most frequent diagnosis [32]. Data on HIV associated malignancies are scarcely documented suggesting that the reported incidence of NHL in South Africa may be lower than the actual incidence. This may be a reflection of under recognition and failure to diagnose and/or document some cases [31, 32].

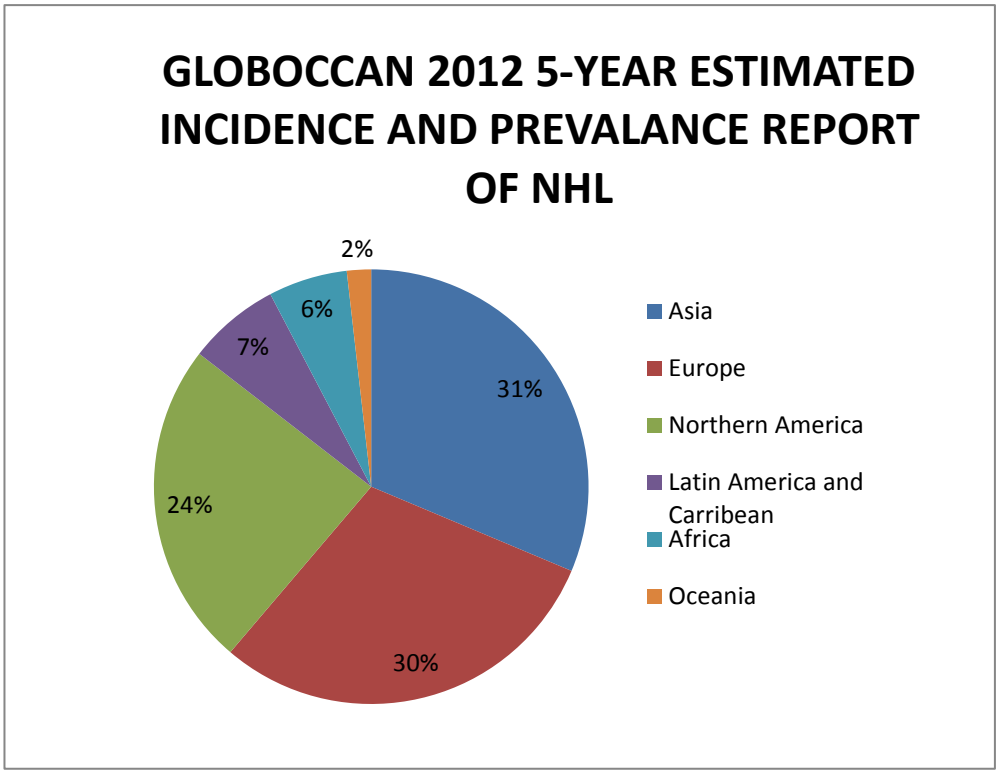

Figure 1.2: The 5-year prevalence of Non-Hodgkin lymphoma is not as high in Africa compared to other continents. [Adapted from GLOBOCAN Estimated Cancer Report, 2012].

1.4. HIV infection leads to an increased risk of lymphoma

The World Health Organization (WHO) classifies HIV-associated lymphomas into three groups: lymphomas occurring in immunocompetent patients, lymphomas occurring in HIV-positive patients and lymphomas occurring in patients with other forms of immunosuppression [33-36]. In HIV positive individuals, approximately ~70-90% of lymphomas are aggressive high grade B-cell malignancies [27, 37, 38], and include BL, DLBCL, Plasmablastic lymphoma (PL) of the oral cavity, Primary effusion lymphoma (PEL) and its solid variants and large cell lymphoma arising in Human herpesvirus-8 (HHV8) associated multicentric Castleman's disease [34, 39-41]. The WHO has recently assigned a provisional category of B-Cell Lymphoma unclassifiable, with features intermediate between DLBCL and BL. This intermediate group share morphological, immunophenotypic and molecular/cytogenetic features with both DLBCL and BL which make the diagnostic distinction difficult. There has been an increase in the diagnosis of highly aggressive B-cell lymphoma intermediate group [42].

1.5. Subtypes of high grade B-cell NHL Subtypes seen in HIV positive individuals

1.5.1 Burkitt Leukaemia/Lymphoma

Burkitt Leukaemia/Lymphoma is a mature lymphoproliferative disorder and is a high-grade aggressive NHL characterised by a high proliferation rate [43]. BL is also seen in immunocompetent individuals, predominantly children in the endemic BL areas where it often presents as a jaw mass. In adults, it is one of the most common AIDS-defining malignancies [44-47]. The disease in HIV-infected individuals clinically presents with a bulky tumourigenic mass localized in the lymph nodes and bone marrow but may include other extra-nodal sites such as the central nervous system (CNS) [44, 45]. BL comprises monomorphic medium sized B-cells which contain densely-packed chromatin and prominent nucleoli. Intermingled macrophages and/or phagocytes containing debris of apoptotic cells give the illusion of a starry sky pattern (Figure 1.3) [48-50]. The translocation of the *c-MYC* gene located on chromosome 8 to the immunoglobulin locus (most commonly the heavy chain locus on chromosome 14) , is a distinct genetic characteristic of BL [43, 51]. BL presents with a mature B-cell immunophenotype expressing CD19 and CD20 as well as CD10 reflecting the germinal centre cell of origin. The cells generally show light chain restriction and PCR reactions for antibody rearrangements are monoclonal [46, 52]. The high proliferation rate is indicated by the cell turnover marker, Ki-67, which shows an index approaching 100% [44, 49].

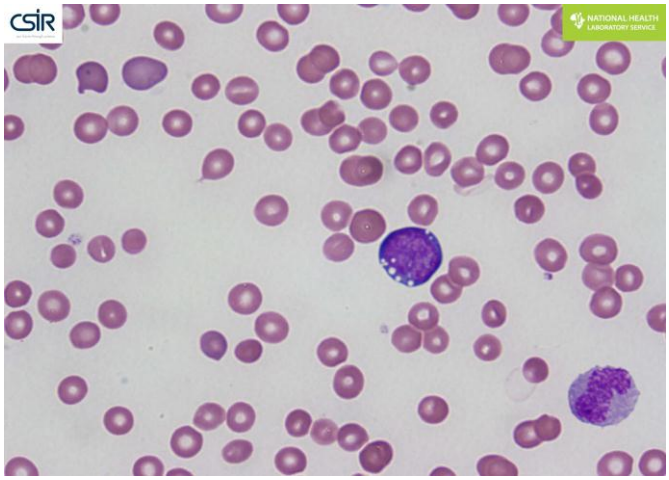

Figure 1.3: A) Microscopic haematoxylin and eosin stain of a Burkitt Leukaemia/Lymphoma resembling a starry sky pattern. [Taken from the NHLS/ CSIR Database]

1.5.2. Diffuse Large B-cell Lymphoma, not otherwise specified (DLBCL, NOS)

Diffuse Large B-cell lymphoma, not otherwise specified is the second commonest AIDS-defining malignancy [53, 54]. Clinical manifestations include an enlarging tumour mass primarily involving the gastrointestinal tract (GIT), (Figure 1.4), but can include other extra-nodal sites such as the CNS, oral cavity and salivary glands, breast and nose [55-57]. Patients often present with B-cell lymphoma symptoms, including night sweats, unintended weight loss and fevers, reflecting the aggressive nature of this tumour [36, 55]. Morphologically, the cells are larger with prominent nucleoli and fewer mitoses. Variants of HIV related DLBCL are morphologically categorised as centroblastic, immunoblastic and/or anaplastic [48, 58].

The most common chromosomal translocation seen in DLBCL is that of the *B-cell CLL/lymphoma 6 (Bcl6)* gene (3q27) which occurs in approximately ~25-40% of patients, and may be IgG or non-IgG related [43, 59]. *Bcl6* modulates B-cell responses to the growth factor, IL-4 and is also associated with germinal centre B-cells [60, 61]. Other genetic anomalies associated with HIV-associated DLBCL include mutations of various proto-oncogenes such as *PAX5*, *PIM1* and *MYC* [62]. DLBCL show features similar to BL with B-cell markers, CD19 and CD20 and light chain restriction. In addition, DLBCL may express the germinal centre marker, CD10. Typically, it shows a lower proliferative index (a Ki67 of less than 90%) although this may be higher in HIV-associated tumours [43, 44, 49, 51].

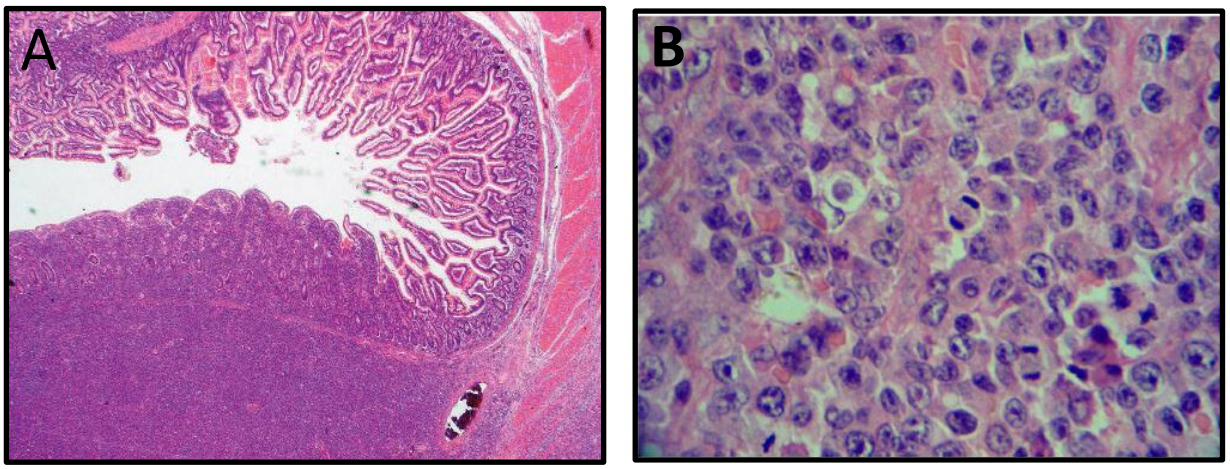

Figure 1.4: Representation of microscopic haematoxylin & eosin stains of Diffuse Large B-cell lymphoma. A) Histological representation of a HIV-associated DLBCL extra-nodal case in the GIT. **B)** Shows a polymorphic centroblastic lymphoma, 40x magnification. [Taken from Romero-Guadarrama *et al*, 2012.*Open J of Path;* Grogg *et al*, 2007. *J. Clin. Path*]

1.5.3. B-cell lymphoma unclassifiable with features intermediate between Burkitt and Diffuse Large B-cell Lymphoma

B-cell lymphoma unclassifiable with features intermediate between DLBCL and BL is a provisional category assigned by the WHO to aggressive high grade lymphomas displaying a variable spectrum of morphologic, immunophenotypic and molecular/cytogenetic features resembling and characteristic of BL and DLBCL [42]. These tumours show immunophenotypic features suggestive of a germinal centre cell of origin and often show *MYC* Ig gene translocations. [43, 48, 51]. Morphologically, the cells often resemble BL cells and the proliferative index can be high.

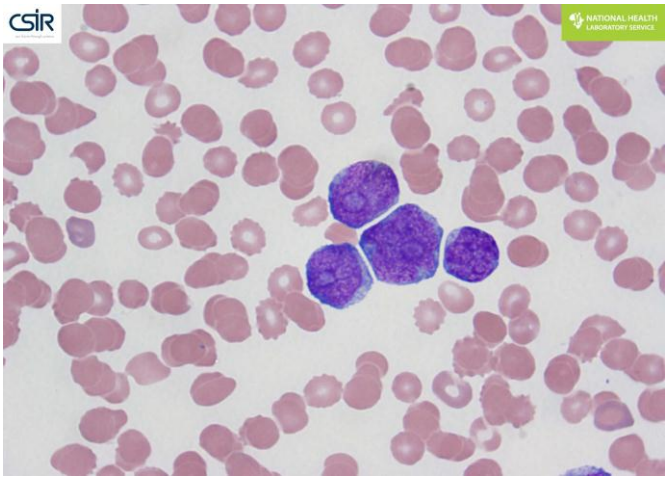

Figure 1.5: Microscopic representation of a triple hit B-cell lymphoma unclassifiable with features intermediate between DLBCL and BL. [Taken from the NHLS and CSIR Database].

1.5.4. Other high grade lymphoma subtypes arising in HIV positive individuals

Primary effusion lymphoma, plasmablastic variant of DLBCL and large cell lymphoma arising in HHV8 associated multicentric Castleman's disease, are also associated with development in HIV infection.

PEL occurs less frequently than the fore a mentioned lymphomas, accounting for 4-5% of all HIV-associated NHL [27, 63]. Pre-existing or co-infection with other oncogenic gamma herpesviruses predominantly HHV8 and/or Karposi's sarcoma herpesvirus (KSHV) as well as EBV are concomitant infections in patients with HIV-associated PEL [43, 63]. The disease generally manifests in body cavities such as the pericardium, pleural and peritoneum [64].

Immunophenotypically, PEL are void of the expression of most B-cell lineage markers but express other markers usually associated with plasma cell activation and differentiation. These include CD138, MUM1 and CD71 to mention a few [65-67].

Plasmablastic lymphoma is a subtype of DLBCL as described by the WHO [27, 42]. PL occurs commonly in the oral cavity and jaw of HIV positive individuals as well as in immunocompetent HIV negative individuals [68, 69]. The lymphoma arises from activated B-cells or a plasmablast that has already undergone affinity maturation by somatic hypermutation and class switching. Morphologically it has similar features to BL and DLCBL. These cells express plasma cell markers CD38, CD138, Ki67, BLIMP-1 (plasma cell differentiation marker) and MUM/IRF4 [68, 70].

Large cell lymphoma arising in HHV8 associated multicentric Castleman's disease is usually EBV negative, but is positive for Kaposi' sarcoma herpesvirus [71]. These lymphomas arise from naïve B-cells and lack immunoglobulin gene mutations [71,72]. Germinotropic lymphoproliferative disorder is a variant of the latter where germinal centre B-cells are co-infected with gamma herpesviridae [40, 69]. These lymphomas will not be further discussed in this study as they were not included in the study cohort.

1.6. Biomarkers: Definition and function in HIV-associated Non-Hodgkin Lymphoma

New methods are continuously being pursued to improve the diagnosis and monitoring of disease. Biomarkers can be measured and investigated as potential indicators of biological, pathogenic and pharmacological processes and/or responses to therapeutic strategies [73, 74] and are used to diagnose and prognosticate in a number of diseases including cancer, infectious diseases and autoimmune diseases. These biomarkers can be proteins (chemokines, growth factors, cytokines or hormones) or complex carbohydrates and lipids [38], and can be detected in a vast number of specimen types such as serum, plasma, whole blood and/or cell culture supernatant [20]. Cancer-associated biomarkers may be increased as a direct result of malignancy or as an indirect effect of the malignant processes [20]. Identification of novel and existing biomarkers in cancer patients could provide insight into prognosis and could be useful in guiding treatment [20, 75, 76].

The exact mechanisms of B-cell oncogenesis in HIV are uncertain. Infection with HIV causes severe defects in cell-mediated immunity, with decreased CD4+ T-cell counts as well as impaired cytotoxic T-cell killing associated with dysregulated immunosurveillance against tumours and virally infected cells, consequently disturbing the homeostasis of B-cells [77-79]. HIV also targets key molecules of the immunological synapse (like CD4) which results in incomplete signalling, thereby altering the terminal differentiation and affinity maturation processes of B-cells [80, 81].

Polyclonal B-cell activation is also characteristic of HIV infection which is often associated with a number of non-specific B-cell activation features [82]. These include non-specific hypergammaglobulinaemia (an increase in Ig secreting cells), the presence of auto-reactive antibodies as well as and a number of antibody mediated autoimmune phenomena like immune-mediated thrombocytopaenia [23, 81-83]. There is persistent infection and failure to clear a number of B-cell tropic viruses which are associated with B-cell transformation, such as oncogenic gamma-herpesviridae, EBV and KSHV [71]. Viral proteins from both of these viruses have been directly implicated in HIV associated lymphomagenesis and represent a diagnostic criteria for conditions like primary effusion lymphoma [72]. Lastly, evidence exists that there is direct antigenic stimulation of B-cell by the virus itself mediated by antigenic components incorporated into the viral envelope [27, 84, 85].

Aberrant expression of B-cell stimulatory biomarkers in HIV-associated NHL has been previously studied [38, 76, 83, 86]. These studies found that serum levels of several B-cell stimulatory cytokines and immune markers associated with B-cell activation are elevated prior to development and diagnosis of HIV-associated NHL. These include cytokines interleukin-6 (IL-6), IL-10 and tumour necrosis factor alpha (TNFα), soluble factors CD23, CD27, CD30, C reactive protein (CRP), chemokine CXCL13 and immunoglobulin free light chains (FLC) [1, 40, 41(83, 86)].

Expression of these biomarkers in HIV positive patients with NHL in low and middle income countries, like South Africa, have been poorly documented. There are likely to be differences to biomarkers reported in patients from Europe and the USA, considering a higher burden of chronic infectious diseases (excluding HIV) and relatively advanced stage of immunodeficiency seen in South African patients, as well as the late presentation of patients to oncology services [31, 87].

1.7. B-cell stimulatory molecules and their role in B-cell lymphomagenesis

Cytokines are a heterogeneous group of low molecular weight, extremely potent, secreted proteins that act as messengers of the immune system that exert effects upon a target cell [38, 88]. These molecules have multiple roles in immune cell function and development, and exert their functions in either a paracrine manner to modulate the activity of surrounding or nearby cells, or in an autocrine fashion affecting the cell responsible for the production of the cytokine [89]. The following biomarkers will be investigated in this study based on their involvement in the development of HIV-associated NHL, as reported in the literature: B-cell growth factors IL-4, IL-5 and IL-6. The immunoregulatory and/or anti-inflammatory cytokine IL-10, chemokines (CXCL8) IL-8, stromal derived factor 1 alpha (SDF-1α), pro-inflammatory cytokines IL-1β, and IL-2, TNFα and interferon gamma (IFNγ). In addition, this study will consider soluble factors associated with B-cells namely sCD23 and sCD30.

1.7.1. B-cell growth factors

IL-4 is a multi-functional T helper (Th) cell 2 cytokine and growth factor of mature B-cells [9, 90]. IL-4 also induces its effects on various targets such as haematopoietic cells, tumour and endothelial cells [91]. Along with co-stimulatory molecules CD40 ligand (CD40L) and lipopolysaccharide (LPS), IL-4 has the capacity to induce B-cell proliferation, differentiation and antibody secretion [92, 93]. It is predominantly responsible for the class switching of antibody secreting B-cells to IgE [94, 95]. T follicular helper cells (T_{FH}) located in the germinal centre, also produces IL-4. The germinal centre is where most NHL tends to develop indicating a role in lymphomagenesis [84].

IL-5 is another cytokine produce by Th2 as well as mast cells [92]. It acts on various targets binding to its receptor to induce cell survival and differentiation of B-cells as well as eosinophil's and basophils [96, 97]. IL-5 also stimulates the maturation of CD40 activated B-cells and IgG1 secretion in B-cells stimulated with LPS [92, 93].

The pleiotrophic pro-inflammatory cytokine IL-6 is a potent autocrine B-cell growth factor [83, 98, 99]. IL-6 has been extensively studied for its role in both HIV infection and HIV related NHL [100, 101]. Increased IL-6 levels are well-documented in HIV positive patients and show a strong correlation with mortality in patients with low CD4+ T cell counts [102]. IL-6 may reflect the chronic and recurrent inflammatory process underlying chronic HIV infection and many other disease states [103].

It is a non-redundant cytokine associated with terminal B-cell differentiation, specifically plasma cell differentiation, and assists in antibody production subsequently enhancing the development of lymphoma [94]. The role of IL-6 in B-cell lymphomagenesis is well documented in chronic viral infections and IL-6 mimetic agents are associated with infection with agents such as KSHV [104, 105]. Numerous studies have shown that IL-6 levels are elevated in HIV infection facilitating its replication cycle as well as significantly elevated preceding the development of HIV associated NHL [106-108].

1.7.2. Immunoregulatory cytokine IL-10

IL-10 is an anti-inflammatory and/or regulatory cytokine produced by regulatory T cells [107]. It mainly functions as an immunosuppressive cytokine converting the immune system from a Th1 pro-inflammatory state to an anti-inflammatory state [9]. IL-10 also induces the production of cytotoxic T-cells and the expression of IFNγ thereby promoting tumour killing [109].

Normal and malignant B-cells produce and respond to IL-10, thereby promoting B-cell growth and lymphomagenesis in an autocrine manner [98, 99]. In addition, it also promotes the differentiation of B-cells into plasma cells that secrete IgM, IgG and IgA [94, 110, 111]. A single nucleotide polymorphism (SNP) within the promoter region of IL-10 with the genotype (-592 C/C) has been associated with development of HIV related lymphoma [79]. Pre-diagnostic serum levels of IL-10 have also been shown to be elevated in patients with HIV associated NHL [83, 108, 112].

1.7.3. Chemokines implicated in B-cell physiology and NHL development

Chemokines are chemoattractrant cytokines that are responsible for trafficking of a number of immunologically active cells including B-cells and T-cells. They have an important homeostatic function in lymphocyte maturation and movement, and are up-regulated in response to inflammatory processes and result in cell cycle arrest [113, 114].

IL-8 is a chemokine of the CXC family and function as an activator and chemokine attractant of neutrophils [115]. IL-8 is also a pluripotent pro-inflammatory cytokine that has been reported to be related to the progression of tumours, prognosis and survival in various types of cancers [116]. In the tumour microenvironment, IL-8 is known for its angiogenic properties attracting neutrophils and macrophages to tumour site which may produce other cytokines and growth factors further promoting tumour growth and angiogenesis [115, 117].

It is also produced by tumour cells and acts in a paracrine manner on nearby cells in the tumour microenvironment to exert its function and support tumour growth, proliferation and metastasis [115]. Studies examining the relationship between IL-8 and NHL development have demonstrated that IL-8 was elevated in conjunction with creatinine in the urine of patients with NHL [116]. Other studies showed that IL-8 was expressed in several AIDS-NHL cell lines and was also significantly expressed in patients with NHL with or without HIV infection [118-120].

Another cytokine, SDF-1 or more commonly referred to as CXCL12, functions as a chemoattractant that homes haematopoietic stem cells, specifically B-cell and its precurosrs, to the bone marrow microenviroment [113, 114, 121]. In HIV infection, SDF-1 mediates the hyperproliferation of B-cells and shares a co-receptor or ligand with HIV, CXCR4, which has been shown to be highly expressed in hematological malignancies [121, 122].

Individuals with a single nucleotide polymorphism within SDF-1 (SDF1-3'A) have an increased risk of HIV associated NHL [121, 122].

1.7.4. Th1 cytokines

IL-1β, IL-2, IFNγ and TNFα are a collective group of pro-inflammatory cytokines. IL-1β is produced by activated macrophages and monocytes and mediates inflammation in conjunction with other mediators of inflammation affecting nearly all cell types [123].

IL-1β is produced by activated macrophages and monocytes and mediates inflammation in conjunction with other mediators of inflammation affecting nearly all cell types [123].The role of IL-1β in HIV-associated NHL is not known and has not been fully described. In HIV infection, however, IL-1β induces the expression of HIV-1 in U1 cells, a chronically infected cell line and is speculated to play a role in the pathogenesis of HIV along with IL-6 [107, 124].

IL-2 is produced by Th1 cells but is also a haematopoietic growth factor involved in the differentiation of naïve T-cells into Th1 cells [125-127]. IL-2 receptors are also located on normal B-cell and in malignant B-cell lines such as BL, chronic lymphocytic leukaemia and hairy cell leukaemia [125, 128]. In B-cell development, IL-2 enhances the differentiation of and Ig production by activated B-cell with other cytokines [94, 129, 130].

T helper cells 1 secrete a number of B-cell stimulatory cytokines such as IFNγ and TNFα which skew the adaptive immune response from a B-cell predominant humoral response to a

cell-mediated response [9, 107]. IFNγ is a type II interferon that has both anti-viral and anti-tumour properties as well as protecting the immune system against intracellular pathogens [131]. It primarily differentiates naïve T-cells into Th1 cells (along with IL-2) and aids in tumour surveillance by inducing the activity of cytotoxic lymphocytes. In addition, it also has the ability to induce class switching of B-cells [132, 133]. Studies have shown that IFNγ is increased in patients with HIV-associated NHL compared to HIV positive controls [120].

TNFα exerts its function indirectly on B-cells in an autocrine manner by augmenting the secretion of various cytokines (such as IL-6, IL-8 and IL-10), which in turn enhance B-cell functioning [134-137]. Increased serum levels of TNFα have also been associated with HIV-1 induced polyclonal B-cell activation as well as IL-4 induced antibody production., suggesting a possible role in lymphomagenesis (134, 138). Its pathogenic role in HIV-1 infection involves activation of nuclear factor κB (NFκB), stimulating apoptosis of T-cells and reducing HIV-1 viremia [137].

1.7.5. Soluble factors CD23 and CD30 in lymphomagenesis

Other markers have also been associated with B-cell activation and NHL development. Soluble CD23 is a low affinity receptor for IgE and is expressed on resting mature B-cells as a differentiation and/or early activation marker. sCD23 is cleaved from the cell surface of activated B-cells [139, 140], and has been linked to various other disease states in persons with B-cell hyperactivation and augmented humoral immunity, allergies as well as elevated IgE levels [141-143]. sCD30 is a member of the TNF receptor superfamily which are markers of B- and T-cell activation [84]. sCD30 is a known marker of chronic B-cell stimulation which may be involved in B-cell proliferation that could lead to genetic error accumulations and possibly cause lymphoma [144-146].

A comprehensive list of B-cell stimulatory molecules associated with B-cell malignancies in HIV is appended. (See Appendix A, Table 2). Although, it is clear that these molecules may contribute to the development and subsequent growth of malignant lymphomas, the actual role of individual cytokines and stimulatory molecules has not been fully elucidated [27, 84, 85].

AIM & OBJECTIVES:

The aim of this study was to detect and quantify specific biological biomarkers in the plasma of patients with HIV-associated high grade B-cell lymphoma and investigate their contribution to lymphomagenesis within a South African population/setting.

Objectives

1. To determine the expression of cytokines, chemokines and soluble factors in the plasma of patients with HIV-associated high grade B-cell lymphoma using the Bio-Rad Luminex xMAP™ Technology cytokine/chemokine panels and ELISA soluble factor kits.
2. To investigate the effects of various cytokines on a lymphoma cell culture by measuring markers of activation CD38+, proliferation Ki-67 and/or ploidy and Annexin V-FITC apoptotic assay, by immunophenotyping and flow cytometry.

CHAPTER 2.0

MATERIALS AND METHODS

2.1. Ethics Statement

This study was approved by the Medical Human Research Ethics Committee (HREC) of the University of the Witwatersrand and provided with the clearance number HREC M140395 (Appendix A). This project was conducted in accordance with the guidelines of the ethics committee.

2.2. Study Cohort and sample collection

This study used residual peripheral blood samples collected in ethylenediaminetetraacetic acid (EDTA) tubes from HIV positive patients diagnosed with high-grade mature B-cell NHL by histology or by immunophenotyping (n=36). The B-cell NHL subtypes included in this study were Burkitt Leukaemia/lymphoma, Diffuse Large B-cell Lymphoma and DLBCL not otherwise specified with features intermediate between Burkitt and Diffuse Large B-cell lymphoma. Samples from patients with PEL, PL and large cell lymphoma arising in HHV8 multicentric Castlemen's disease were not included or further studied. Samples were collected between 2013 and 2015 referred from the Wits Academic Hospital Complex (Chris Hani Baragwanath Hospital, Helen Joseph Hospital and Charlotte Maxeke Johannesburg Academic Hospital) referred to the immunophenotyping laboratory at Charlotte Maxeke Johannesburg Academic hospital (CMJAH) (National Health Laboratory Service). All adult patients between the ages of 18-80 years with confirmed HIV infection and diagnosis of NHL with sufficient residual sample for testing were originally included. Clinical data including diagnosis, age, gender, CD4 counts, and viral loads were collected from the NHLS TrakCare Lab database system. A small number of samples (n=4) had later to be excluded because of co-existing confounding diseases, specifically co-infection with *Mycobacterium tuberculosis*. Patients with NHL were matched by age (5 years) and gender to HIV-positive patients with no laboratory evidence of B-cell NHL (n=32).Peripheral blood was centrifuged at 3500 revolutions per minute (rpm) for 15 minutes to separate plasma. Plasma was stored for all patients at -80^0C until further use.

2.3. Initial characterization of HIV-associated high grade B-cell lymphomas

The diagnosis of HIV-associated high grade mature B-cell lymphoproliferative disorders was made by histology and subtyping was confirmed immunophenotypically (by flow cytometry or by immunohistochemistry) on an appropriate sample according to the WHO classification of haematological lymphoid malignancies [34]. The following markers were used to characterize and identify patients with HIV-associated high grade B-cell lymphomas: B-cell lineage markers CD19, 20, germinal center marker CD10, and FMC7 which is an epitope for CD20 [46, 55]. Clonality was determined by kappa or lambda Ig light chain restrictions or by PCR analysis of IgH gene rearrangements [48]. If indicated, additional markers were added including CD38, CD138 and BLIMP-1 to indicate plasma cell differentiation [68, 70]. In all cases, samples were obtained before patient's commenced chemotherapy.

2.4. Detection and quantification of cytokines

Luminex multiplex assays are fluorescent magnetic bead based immunoassays that allows for the quantifiable detection of multiple markers within a single sample, simultaneously [147]. The principle of the method is based on the concept of the sandwich ELISA where the antigen in question present in the sample is sandwiched between two antibodies conjugated to beads [148, 149]. The concentration of the protein is proportional to the amount of streptavidin-RPE fluorescence detected.

Experiments were conducted on plasma of HIV-positive patients diagnosed with high grade B-cell NHL (n=32) and HIV positive individuals without overt evidence of lymphoma (n=32). The cytokines assessed were selected based on the relevant literature and the human cytokine 10-plex panel (Invitrogen, Life Technologies Corporation, USA) was selected as the most appropriate to measure the relevant cytokines. The following analytes were measured: IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, granulocyte-macrophage colony stimulating factor (GM-CSF), interferon gamma (IFNγ) and TNFα. All standards and plasma samples were set up in duplicate and reagents as well as were allowed to come to room temperature before proceeding with experiments. All standards, plasma samples and buffers (wash solution, antibody mixes, and streptavidin-RPE) were diluted with the appropriate diluent prior to the experiment (see Appendix B). Standards and plasma samples were incubated with conjugated capture antibodies beads in a 96 well plate for 2 hours at room temperature in the dark on a rotating platform. After incubation, the plate was washed with wash solution to remove excess unbound antibody.

A secondary biotinylated detector antibody was added to all wells and the samples were incubated for an additional 1 hour. The wash step was repeated post incubation to remove excess biotinylated antibody. Conjugated Streptavidin-RPE, was added to all wells and the samples were incubated for a further 30 minutes [150, 151]. The beads were resuspended in wash solution and standards and samples were acquired using the Luminex xMAP™ technology (Bio-Rad Laboratories Inc., USA) and xPONENT 3.1 acquisition software (Luminex Corporation, Austin, Texas).

2.5. Quantification of Stromal Derived Factor-1 alpha (SDF-1α) by Luminex Technology

Recent literature has suggested that SDF-1α levels were significantly increased in the plasma of HIV positive patients with NHL [113]. Patients with residual sample were therefore tested subsequent to the cytokine analysis for SDF-1α levels. Unfortunately, only 20 patients had sufficient residual samples for assessment. An SDF-1α simplex kit was selected which uses similar principles to the cytokine kit but utilises magnetic beads.

A magnetic 96-well plate was coated with magnetic SDF-1α antibody beads and allowed to rest for 2 minutes on a magnetic hand held washer for the accumulation of the beads before the liquid was discarded. Standards and plasma samples were incubated for 2 hours at room temperature in the dark on a rotating platform. The liquid was then discarded and the magnetic wash step repeated twice to remove unbound antibody. Detection antibody was added to all wells and incubated for another 30 minutes. The wash step was repeated twice and streptavidin-PE was added to all wells followed by a final incubation period of 30 minutes. The beads were resuspended in reading buffer and acquired using the Luminex xMAP™ technology (Bio-Rad Laboratories Inc., USA) and xPONENT 3.1 acquisition software USA).

2.6. Quantification of soluble factors sCD23 and sCD30 in plasma by Enzyme-linked Immunosorbent Assay (ELISA)

Previous studies have demonstrated increased serum levels of sCD23 and sCD30 in HIV positive individuals who develop lymphoma and NHL several months before diagnosis [84, 152]. This may show utility for early diagnosis and monitoring of response. To determine levels in this study cohort, human soluble CD23 (sCD23) EASIA (BioSource Europe S.A., Belgium) and CD30 (sCD30) ELISA kits (Invitrogen, Life Technologies Corporations, USA) were used to perform the relevant assays.

All standards, controls and plasma samples were set up in duplicate by vertical alignment as suggested per manufacturer's protocol. Microwell strips (96) pre-coated with capture monoclonal antibodies against sCD23 and sCD30 were provided with the each individual kit. In addition, lyophilized standards (1-6) and lyophilized controls (1 and 2) in human plasma with preservatives were provided with the sCD23 kit (see Appendix B for control and standard concentrations).

2.6.1. sCD23 detection by ELISA

The sCD23 assay is a chromogenic ELISA which utilises tetrametylbenzidine (TMB) to allow visualisation. Standards, controls and patient samples were incubated with anti-sCD23 conjugate for 2 hours at room temperature on a rotating platform. Post incubation, the plate was washed three times with wash solution. TMB was added to all wells and incubated for another 30 minutes. Stop solution was added to all wells when the highest standard developed a determined degree of chromogenicity. The absorbance was read at 450nm [153] on the NEXGEN FOUR fully automated ELISA machine and Open Lab® Adaltis software (Adaltis, Italy). Additional filters of 620nm and 405nm were included for plastic interference and over-range filter respectively.

2.6.2. sCD30 ELISA

Plasma samples and standards were incubated with diluted horseradish peroxidase (HRP) conjugate for 3 hours at room temperature on a rotating platform. All wells were emptied and washed three times to remove excess unbound antibody. TMB substrate solution was added to all wells and incubated for an additional 10 minutes. Stop solution was added to all wells when the highest standard concentration developed a dark blue colour [154].

The absorbance was read at 450nm on the NEXGEN FOUR fully automated ELISA machine and Open Lab® Adaltis software (Adaltis, Italy). Additional filters of 620nm and 405nm were included for reference wavelengths, with a range of 610-650nm as acceptable).

2.7. Optimization of the *in vitro* cell culture of HIV associated NHL cases

2.7.1. Cryopreservation and thawing of cells

PBMC or (BMC) cells isolated via Ficoll-Histopaque density gradient centrifugation method were stored in cell culture freezing media (Millipore Corporation, USA) at -80^{0}C until further use. The cells were thawed in complete culture media composed of Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with L-glutamine and Sodium hydrogen carbonate ($NaHCO_{3)}$ (Sigma- Aldrich, Steinheim) with 5% fetal bovine serum (FBS) (Highveld Biological, SA) and 1% penicillin/ streptomycin (Gibco Life Technologies, USA). The cells were thawed in a water bath at 37^{0}C, resuspended in culture media and centrifuged at 3000 rpm to wash off residual freezing medium. The total numbers of cells were counted and the viability was checked under an inverted light microscope (Alltion®(Wuzhou) Co.,Ltd. China) using the Neubauer haemocytometer counter chamber (Hausser Scientific, USA) and trypan blue exclusion dye (Sigma-Aldrich, Steinheim) [155, 156]. Trypan blue exclusion dye was used to determine the number of live and dead cells, as well as cell viability. Live cells were impermeable to the dye because of their intact plasma membrane and remained unstained, whereas dead cells absorbed the dye into the cytoplasm as a result of the loss of their membranes and were stained blue [157, 158].

The volume of culture media was adjusted in order to resuspend the cells at a concentration of 1 x 10^6 cells/ml (see Appendix C for calculations). The cells were then incubated for 24 hours at 37^{0}C with 5% CO_2 until confluent [155]. Cells were then seeded at a concentration of 1 x 10^{6}cells/ml and treated with 200-1000 pg/ml of recombinant human IL-6 and further incubated for 2 hours at 37^{0}C with 5% CO_2 in air.

2.8. Antibody titration

Antibody titration was performed on various MAb to determine the optimal concentration or volume that would produce the highest and lowest fluorescent intensity of the positive and negative populations of interest respectively, while simultaneously reducing the amount of background signal or noise and non-specific binding of the antibody to the population of interest [159]. Peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood collected in EDTA tubes by Ficoll-histopaque density gradient-centrifugation. MAb (Appendix D) were incubated with PBMCs for 15 minutes in the dark at room temperature. An unstained tube containing cells only was also included.

Unbound antibody was then washed with PBS and the cells were resuspended in PBS and were acquired on the LSRII flow cytometer (BD-Biosciences, USA) using BD FACSDiva v6.1 acquisition software (BD-Biosciences, USA).

2.9. Flow cytometry to determine the role of significant biomarkers in lymphomagenesis

Flow cytometry was performed to investigate the effects of various biomarkers, selected from the previous Luminex and ELISA assays, on the cultured lymphoma cells. Parameters measured included; CD38 which determined the percentage of activated tumour cells, Ki-67 which is a marker of proliferation, and propidium iodide (PI) which measured the ratio of live-to-dead cells. All samples used had tumour burdens in excess of 90% of cells on initial flow cytometric analysis and it was not considered necessary to add either B-cell or T-cell discriminatory markers.

Cells were harvested and resupended in culture media. Cells were added to the appropriate tubes with MAb (Appendix E) and incubated for 30 minutes in the dark at 2 - 8°C.The cells were washed twice with cell staining buffer to wash off residual culture media (BioLegend, USA). The cells were then resuspended in cell staining buffer and acquired on the LSR II Flow cytometer using the BD FACSDiva v6.1 software (BD-Biosciences, USA). A minimum of 100000 events were recorded.

2.10. Propidium Iodide Live/Dead staining

Propidium Iodide is a viability dye which stains the nucleus of a cell by intercalating between the nucleic acids of double stranded DNA. Viable cells have intact cell membranes and are impermeable to the dye, whereas dead cells are permeable to the dye [160]. Cultured PBMCs were harvested and washed in PBS and resuspended in cell staining buffer. Cells were stained with 10 µl of PI followed by an incubation period in the dark at room temperature. The cells were then acquired on the LSRII Flow cytometer using BD FACSDiva v6.1 software (BD-Biosciences, USA) with a minimum of 1 million events recorded. An unstained control was used to set the voltages for the FSC and SSC scatter dot plots in acquisition mode. Analysis was performed using FlowJo gating software (FlowJo v9.2, ©Tree Star Inc.).

2.11. Instrument Calibration

Please see Appendix F for calibration of the LSRII Flow cytometer and Bio-Rad Luminex200.

2.12. Statistical Analysis

1. Statistical methods

Normality or frequency distribution was measured using Shapiro Wilk normality test and computed using GraphPad prism (GraphPad Prism 5™, San Diego, California, USA). If the data were normally distributed the mean, standard deviation and Pearson's correlation was used. Non-parametric parameters and measures of association such as the median, range and Spearman's rank correlation respectively, were computed if the data were not normally distributed. HIV-associated NHL and HIV positive controls without lymphoma data were computed and compared to one another using the non-parametric Mann-Whitney test. Where the data were normally distributed, parametric unpaired student's t-test was computed. All box and whisker plots are representative of the minimum and maximum values or range depicted by the bars and/or whiskers, 25^{th} and 75^{th} percentiles represented by the body and/or box itself and the median, as shown by the horizontal line within the box. P values of * = $p < 0.05$; **= $p < 0.001$ and ***= $p<0.0001$ were considered to be statistically significant.

2. Luminex and ELISA Data Analysis

The mean concentration and mean fluorescent intensities (MFI) were collected for all analytes acquired on the Luminex xMAP™ technology (Bio-Rad Laboratories Inc., USA). The average optical density (450nm) and plasma concentration (U/ml) were recorded for both sCD23 and sCD30 ELISA assays. Cytokine plasma concentrations (pg/ml) were calculated and extrapolated from a 5-point logistic standard curve for all samples (See Appendix G). Soluble factor concentrations (U/ml) were calculated and extrapolated from a 4-point logistic standard curve for all samples (See Appendix G).

3. Flow Cytometric Data Analysis

Data from the flow cytometric analysis was analysed using FlowJo software (FlowJo v9.2, ©Tree Star Inc.). Populations of interest were gated along a forward (FSC) vs. side scatter (SSC) dot plot using BD FACSDiva Software v6.1 (BD Biosciences, USA). Propidium Iodide plots were represented as histograms in the linear scale. The non-viable cells were also gated. The (MFIs) and/or geometric means were recorded for the antibody titrations curve using FlowJo software and plotted against the respective volumes on a xy graph using Microsoft Excel.

The highest point or plateau area on the graph indicated the optimal concentration or amount of antibody to use for optimal fluorescent intensity and cell positivity for experimentation (Appendix F).

4. Demographic Data

The median was measured and computed for demographic data such as the age (range ±5 years), CD4+ T cell ($x10^6$L) counts and HIV viral loads (copies/ml) were data was available.

CHAPTER 3.0

RESULTS

3.1. Sample Collection of Study Cohort

Residual blood was collected from HIV positive patients with confirmed NHL (n=36) and compared with HIV positive age- and gender-matched controls without any evidence of lymphoma (n=32). Of these HIV-positive NHL cases, 19 were diagnosed with DLBCL, 5 with BL, 4 had a diagnosis of unclassifiable lymphoma with features intermediate between DLBCL and BL and 4 cases were not classified by subtype [36, 42]. None of the patients assessed had documented plasmablastic variant lymphoma, primary effusion lymphoma or HHV8 and therefore was not examined. The majority of the HIV-associated NHL tumours were initially localized in extra-nodal sites. Diffuse large B-cell lymphoma tumours were localized to the GIT and head and neck masses and to nodal sites including the cervical and axillary nodes. Burkitt lymphoma cases presented mainly in the CNS and the axillary. The intermediate group, demonstrated lymphoma masses mainly in the pelvic or cervical nodes. Primary tumour location could not be determined in n=13 cases. In many of these cases, the patients presented with widely disseminated disease or leukaemia. The aforementioned are described in Table 3.1 which outlines the site or location of the tumour per NHL subtype, while Table 3.2 depicts demographic information for all patients including age, CD4 count (x 10^6L) and HIV viral load (copies/ml).

In HIV-associated NHL cases, the median age was 39 years and the CD4+ T cell count was significantly lower compared to the HIV positive controls (median 101 vs. 356.77 x 10^6L, $p<0.0001$). The median CD4+T counts of patients DLBCL (131 x 10^6L) were slightly higher ($p= 0.8699$) than patients diagnosed with BL (41 x 10^6L), intermediate lymphoma (86 x 10^6L) and NHL cases not specified (79 x 10^6L). The majority of the study cohort (n=23) and controls (n=25) had a viral load below the limit of detection. Of those patients who had detectable viral loads, the median viral load was higher in HIV-associated NHL cases compared with controls (401 275 vs. 73.50 copies/ml).

Table 3.1: Characteristics of HIV-Associated NHL Subtypes with location and CD4 count

HIV-associated NHL subtypes	Site of lymphoma	Median CD4 count ($X10^6L$)
Burkitt Leukaemia/Lymphoma	3 disseminated 1 CNS 1 Axillary mass	41
Diffuse large B-cell Lymphoma	Unknown primary site (n=6) Head and neck masses (non-nodal) Nasopharyngeal masses (n=2) Para nasal mass (n=1) Posterior nasal space (n=1) Gastrointestinal tumour Small intestine (n=1) Colon (n=1) Nodal sites Sub-mandibular and cervical nodes (n=3) 1 Cervical node 1Inguinal node (n=1) 3 Axillary nodes (n=3)	131
B-Cell Lymphoma unclassifiable, with features intermediate between diffuse large B-cell lymphoma (DBCL) and Burkitt lymphoma (BL)	Pelvic mass (n=1) Mediastinal mass (n=1) Unknown primary site (n=1) 1 Cervical node (n=1)	86
Not specified	Unknown primary site (n=3) 1 cervical node (n=1)	79

Table 3.2: Characteristics of the cohort

Parameter	HIV-associated B-cell NHL	HIV positive controls
Sample size (n)	32	32
Age (years) range	18-58	19-61
Median age (5 years)	39.00	40.50
Average age± Std deviation	39.42±9.354	39.37±11.61
Gender	15 F;17 M	15 F;17 M
CD4 Count ($X10^6L$) Range	13-747	69-830
CD4 Count ($X10^6L$) Median	101	356.77
Average CD4 Count± Std deviation	159±195.3	378.27±206.07
HIV Viral load (copies/ml) Median	401 275 (n= 9)	73.50 (n=7)
Treatment status (n)		
On HAART	n= 11	32

3.2. Initial characterization of HIV-associated high grade B-cell lymphomas

Flow cytometric plots of a normal B-cell population and a high grade DLBCL NOS case are presented in Figure 3.1 below. Lymphocytes were defined by their forward and side scatter characteristics. The B-cells were identified by expression of the pan-B-cell marker CD19. Clonality was determined by the ratio of expression of kappa to lambda light chains (with a ratio of 1:1 to 3:1 considered normal). DLBCL cells were identified by their size (larger than normal lymphocytes), the monoclonal expression of either kappa or lambda light chains and by the expression of B-cell markers. The expression of CD20 and the epitope FMC-7 with surface IgM expression was used to assess maturity and CD10 expression was considered an immunophenotypic indication in mature cells of germinal centre origin. A representative scatter plot of a DLBCL tumour is shown in Figure 3.2 and a polyclonal B-cell population in Figure 3.3. The normal lymphocytic population highlighted in green on the FSC vs. SSC in Figure 3.1.

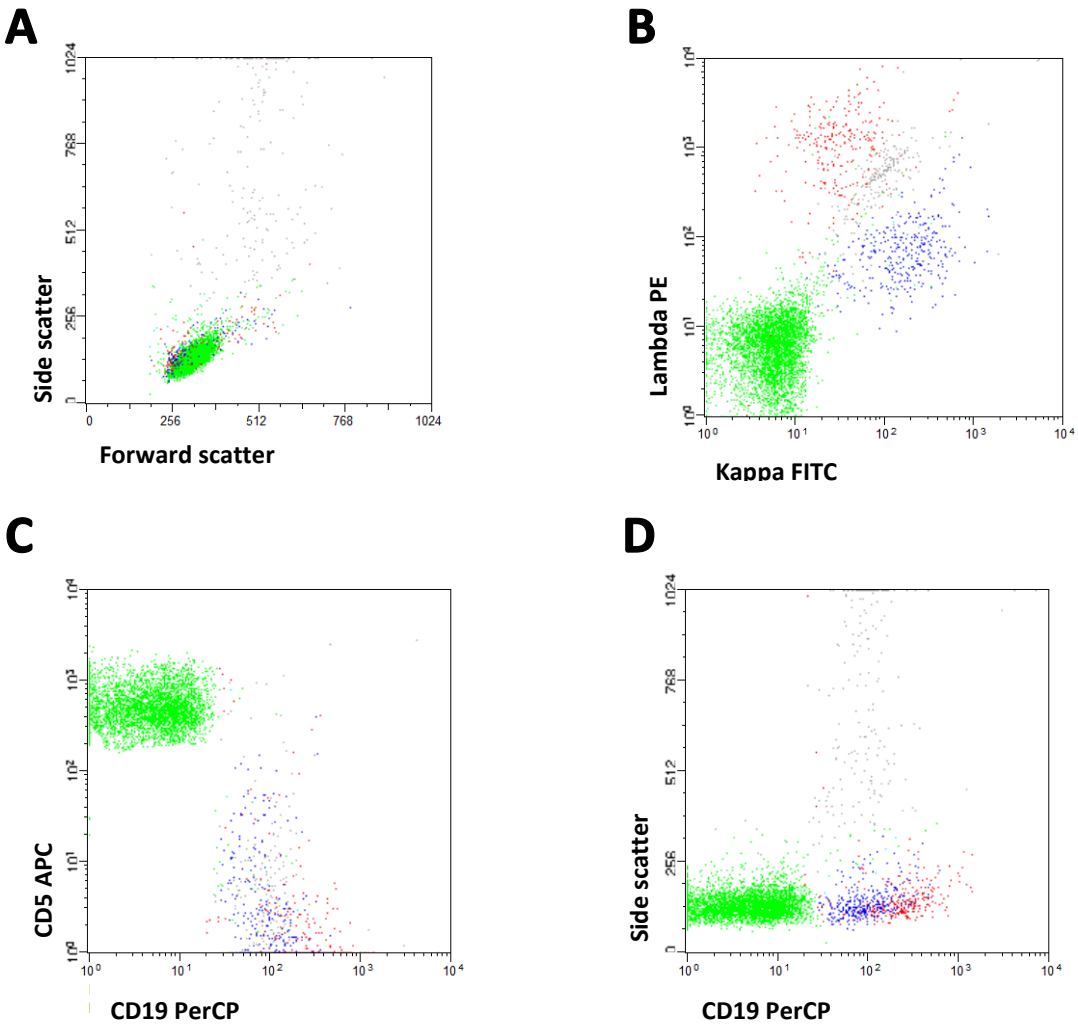

Figure 3.1: Flow cytometric immunophenotypic diagnosis of a normal polyclonal case. **A)** FSC vs. SSC dot plots illustrates lymphocytes, **B)** The cells demonstrate a normal polyclonal light chain ratio (anti-Kappa (FITC) on the x-axis) Lambda (PE) on the y-axis). **C)** CD5 (APC) vs. CD19 (PerCP) shows CD5+ B cells and **D)** CD19+ B-cells.

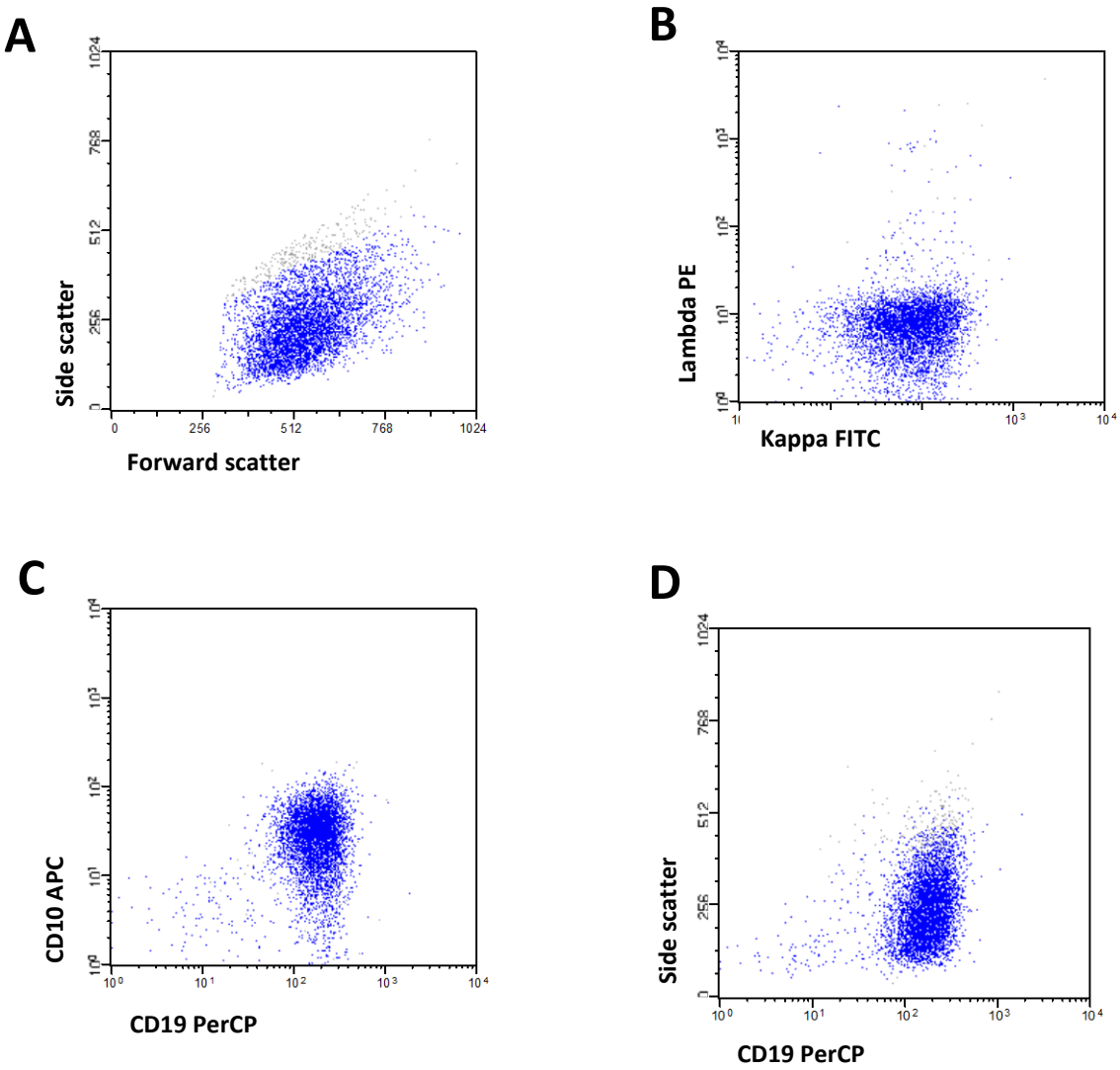

Figure 3.2: Flow cytometric immunophenotypic diagnosis of a high grade DLBCL case. **A)** The cells are larger than typical lymphocytes **B)** the cells demonstrate lambda light chain restriction [Kappa (FITC) vs. Lambda] **C)** and **D)** the cells express both the germinal centre marker, CD10, and positivity for the pan-B cell marker CD19.

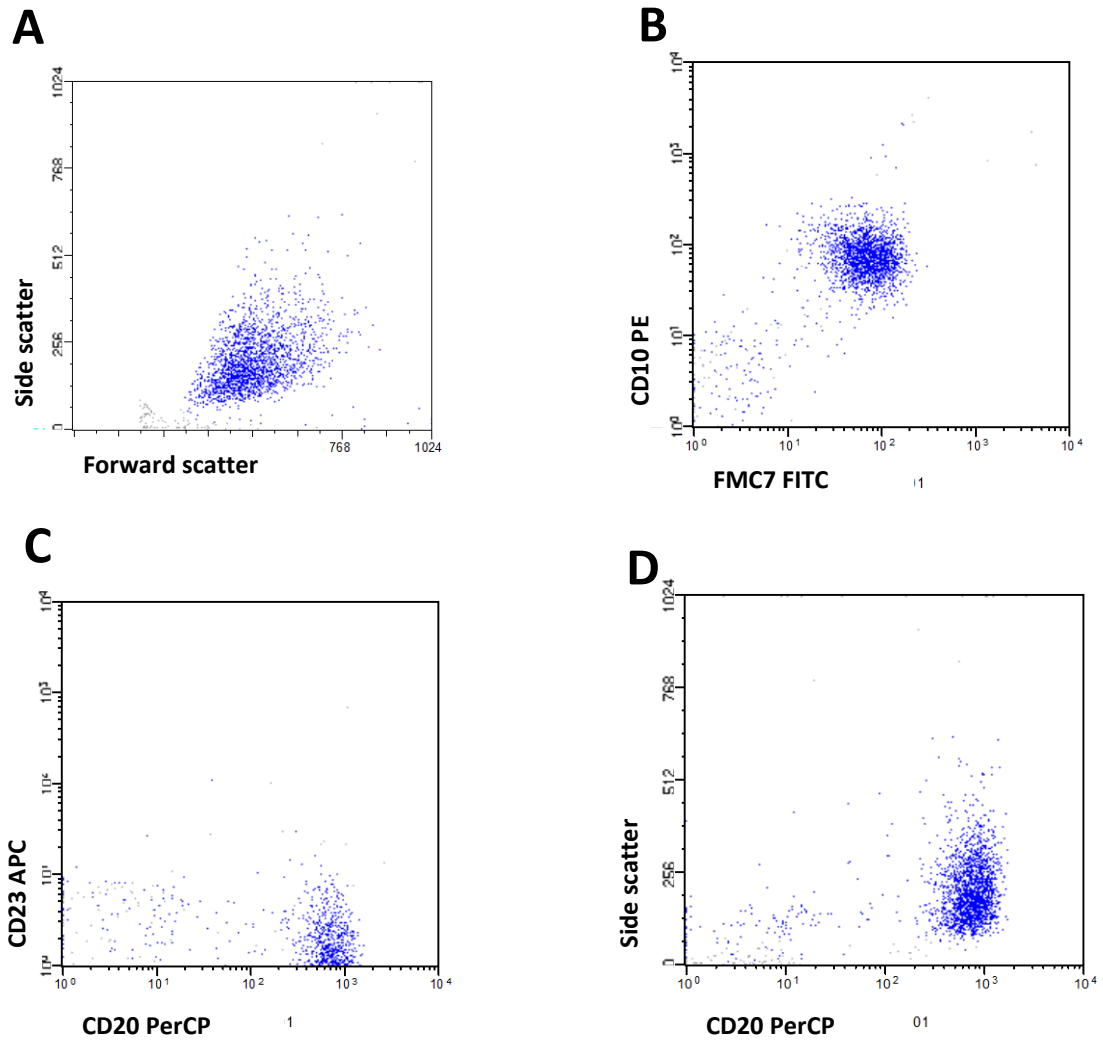

Figure 3.3: Immunophenotypic diagnosis of a high grade DLBCL case. Cells show **B)** maturation and bright CD20 expression **(**FMC7 (FITC)) and express the germinal centre marker CD10 (PE), **C)** and D) bright CD20 expression (PerCP).

3.3. Cytokine expression in HIV positive lymphoma patients

This study aimed to examine the circulating cytokine expression in the plasma of HIV positive NHL patients and was compared to HIV positive patients with no evidence of NHL as it was hypothesized that these levels would differ between the two groups. Plasma samples were assessed for 10 cytokine analytes namely: IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IFNγ, GM-CSF and TNFα as well as SDF-1α using the Luminex analyser with xPONENT 3.1 software. Cytokine analytes will be discussed in functional groups; B-cell growth factors, anti-inflammatory and/or immunoregulatory cytokines, chemokines and pro-inflammatory cytokines.

3.3.1. Circulating levels of B-cell growth factors

IL-4, -5 and -6 were the B-cell growth factors measured in this study. IL-6 is a pleiotropic cytokine that is involved in the terminal differentiation of B cells and aids in antibody production [83, 99]. The median IL-6 concentration was significantly elevated in HIV positive patients with NHL compared with HIV positive controls without evidence of lymphoma (median concentration, 16.70 vs. 8.892 pg/ml respectively, $p<0.0001$) (Figure 3.4). IL-4 is a B-cell haematopoietic growth factor and facilitates proliferation in the presence of co-stimulatory molecules such as CD40 or lipopolysaccharides (LPS) [95], whereas IL-5 directly promotes the differentiation and proliferation of B-cells [92]. In this study it was observed that the median IL-4 concentration levels were slightly higher in HIV positive patients compared with controls, however these results were not statistically significant (median concentrations 67.46 vs. 42.29 pg/ml respectively, $p= 0.4211$). No differences were noted between HIV-positive patients with NHL and controls for IL-5 (median concentrations 18.52 vs. 18.43 pg/ml respectively, $p= 0.1642$) (Figure 3.4).

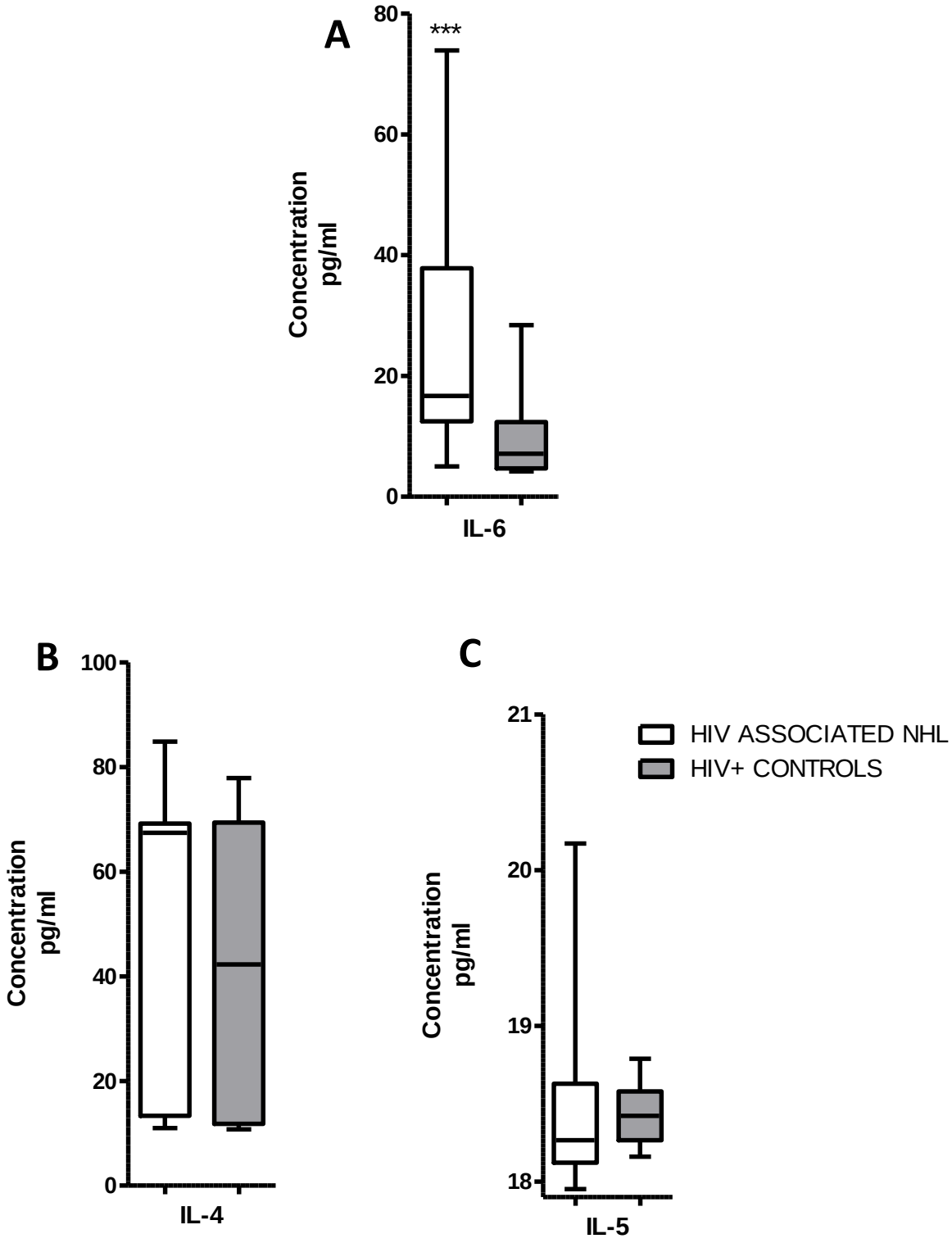

Figure 3.4: B-cell growth factors expressed in HIV positive patients with documented NHL. A) IL-6 median concentration was significantly elevated in HIV-associated NHL cases (median 16.70 pg/ml) compared to HIV positive controls (median 8.892 pg/ml), $p<0.0001$. **B)** IL-4 median concentration was slightly elevated in HIV-associated cases (median 67.46 pg/ml) compared with controls (median 42.29 pg/ml) but was not significant. **C**) No statistically differences in the median concentration levels of IL-5 between HIV-associated NHL cases and controls (median 18.52 vs. 18.43 pg/ml respectively).

3.3.2. Immunoregulatory cytokine levels were elevated in HIV-associated NHL

IL-10 is an anti-inflammatory and/or regulatory cytokine which suppresses tumour specific responses and also serves as a growth factor for non-malignant and malignant B-cells in an autocrine manner thus contributing to lymphomagenesis [98, 99]. It was observed that the median IL-10 plasma concentration was significantly elevated in HIV positive patients with NHL compared to HIV positive controls without evidence of lymphoma (median concentration, 42.46 vs. 21.34 pg/ml respectively, p= 0.0010) (Figure 3.5).

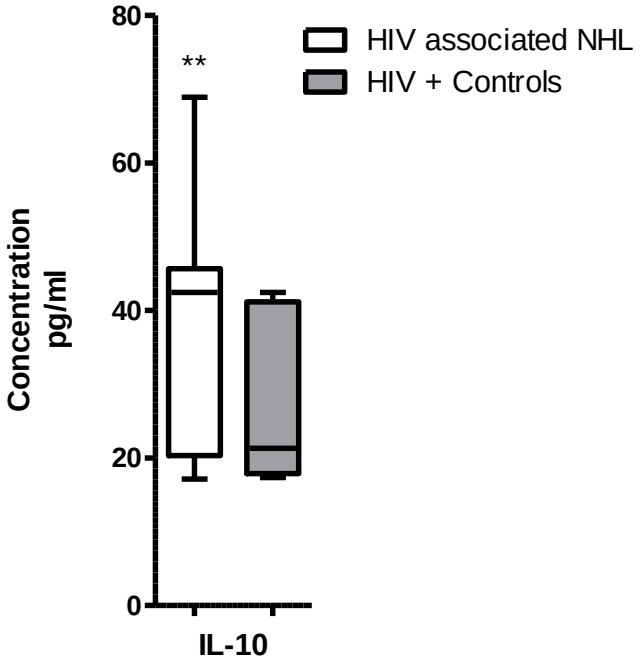

Figure 3.5: Immunoregulatory cytokine expression was elevated in HIV-associated NHL. IL-10 median concentration was significantly higher in HIV positive individuals with NHL (median 42.46 pg/ml) compared to HIV positive controls without lymphoma (median 21.34 pg/ml), p= 0.0010.

3.3.3. Chemokines detected in plasma of HIV-associated NHL patients

IL-8 is a pluripotent chemokine of the CXC family and angiogenic cytokine that has been reported to be related to the progression of tumours, prognosis and survival in various types of cancers [116]. IL-8 median concentrations were significantly elevated in plasma of HIV positive patients with NHL compared with HIV positive controls (median concentration, 262.1 vs. 82.43 pg/ml respectively, p= 0.0098 (Figure 3.6).

SDF-1 is a chemoattractant for homing haematopoietic stem cells, specifically the B-cell and its precursors, to the bone marrow microenviroment [114]. Differences in the medians of the plasma concentrations of SDF-1α were noted and elevated in HIV positive patients with NHL cases compared to HIV positive controls without lymphoma. These results were not, however, statistically significant (median concentration, 347.1 vs. 318.1 pg/ml respectively, p= 0.3513) (Figure 3.6).

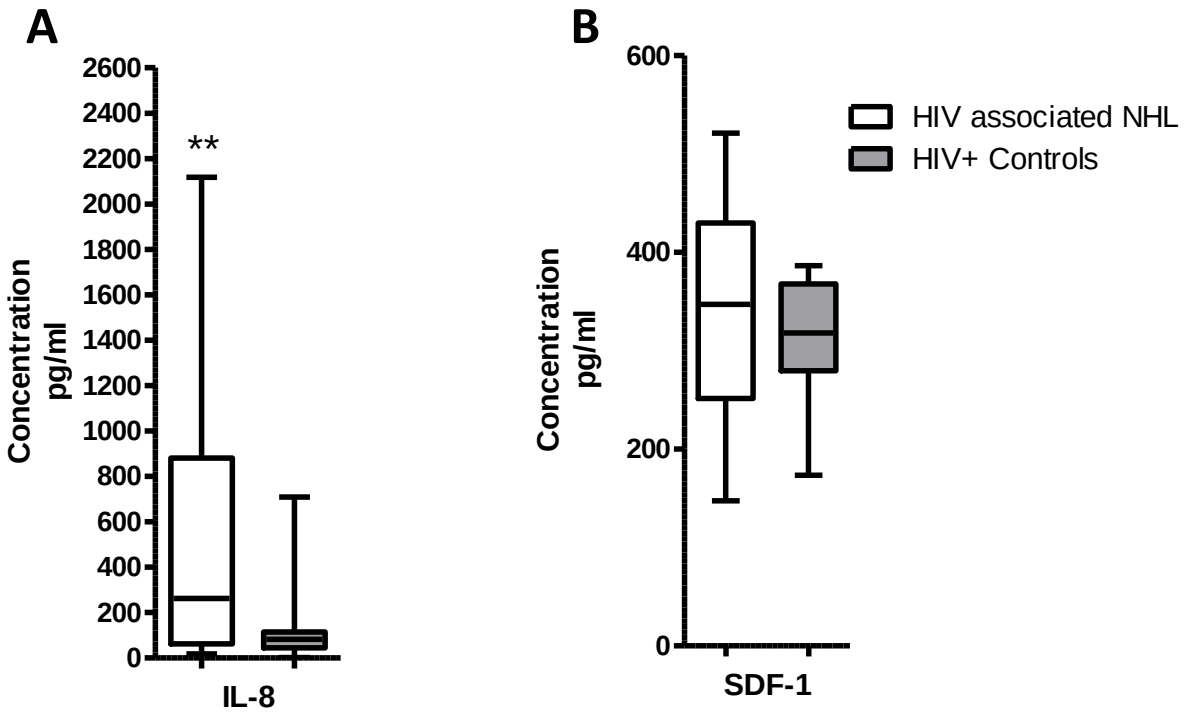

Figure 3.6: Circulating chemokine IL-8 was increased in HIV-associated NHL. A) IL-8 was significantly elevated in HIV positive individuals with NHL (median 262.1 pg/ml) compared to HIV positive controls (median 82.43 pg/ml), p = 0.0098. **B)** Differences SDF-1α in medians were noted between HIV positive patients with NHL (median 347.1 pg/ml) and controls (318.1 pg/ml) but were significant, p=0.3514.

3.3.4. Pro-inflammatory cytokines measured in HIV-associated NHL

Pro-inflammatory cytokines were assayed in HIV positive individuals with NHL (Table 3.3). Each of these cytokines is associated with aspects of B-cell development. IL-2, along with other cytokines, assists with the differentiation of activated B cells [(94)]. TNFα provides co-stimulus for the differentiation of B-cells induced by CD40L and IL-4. IFNγ promotes the survival and differentiation of B-cell [(94)] and GM-CSF is an essential haematopoietic growth factor and immune modulator [(161)]. No statistical differences were noted between the median concentrations of HIV-associated NHL cases compared to HIV positive controls without evidence of lymphoma for the cytokines levels of IL-1β, IL-2, IFNγ, TNFα and GM-CSF. The median concentrations are tabulated below for each analyte.

Table 3.3: Pro-inflammatory cytokines and GM-CSF statistical data identified in HIV-associated NHL cases vs. HIV positive controls

Cytokine	Median concentration (HIV positive NHL cases vs. HIV positive controls without lymphoma)
IL-1β	15.84 vs. 15.42, p = 0.8676
IL-2	18.93 vs. 16.48 pg/ml, p= 0.5732
TNFα	15.71 vs. 14.02 pg/ml, p= 0.9667
IFNγ	23.21 vs. 21.62 pg/ml, p = 0.9267
GM-CSF	43.75 vs. 29.21 pg/ml, p = 0.6150

3.4. Correlation between significant levels of cytokines expressed in HIV-associated NHL

IL-6, IL-8 and IL-10 were found to be significantly elevated in HIV-associated NHL when compared to HIV positive controls. We determined whether there was any correlation between these cytokines in HIV positive patients with NHL No significant correlation was noted between cytokine concentration levels of IL-6 and IL-8 (Spearman's correlation, r=0.01128, p= 0.9564), or between IL-8 and IL-10 (Spearman's correlation, r= -0.2278, p = 0.2630) (Figure 3.7).The latter cytokine concentrations also showed a negative correlation. Cytokine concentration levels of IL-6 and IL-10, however, were significantly correlated with one another (Spearman's correlation, r= 0.6280, p<0.0002) (Figure 3.7).

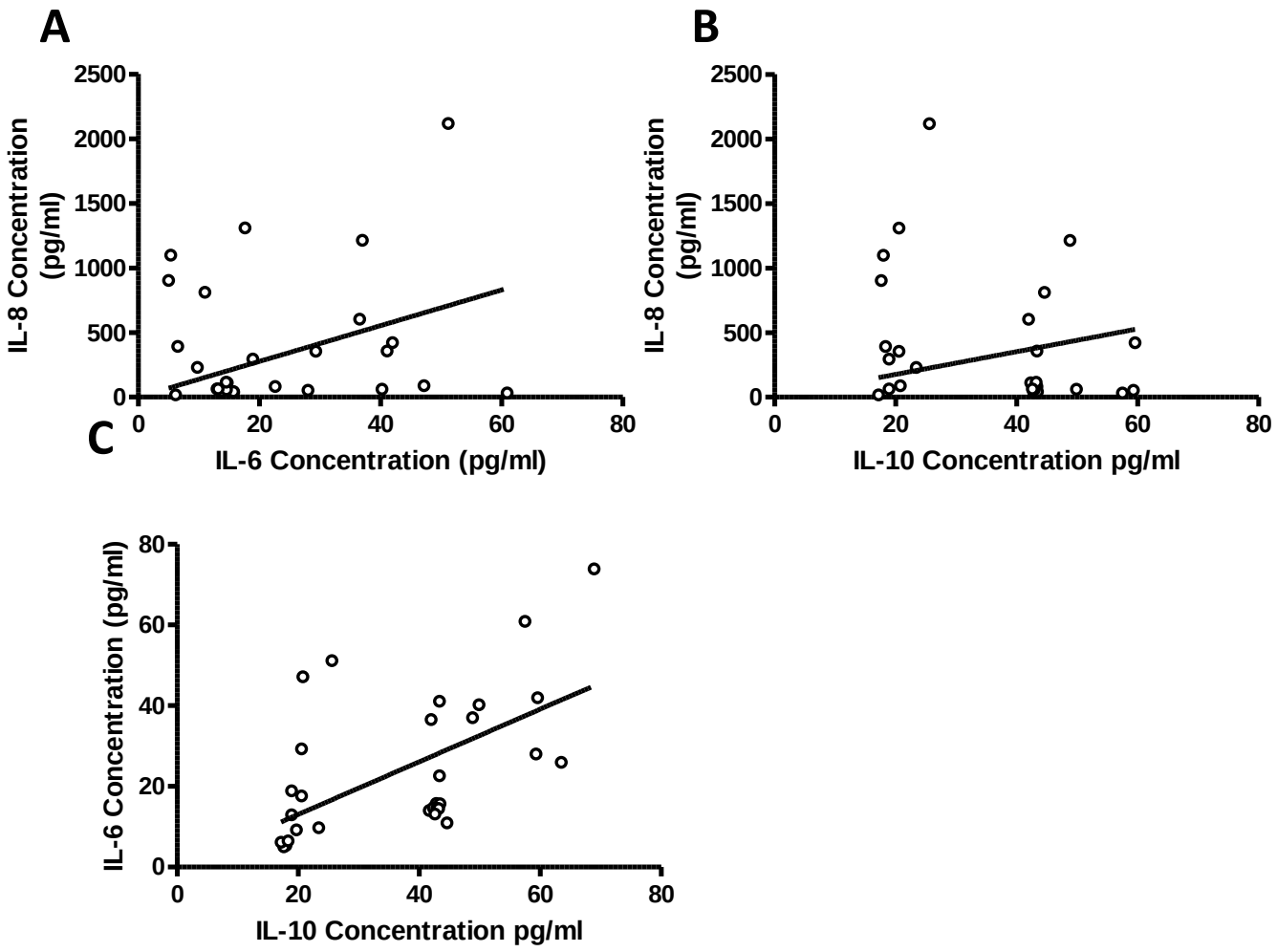

Figure 3.7: IL-6, -8 and -10 Correlations. IL-6 and IL-10 showed significant correlation, whereas no correlation was shown between IL-6 and IL-8, or IL-8 and IL-10. **A)** IL-6 vs. IL-8 (r=0.01128, p = 0.9564), **B)** IL-8 vs. IL-10 (r= -0.2278, p = 0.2630). and **C)** IL-6 vs. IL-10 (r= 0.6280, p<0.0002).

3.5. Circulating levels of soluble factors sCD23 and CD30 in HIV-associated NHL

Soluble factors CD23 and CD30 were assayed by ELISA as it has been previously reported that they may play a role in B-cell malignancy in HIV positive patients [84, 112]. CD23 is a low affinity receptor for IgE and is expressed on resting mature B-cells as a differentiation and/or early activation marker [139, 140]. Soluble CD30 is a known marker of chronic B-cell stimulation and proliferation possibly leading to lymphoma [144-146].

Our results showed that the plasma levels of sCD23 (median concentrations 1.972 vs. 1.583 U/ml, respectively, p= 0.0759) and sCD30 (median concentrations, 23.14 vs. 10.67 U/ml respectively, p= 0.2045) were elevated in HIV positive patients with NHL cases compared to the HIV positive controls, however these results were not statistically significant (Figure 3.8).

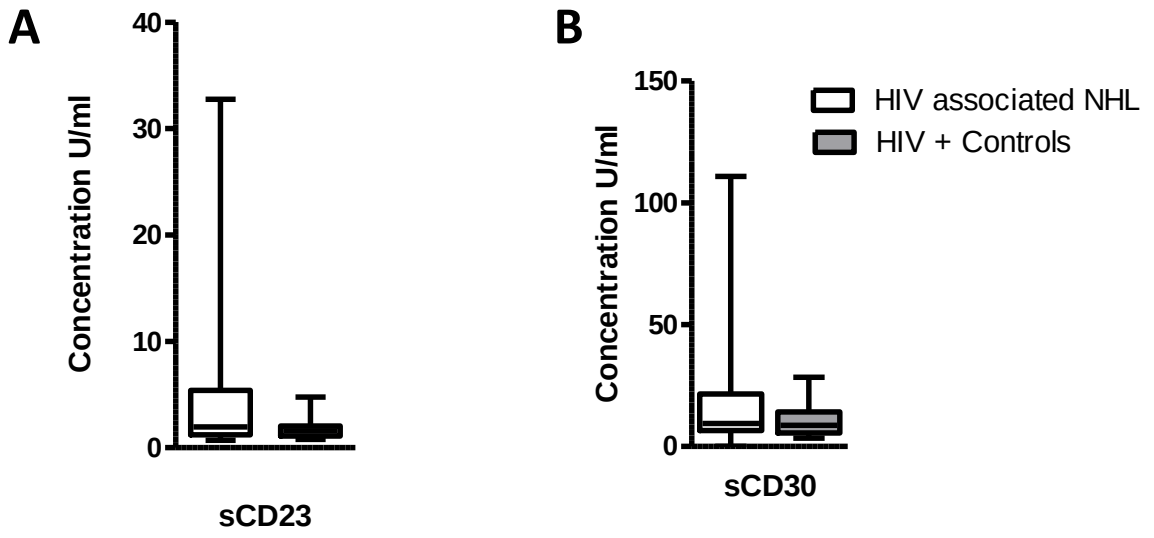

Figure 3.8: Soluble Factor expression in HIV-associated NHL. **(A)** sCD23 and **B)** sCD30 were elevated in HIV positive patients with NHL but were not statistically different to controls (p= 0.0795 and p= 0.2045 respectively).

3.6. *In vitro* cell culture of HIV-associated NHL

In order to determine the effects of various B-cell stimulatory biomarkers on a lymphoma cell culture, PBMCs from a confirmed HIV positive patient with BL were cultured for 24 hours in complete RPMI culture media supplemented with antibiotics and serum.

Compared with a normal healthy control, the BL cells failed to successfully grow in culture and were not viable for further experimentation (data not shown). The remainder of the HIV-associated NHL samples (n=2) were tested for viability using the PI live/dead flow cytometric assay prior to culture. The unstained normal control tested negative for PI and demonstrated a population of viable cells and a smaller population of non-viable cells (0.5%) (Figure 3.9a). Whereas the patient cells were positive for PI and demonstrated a larger population of non-viable cells (31.5%). (Figure 3.9b). Furthermore, fewer number of cells were available for acquisition for the patient sample compared to the normal control (1200 vs. 3000 cells respectively).

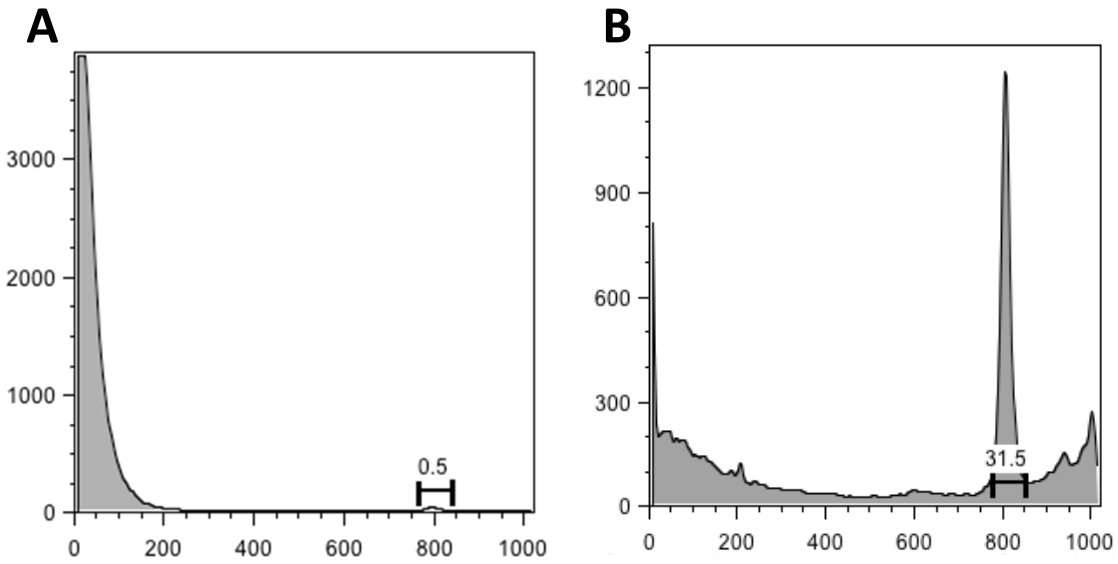

Figure 3.9: Flow cytometric histograms of Propidium Iodide viability assay. A) A healthy sample showing exclusion of propidium iodide staining (0.5% dead cells). **B)** Patient sample showing cell death by inclusion of propidium iodide staining (31.5%).

CHAPTER 4.0

DISCUSSION OF RESULTS

4.1. General Background

B-cell NHL is an AIDS defining condition [23]. In South Africa, where HIV infected patients present late and with high levels of immune dysregulation , HIV associated NHL is less common than would be anticipated given the high prevalence of infection. This probably does not reflect the true incidence but rather is indicative of under diagnosis and under reporting, as well as the high mortality rates associated with this aggressive cancer. It becomes important to ascertain both the underlying pathogenesis and explore new biomarkers which may improve early detection of malignancy.

A number of potential oncogenic mechanisms in HIV associated lymphoma have been described. These include immune dysregulation associated with CD4+ T cell depletion and aberrant cytokine production which may drive uncontrolled B-cell proliferation and ultimately, transformation [84, 86, 162]. Studies in other settings have found that the levels of a number of different cytokines and other biomarkers are increased in the plasma or serum of patients with HIV-associated NHL, both prior to, and after, the diagnosis of the malignancy [17, 37, 108, 163]. Some of these may show diagnostic utility and predict the development of lymphoma and others play a pathogenic role in B-cell transformation [84, 85]. Some of these markers include IL-6, IL-10, soluble CD23, CD27, CD30, CD44, CXCL13, TNF, IFNγ, as well as serum free light chains [17, 112, 164]. This study aimed to examine the levels of a select number of these markers in a South African HIV population.

4.2. Epidemiology of the study cohort

Diffuse large B-cell lymphoma followed by Burkitt lymphoma were the most common HIV-related NHL subtypes amongst this study cohort. This is consistent with data from both the Gauteng and Western Cape provinces of South Africa that reported increased numbers of the latter cases [32, 87].

There was no gender bias in this cohort with almost equal male: female ratios. In developed countries, a male preponderance has been described and this may reflect the HIV epidemic in South Africa which affects more females than males [165, 166]. Most of the studies conducted on HIV-associated NHL in other settings, generally consisted of cohorts of men-who-have-sex-with-men, whereas in South Africa, the major risk group is the heterosexual population [87]. This may also reflect the male bias seen in HIV-associated NHL that has been described in developed countries and the different male: female ratio found in this study [28, 166]. The median CD4 count was significantly lower ($p<0.0001$) in HIV–associated NHL cases at diagnosis compared with HIV positive controls. This deviates from the literature documenting higher CD4 counts in patients with HIV-related NHL from Sub-Saharan Africa (Table 3.2) [165]. This may reflect previous reporting biases as patients who attend clinics regularly for ARV therapy may possibly undergo more regular screening for known complications of HIV infection, like lymphomas. HIV positive patients with DLBCL had a moderately higher CD4 counts compared to those patients with BL, intermediate lymphoma and unspecified NHL ($p = 0.8699$). Both BL and DLBCL occur in immunocompetent as well as immunocompromised individuals and the literature supports that patients with BL generally present with higher CD4+ T cell counts (Table 3.2) [165, 167]. Our results differ from the literature and could be a direct result of DLBCL being the most common subtype diagnosed within this cohort compared to BL, intermediate lymphoma and unspecified NHL cases.

The HIV viral loads in the controls were considerably lower compared to the NHL cases given the fact that all the controls were on anti-retroviral and/or HAART treatment which normalizes the viral load at a level lower than detectable (Table 3.1). Clinical data points were also not available for all HIV positive patients with documented NHL, namely; CD4 counts, treatment status and viral load which could possibly skew the demographic data of this study. Patient clinical history prior to 2013 was not also available, hence making it difficult to establish what previous diseases or disease exposures the patient might have encountered which could have influenced the results (see Table 3.1).

4.3. Circulating biomarkers elevated in HIV positive patients with NHL

We hypothesized that several B-cell stimulatory cytokines would be elevated in the plasma of HIV-associated NHL, driving the proliferation of B-cells towards the development of HIV-associated NHL, as based on the findings of the literature [17, 98, 112, 168].

IL-6 is mainly involved in the terminal differentiation of B-cells into plasma cells and enhances the survival of and IgG antibody secretion by plasma cells [169-171]. It also enhances the growth of malignant and non-malignant B-cells in an autocrine manner [100]. In this study, HIV positive patients with documented NHL demonstrated significantly elevated plasma levels of factor IL-6 (Figure 3.4). This result is consistent with studies reporting increased IL-6 protein expression in plasma several years prior to the development of HIV-related NHL as well as in HIV positive individuals without lymphoma [83, 84, 112]. The fact that IL-6 is significantly elevated in HIV-associated NHL cases suggests that IL-6 may be produced and/or up-regulated in excess by the malignant B-cells themselves, thereby supporting the growth and survival of the tumour [172].

IL-4 and IL-5 promote the proliferation and differentiation of mature B-cells and induce immunoglobulin class switching of differentiated antibody secreting plasma B-cells [92, 95]. The median IL-4 plasma concentration was slightly increased in HIV positive patients with NHL compared to HIV positive controls; however this was not statistically significant (Figure 3.4). This deviated from the literature demonstrated by Flepisi *et al.* who showed significantly increased levels of IL-4 in HIV–related NHL patients within a South African cohort [120]. The slight increase in IL-4 protein concentration levels may be attributed to it being secreted by T_{FH} cells within the germinal centre [84], although IL-4 usually functions post germinal centre to induce class switching of plasma B-cells to IgE [94, 95]. Furthermore, IL-4 also induces the proliferation, differentiation and antibody secretion of B-cells in the presence of co-stimulatory molecules CD40L and LPS [92, 93]. This suggests that IL-4 requires additional help from the latter co-stimulatory molecule to exert its function in B-cells and contribute to lymphomagenesis.

No significant differences were noted in the plasma concentrations of B-cell growth factor IL-5 between HIV-associated NHL cases and controls (Figure 3.4). This result was similar to the literature, although the association between IL-5 and the development of HIV-associated NHL has not been fully elucidated in the literature. IL-5 also stimulates the maturation of CD40 activated B-cells and IgG1 secretion in B-cells stimulated with LPS [92, 93]. Our result indicates

that IL-5 may not contribute to the development or progression of the tumour burden in HIV-associated NHL and may also require additional help from stimulatory molecules such as LPS.

4.4. Immunoregulatory cytokine IL-10 was significantly elevated in HIV-associated NHL

IL-10, an anti-inflammatory cytokine, is produced by Th2 CD4+ T cells, macrophages and B-cells [173]. It functions to suppress or inhibit pro-inflammatory responses of the immune system and promote humoral immunity including B-cell proliferation and differentiation [174-176]. It was hypothesized that IL-10 would be elevated in HIV-associated NHL as described in the literature. In this study, IL-10 was significantly higher in HIV positive patients with confirmed NHL cases compared to HIV positive controls without lymphoma (Figure3.5). The results are consistent with previous studies which have demonstrated increased levels of plasma IL-10 prior to the onset of HIV-related NHL acting as a prognostic marker for the risk of development of NHL [98, 99]. Similarly to IL-6, IL-10 has also been increased in HIV infection [137]. This suggests IL-10 serves as a growth factor for B-cell lymphomas in an autocrine manner [98]. It also suggests that IL-10 is involved in the interplay between tumour cells, oncogenic viruses (like KSHV) and the HIV virus and HIV infection supporting the hypothesis that B-cell stimulatory molecules drives the transformation of B-cells into malignancy.

4.5. Th1 cytokines were not implicated in HIV-associated NHL

The majority of the inflammatory mediators such as IL-1β, IL-2, TNFα and IFNγ have been implicated in the pathogenesis and control of HIV-1 infection and viremia respectively [107, 137]. Granulocyte Macrophage-Colony stimulating factor is an essential haematopoietic growth factor and immune modulator [161]. In this study, however, no differences in protein concentrations were noted between HIV positive patients with confirmed NHL cases compared to HIV positive controls without lymphoma for analytes: IL-2 and GM-CSF (Table 3.3). These results indicate that mediators of inflammation and GM-CSF are not required for lymphomagenesis. Similarly, there were no differences in the protein concentration of IL-1β, TNFα and IFNγ between HIV-associated NHL and HIV positive controls in this study, which differed from the literature which reported elevated levels of TNFα and IFNγ in the serum and/or plasma in HIV-associated NHL [108, 112, 120, 177]. It is not entirely clear why our results differ as TNFα reportedly stimulates the proliferation of B-cells and Ig secretion in HIV positive and HIV uninfected individuals [178]. TNFα has also been shown to up-regulate the expression of IL-10, which in turn inhibits its pro-inflammatory effects [137].

IFNγ also has the capacity to induce Ig class switching in B-cells[131]. IL-1β , IFNγ and TNFα are also Th1 dominant cytokines and their levels may be suppressed and/or inhibited by IL-10, a regulatory cytokine [174, 179] or as a subsequent result of the reduced CD4+ T –cell count.

4.6. IL-8, but not SDF-1α, was significantly elevated in HIV-associated NHL

IL-8 is also a pluripotent neutrophil chemokine and an angiogenic pro-inflammatory chemokine that is secreted by and expressed on both normal and tumourigenic cells [115], and has been implicated in the progression and metastasis of tumours [115, 116]. Numerous studies have demonstrated that IL-8 was in several AIDS-NHL cell lines and were also significantly expressed in patients with NHL with or without HIV [118-120]. In this study, IL-8 was significantly elevated in HIV-associated NHL when compared to HIV positive controls (Figure3.6). This finding is in keeping with a similar study performed in a cohort of South African patients demonstrating increased levels of IL-8 in the plasma of HIV-associated NHL [120]. IL-8 is also produced by tumour cells and acts in a paracrine on nearby cells in the tumour microenvironment to exert its function and support tumour growth, proliferation and metastasis [115]. This indicates that IL-8 may play a supporting role in the progression of NHL itself, by recruiting neutrophils and macrophages to the tumour site which may be secreting additional cytokines and growth stimulants further enhancing tumour growth [115, 117]. IL-8 could possibly be produced by the tumour cells themselves increasing their levels in HIV-associated NHL and not in HIV controls without evidence of lymphoma, irrespective of the viral load.

SDF-1α is a chemoattractant homing haematopoietic stem cells, specifically B-cell and its precursors, to the bone marrow microenviroment [114]. In HIV infection, SDF-1α mediates the hyperprolifertaion of B-cells and shares a co-receptor or ligand with HIV, CXCR4, which has been shown to be highly expressed in haematological malignancies [121, 122]. Although the median concentrations of SDF-1α were slightly higher in the plasma of HIV positive patients with NHL when compared to controls, these results were not significant (Figure 3.6). These results could also be attributed to the smaller sample size available for measurement (n=20), and a larger cohort of patients should be studied in the future. Because of its function as a homeostatic chemokine which condones trafficking of precursor B-cell and stem cells SDF-1α may be more permissive of tumours arising in earlier precursor stages of B-cell development in the bone marrow rather than more mature lymphomagenesis.

The majority of HIV-NHLs usually manifest at the germinal centre or post germinal centre maturation stages of B-cell development [1, 19].

Taken together, these results how that elevated levels of cytokines IL-6, IL-8 and IL-10 may play a role in the pathogenesis of HIV-associated NHL, possibly through by stimulating and increasing the proliferation of B-cells in HIV positive patients with NHL. Furthermore, these cytokines are also implicated in the interplay between malignant cells and HIV infection.

4.7. Correlations between cytokine levels of IL-6, IL-8 and IL-10

It was hypothesized that the cytokine expression of IL-6 and IL-10 would be dependent on each other as numerous studies have reported that these two cytokines are able to up-regulate each other's expression [83, 100, 106]. In this study, no correlation was seen between IL-6 and IL-8 or between IL-8 and IL-10 (Figure 3.7) for HIV-associated NHL cases. IL-8 is expressed by various cells types, including tumour cells, within the tumour microenvironment and usually exerts its function in a paracrine manner [115, 117]. Whereas IL-6 and IL-10 are produced by the malignant B-cells in an autocrine manner as well as by monocytes [99, 100]. This confirms that IL-8 is expressed independently of IL-6 and IL-10 and does not function with these cytokines to promote tumour growth. The negative correlation observed between IL-8 and IL-10 further suggests IL-8 is autonomous in its role in tumorigenesis. This is supported by its main function as a neutrophilic chemokine and a pro-angiogenic cytokine [115, 116].

A correlation was noted between cytokine concentration levels of IL-6 and IL-10. Both of these cytokines are produced by B-cells in an autocrine manner suggesting that they may be working together synergistically to enhance B-cell transformation [98, 100]. IL-6 and IL-10 have also been reported to be increased in HIV-1 infection [137]. This suggests that their increased expression is attributed by both HIV and the tumour B-cells and theses cytokines may in turn function together to enhance B-cell growth.

4.8. Soluble factors exhibit an increased trend in HIV-associated NHL

sCD23 is a differentiation and/or early activation marker of mature B-cells and mediates Ig isotype class switching [139, 140]. sCD30 is a known marker of chronic B-cell stimulant and may play a role in B-cell proliferation [144]. In this study, we hypothesized that both soluble CD23 and CD30 would have increased expression in HIV positive patients with NHL as reported in the literature [84, 144, 152, 180]. We found that both sCD23 and sCD30 (Figure 3.8) displayed an

elevated trend in HIV-associated NHL cases compared to controls, although these results were not statistically significant.

These results were similar to other studies conducted on sCD23 and sCD30 in relation to HIV-associated NHL. Yawetz *et al.* showed that sCD23 levels were elevated in patients who developed AIDS- associated NHL compared with patients who had AIDS without lymphoma as well as HIV sero-negative and positive patients [141]. Schroeder *et al.* likewise showed elevated levels of sCD23 in AIDS-associated NHL in the absence of gamma herpesvirus EBV [152] [84].

In this study, sCD30 demonstrated an increase trend in HIV positive patients compared to HIV positive controls without lymphoma. Purdue *et al*. and Breen *et al.* both reported elevated circulating levels of sCD30 in HIV positive individuals who developed NHL as a pre-diagnostic marker of HIV associated NHL[144, 163]. These results may suggest that both of these markers have potential as diagnostic screening markers for patients with HIV-associated NHL. This would need to be validated in a larger cohort.

4.9. Cell culture of HIV-associated NHL

To investigate the effects of B-cell stimulatory biomarkers on lymphomagenesis, an *in vitro* culture of lymphoma cells from a BL patient was made. We hypothesized that HIV-associated NHL would grow vigorously in culture as it has a high rate of proliferation [44, 45, 49]. Burkitt lymphoma has a high proliferation rate with a Ki-67 index of nearly 100% and is characterised by high cellular turnover [43, 44]. The cells, however, failed to grow successfully in culture and were not viable for further testing when compared to a normal healthy control (Figure 3.9). Studies on establishing *in vitro* cultures of lymphoma cells generally used cells isolated from the lymph node or the primary source of the tumour burden [181, 182], whereas the cells use for this study were isolated from peripheral blood**.** This suggests that these cells grow better when in a suitable microenvironment or specific niche which stimulates their growth *in vivo.* As the cells that we were trying to culture were isolated from peripheral blood and not the lymph node, this may have impacted on the survival of these cells. It was suggested that additional growth factors and/or mitogens be added to the culture to enhance B-cell growth *in vitro*. CD40 has been reported to be an efficient stimulant to facilitate the growth of malignant B-cells *in vitro* [183]. Pokeweed mitogen has also been shown to stimulate the proliferation of lymphocytes in culture [184]. Unfortunately, we did not have enough material to culture with these additional growth factors to test this theory, but this would be worth exploring in future studies.

4.10. Other factors impacting the results of this study

Various factors may explain the discrepancies between this study and the published literature. The differences in the geographical subtypes of HIV-1 between published literature and this current study may also have affected the outcome of these results. In high income countries in Europe, the United States and Japan, Clade B is the dominant HIV subtype, whereas Clades A, C, D and E are most common subtypes in low and middle income countries (reference). HIV-1 Clade C is the predominant subtype in Sub-Saharan Africa, India and Brazil, whereas Clade A is predominant in Eastern Europe and Northern Asia [185-187]. Most studies in the literature focus on subtypes A and B, whereas subtype C is the predominant subtype in South Africa [185, 187]. In addition, the majority of the published literature measured these biomarkers retrospectively in stored serum of patients several years prior to diagnosis [37, 86, 164], whereas in this study the biomarkers were assessed prospectively in the plasma of patients recently diagnosed with lymphoma prior to commencement of treatment.

CHAPTER 5.0

LIMITATIONS AND SHORT COMINGS

This study had a few limitations. Not all clinical information could be accessed for all patients. Some patients were lost to follow-up or died prior to intensive investigation. Plasma from all patients could not be assessed for SDF-1α due to insufficient residual plasma sample. It is possible that, had the numbers of patients assessed been higher, these results would have differed significantly as shown in other work. Finally, a small number of patients had to be excluded from the final analysis after concomitant chronic conditions (most notably tuberculosis) were diagnosed. Although the patients in this cohort did not have detectable tuberculosis disease, the impact of latent tuberculosis infection on the biomarkers measured in this study was uncertain. It would be valuable, however, to ascertain the rates of infection with Mycobacterium tuberculosis occurring in patients with HIV-associated lymphoma as it is possible that this infection may be a secondary driver of the malignant process. Patients were not assessed for concomitant viral infections (like EBV and KSHV) but it is likely from the known association between viral infections and lymphoma that the majority had previous or on-going gamma herpesviridae infections.

CHAPTER 6.0.

CONCLUSION & OUTLOOK

In summary IL-6, -8 and -10 were identified as significantly elevated in HIV-associated NHL cases when compared to HIV positive controls without lymphoma. Soluble factors CD23 and CD30 exhibited a trend towards increased expression in HIV positive patients with documented lymphoma. Future work would include repeat Luminex and ELISA assays with increased numbers of samples as well as comparing these results to an additional cohort of HIV uninfected patients. Cell culture experiments need to be repeated with additional stimulants to enhance B-cell growth. This study has demonstrated the link between elevated levels of B-cell stimulatory cytokines IL-6, -8 and -10 which may contribute to the pathogenesis of HIV-associated NHL in a South African cohort. These cytokines may serve as a valuable therapeutic strategy by suppressing or neutralizing its expression within the tumour microenvironment in HIV-associated NHL thereby inhibiting tumour progression, growth and/or transformation of malignant B-cells. Alternatively, they can also serve as a diagnostic screening marker and could be included in the current flow cytometry immunophenotyping panel.

APPENDIX A

R14/49 Ms Lauren Cruywagen and Laryn Wessels

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M140395

NAME: (Principal Investigator)	Ms Lauren Cruywagen and Laryn Wessels
DEPARTMENT:	Molecular Medicine and Haematology Charlotte Maxeke Johannesburg Academic Hospital NHLS
PROJECT TITLE:	Biological B cell stimulation biomarkers in Lymphoma: The potential role of cytokines chemokines, soluble factors in Lymphomagenesis within a South African Population
DATE CONSIDERED:	28/03/2014
DECISION:	Approved unconditionally
CONDITIONS:	
SUPERVISOR:	Cathrine Worsley, Dr ES Mayne
APPROVED BY:	Professor PE Cleaton-Jones, Chairperson, HREC (Medical)
DATE OF APPROVAL:	09/04/2014

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Secretary in Room 10004, 10th floor, Senate House, University.

I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to resubmit the application to the Committee. **I agree to submit a yearly progress report.**

Principal Investigator Signature Date

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

Figure 1: Printout of ethics clearance certificate

TURNITIN REPORT

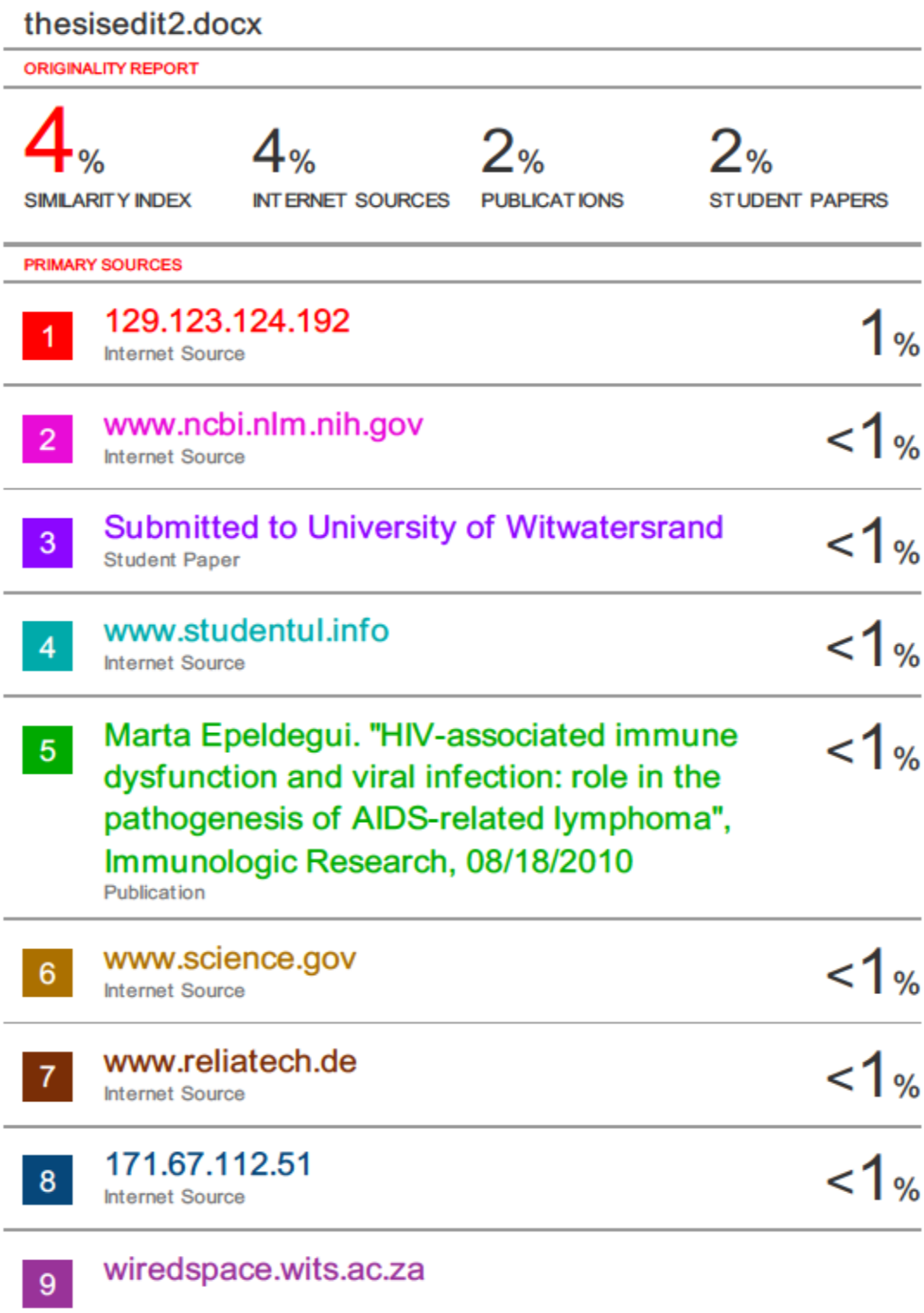

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Figure 2: Turnitin Report

Table 1: A description of B-cell stimulatory molecules and there function in relation to B-cell lymphomas

B-cell stimulatory molecules	Function related to B-lymphocytes and B-cell lymphomas
Serum/Plasma Cytokines	
Interleukin-6	▪ IL-6 is produced by malignant cells and contributes significantly to proliferation [100] ▪ Anti-apoptotic marker for B-cells and may act as a growth factor for HIV-associated lymphoma cells *in vitro* [83]
Interleukin-10	▪ Anti-apoptotic marker for B-cells and may act as a growth factor for HIV-associated lymphoma cells *in vitro*[83] Autocrine B-cell growth factor for B-cell lymphomas [98] ▪ Genetic properties, SNP within the promoter region of IL-10 gene, are related to development of AIDS-associated lymphoma and NHL [79] ▪ *In vivo* production of IL-10 may stimulate proliferation of malignant cells in an autocrine fashion. Its production is triggered synergistically by HIV and EBV [99]
Interleukin-4	▪ Involved in the growth and survival of Non-Hodgkin B-lymphoma cells *in vitro*. ▪ Regulates proliferation and maturation of B-cells [38] ▪ Induces class switching to IgG1, G4 and IgE
Interleukin-5	▪ Promotes cell differentiation and survival of B-cells ▪ Stimulates the maturation of CD40 activated B-cells to IgM and IgG1 secreting cells
Interleukin-13	▪ Involved in the growth and survival of non-Hodgkin's B-lymphoma cells *in vitro*. ▪ Regulates proliferation and maturation of B-cells [38]
CD40 Ligand	▪ Elicits activation and proliferation of B-cells

TNFα	▪ Acts as a co-stimulant promoting B-cell differentiation induced by CD40L and IL-4 [94, 134] ▪ Has the capacity to stimulate B-cell proliferation and the production of IgG by B-cells and associated with inflammation [84] ▪ Salles *et al* reported that elevated plasma levels in lymphoma patients , is associated with disease aggression and may predict the outcome of the patient [188]
TNF ß	▪ Commonly referred to as lymphotoxin [84]
IFNγ	▪ Promotes survival and differentiation of B-cells ▪ Inhibits CD40L B-cell proliferation and IL-4 IgE class switching
Chemokines	
CXCL13	▪ Homeostatic B-cell chemokine also referred to as a B-lymphocyte chemo-attractant [84] ▪ Produced by T follicular helper cells and follicular dendritic cells ▪ Attracts B-cells to secondary lymphoid organs [17]
CXCR5	▪ Also known as Burkitt lymphoma receptor 1 (BCR1) ▪ Expressed on mature B-cells and subsets of T cells ▪ Receptor for CXCL13 [84] ▪ Together with its ligand CXCL13, these chemokines guide B-cells towards secondary lymphoid organs ▪ Single nucleotide polymorphisms associated with risk of NHL ▪ Several polymorphisms has been reported to be associated with risk of NHL [189]
Soluble factors	
sCD23	▪ Expressed on mature resting B-cells ▪ Acts as a B-cell growth factor [190] ▪ Up-regulates the production of monocyte IL-6 [180]
sCD27	▪ Members of the tumour necrosis factor receptor

	superfamily [84] ▪ Expressed primarily on T cells but also on B and NK cells ▪ Various types of malignancies also express CD27 and has apparent correlation to the tumour load, suggesting the tumour itself may possibly be a source of sCD27 [191]
sCD30	▪ Members of the tumour necrosis factor receptor superfamily ▪ Marker of chronic B-cell stimulation which may be involved in B-cell proliferation that could lead to genetic error accumulations and possibly cause lymphoma [144]
sCD44	▪ Elevated preceding diagnosis of NHL and thus may be an indicator of immune dysregulation preceding AIDS-NHL diagnosis [79] ▪ Elevated following NHL, suggesting it may be a by-product of the tumour cells
Other molecules associated with immune system activation and inflammation	
Free immunoglobulin light chains (FLC)	▪ Possibly biomarkers of polyclonal B-cell activation and dysfunction as well as risk of developing NHL in an HIV setting [192]
CRP	▪ Surrogate biomarker for IL-6 and other inflammatory cytokines [193] ▪ Acute phase protein [86]

APPENDIX B

Reagent preparations and standard concentrations

Table 2: Human cytokine 10-plex panel: Cytokine analytes and their respective standard concentrations

Cytokine analyte	**Reconstituted standard concentration pg/ml (provided by package insert)**
IL-1β	8,150
IL-2	10,200
IL-4	36,300
IL-5	9,100
IL-6	5,850
IL-8	13,450
IL-10	20,850
GM-CSF	22,450
IFN$_{\gamma}$	9,550
TNFα	8,850

Table 3: Human cytokine 10-plex panel reagent preparation indicating the appropriate dilution factor and volumes

Reagent	**Dilution factor**	**Volumes (µl)**
Human 16-plex Standards (1-7)	3-fold serial dilution	150ul of assay diluent and 450ul reconstituted standard
Plasma samples of HIV associated NHL and HIV positive individuals without evidence of overt lymphoma	2-fold	50µl of plasma sample and 50µl of assay diluent
Human cytokine 10-plex Antibody bead concentrate (20x) 0.25ml	1:11	220µl of Antibody mix was diluted with 2.2ml of wash solution
Human cytokine 10-plex Biotinylated	1:11	880µl of Antibody concentrate

Antibody concentrate (10x) 1ml		was diluted in 8.8ml of Biotin Diluent
Wash Solution Concentrate (20x) 15ml	1:20	285ml of distilled water (dH_2O) to 15ml of wash solution
Streptavidin-RPE Concentrate (10X) 1ml	1:11	880µl of Strep-PE concentrate was diluted in 8.8ml of Streptavidin-PE Diluent

Table 4: Standard concentrations of the SDF-1α Luminex and ELISA assays

Kit	**Number of standards/ controls**	**Reconstituted standard concentration pg/ml (provided by package insert)**
Human SDF-1α Simplex	7 standards	73,500-17.944pg/ml
sCD23 EASIA ELISA	6 standards	0.00-20.0U/ml
sCD23 EASIA ELISA	2 controls	Control 1: 2.410U/ml Control 2: 9.32.3U/ml
sCD30 ELISA	7 standards	100-1.6U/ml

Table 5: Reagent preparation including the dilution factor and volumes for sCD30 ELISA kit

Reagent	**Dilution Factor**	**Volumes**
Plasma sample	1:4	25 µl of sample + 75 µl of sample diluent
Standards	1:2	100µl of sample diluent + 100µl of standard
HRP-Conjugate, anti-sCD30 monoclonal (murine)	1:100	60µl of conjugate diluted in 5.94ml of assay buffer

antibody.		
Wash Buffer Concentrate(20x) (PBS with 1% Tween 20)	1:20	50ml of wash buffer added to 950ml of dH_2O
Assay Buffer Concentrate (20x) (PBS with 1% Tween 20 and 10% BSA)	1:20	5ml of assay buffer diluted in 95ml of dH_2O

Table 6: Reagent preparation including the dilution factor and volumes for sCD23 EASIA ELISA kit

Reagent	**Volumes**
Plasma samples and standards	100 µl diluent + 100 µ l standards or samples (1:2)
Diluent	Add 8ml of dH_2O
Controls 1& 2 in human plasma with preservatives	Add 1ml of dH_2O
Washing Solution Concentrate (buffer with preservatives)	Add 2ml in 400ml of dH_2O
Concentrated Chromogenic TMB solution	Add 0.2ml into 1 vial of Substrate buffer

APPENDIX C

Cell culture calculations

Total cell count and viability using the Neubauer counting chamber and trypan blue dye (example)

Dilution factor= 2

Average Number of Live cells (n) = 34.375 cells/ml

Average number of dead cells (n) = 3 cells/ml

Total number of cells (n) = 35.75

#Cells/ml= Average # cells x dilution factor x 10^4

= 34.372 x 2 x10^4

= 6.872 x 10^6 cells/ml

Cell viability = Average # live cells/ Average # total cells x 100

$$= \frac{34.372}{35.75} \times 100$$

= 96%

To resuspended cells at a concentration of 1 x 10^6 cells/ml in 2ml of culture media

C1V1= C2V2

6.872 X 10^6 X 2ml = 1 x10^6 V2

= 13.744ml

13.744-2ml= 11.74 ml of culture media

APPENDIX D

Antibody titration tables and graphs

Table 7: Mean Fluorescence Intensity values for antibody titration of CD19 PE

Ancestry Subset Statistic for Lymphocytes (MAb volumes µl)	**MFI CD19 PE**
Unstained control	n/a
2µl CD19	4213
5µl CD19	5198
10µl CD19	6016
15µl CD19	5800

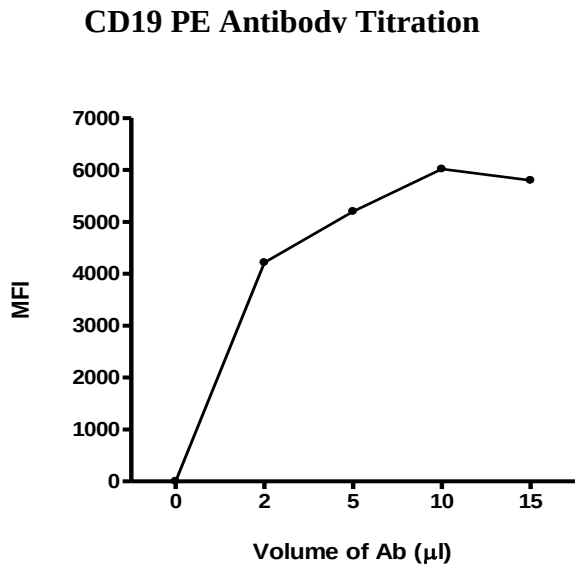

Figure 3: CD19 PE Antibody Titration. MFI plotted against the volume of MAb (µl) to determine the optimal amount of MAb to use for flow cytometry experimentation.

Table 8: Mean Fluorescence Intensity values for antibody titration of CD38 APC

Ancestry Subset Statistic for Lymphocytes (MAb volumes µl)	**MFI CD38 APC**
Unstained control	n/a
2	6427
5	6955
10	6760
15	6817

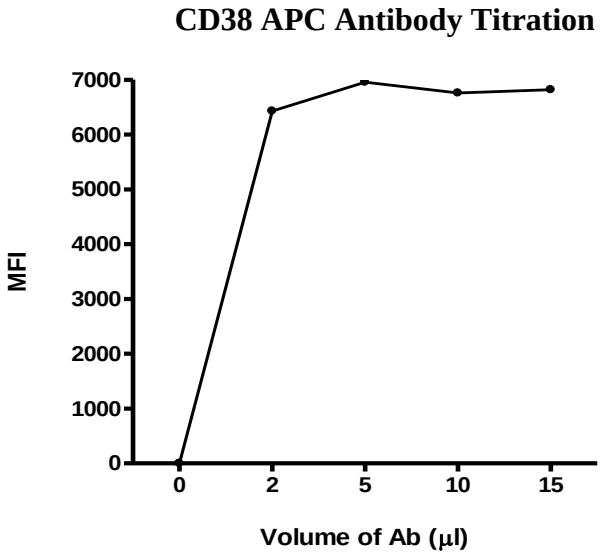

Figure 4: CD38 APC Antibody titration: MFI plotted against the volume of MAb (µl) to determine the optimal amount of MAb to use for flow cytometry experimentation.

Table 9: Mean Fluorescent Intensity values for antibody titration of CD3 FITC

Ancestry Subset Statistic for Lymphocytes (MAb volumes µl)	**MFI CD3 FITC**
Unstained control	n/a
2	18874
5	19534
10	20329
15	20972

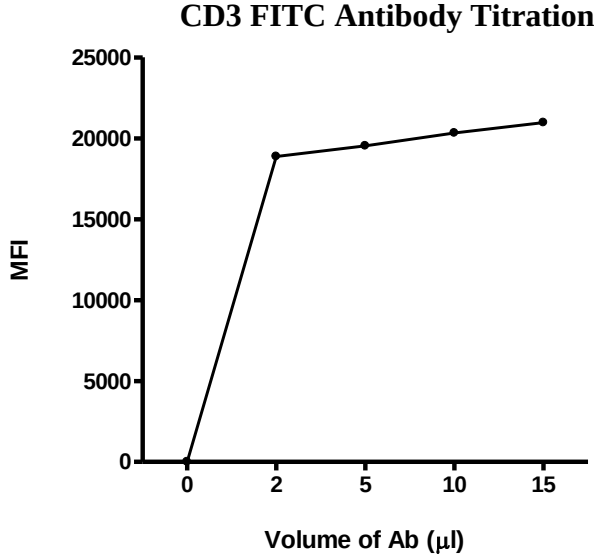

Figure 5: CD3 FITC Antibody titration. MFI plotted against the volume of MAb (µl) to determine the optimal amount of MAb to use for flow cytometry experimentation.

Table 10: Mean Fluorescent Intensity values for antibody titration of CD4 PE

Ancestry Subset Statistic for Lymphocytes (MAb volumes µl)	**MFI CD4 PE**
Unstained control	n/a
2	15862
5	17535
10	17390
15	17666

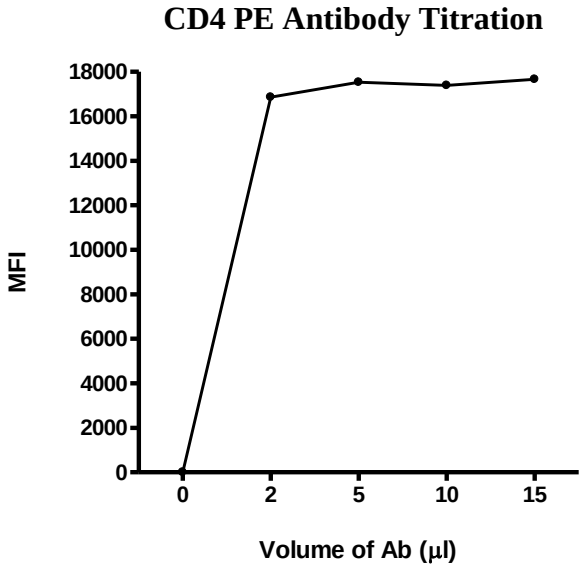

Figure 6: CD4 PE Antibody titration. MFI plotted against the volume of MAb (µl) to determine the optimal amount of MAb to use for flow cytometry experimentation.

Table 11: Mean Fluorescent Intensity values for antibody titration of CD8 Alexa Fluor® 700

Ancestry Subset Statistic for Lymphocytes (MAb volumes µl)	**MFI CD8 Alexa Fluor® 700**
Unstained control	n/a
2	4824
5	4650
10	4800
15	4891

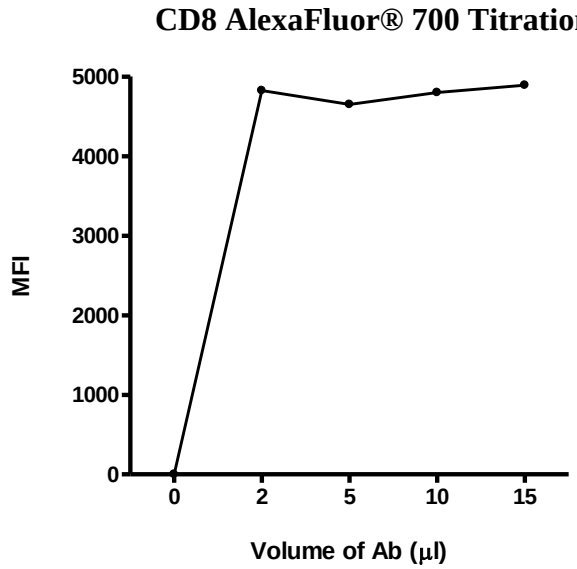

Figure 7: CD8 AlexaFluor® 700 Antibody titration. MFI plotted against the volume of MAb (µl) to determine the optimal amount of MAb to use for flow cytometry experimentation.

APPENDIX E

Table 12: A comprehensive list of MAb panel used to perform antibody titration and set-up of multi-colour compensation

Monoclonal Antibody	**Fluorescent marker**	**Supplier**
Anti-human CD19	PE	eBioscience, US
Anti-human CD3	FITC	eBioscience, US
Anti-human CD4	PE	eBioscience, US
Anti-human CD8	Alexa Fluor® 700	BioLegend, CA, US
Anti-human CD38	APC	eBioscience, US
Anti-human Ki-67	Alexa Fluor® 647	BioLegend, CA, US
Isotype control IgG1 κ (Mouse)	APC PE FITC	eBioscience, US
Isotype control IgG1 κ (Mouse)	Alexa Fluor® 700 Alexa Fluor® 647	BioLegend, CA, US

Flow cytometry experiments

Table 13: Experimental layout for flow cytometry assay

Experiment Layout	**Volume of MAb (µl)Fluorescent marker**	**Function**
Tube 1: CD19 PE CD38 APC	10 5	To determine the percentage of activated f B-cells
Tube 2: CD3 FITC CD4 PE	6 5	To determine the percentage of activated T-cells

CD8 AlexaFluor® 700 CD38 APC	5 5	
Tube 3: CD19 PE Ki-67AlexaFluor® 647	5 10	To determine the percentage of proliferating B-cells
Tube 4: CD3 FITC CD4 PE CD8 AlexaFluor® 700 Ki-67AlexaFluor® 647	6 5 5 5	To determine the percentage of proliferating T-cells
Tube 4: Isotype Control IgG1κ (Mouse) FITC APC PE AlexaFluor® 700	5 µl of each antibody	Negative control

APPENDIX F

Instrument Calibration

1. Calibration of the LSR II Flow cytometer

The LSRII Flow cytometer (BD Biosciences, USA) was calibrated daily and weekly in accordance with the manufacturers and the laboratory's requirements to monitor performance of the optics and fluidics systems. The SPHERO Rainbow Single Peak calibration beads (BD Biosciences, USA) were used daily and the cytometer set-up and tracking beads (CST) (BD-Biosciences, USA) weekly to calibrate the LSRII. .

The Sphero single beads ensured that fluorescent peaks for each fluorescent parameter were within the H gate and the voltages adjusted where necessary in order to align the fluorescent peak within the H gate (appendix E). The CST beads verified the properties of the violet, red and blue lasers by measuring the coefficient of variation (CV) for each laser.

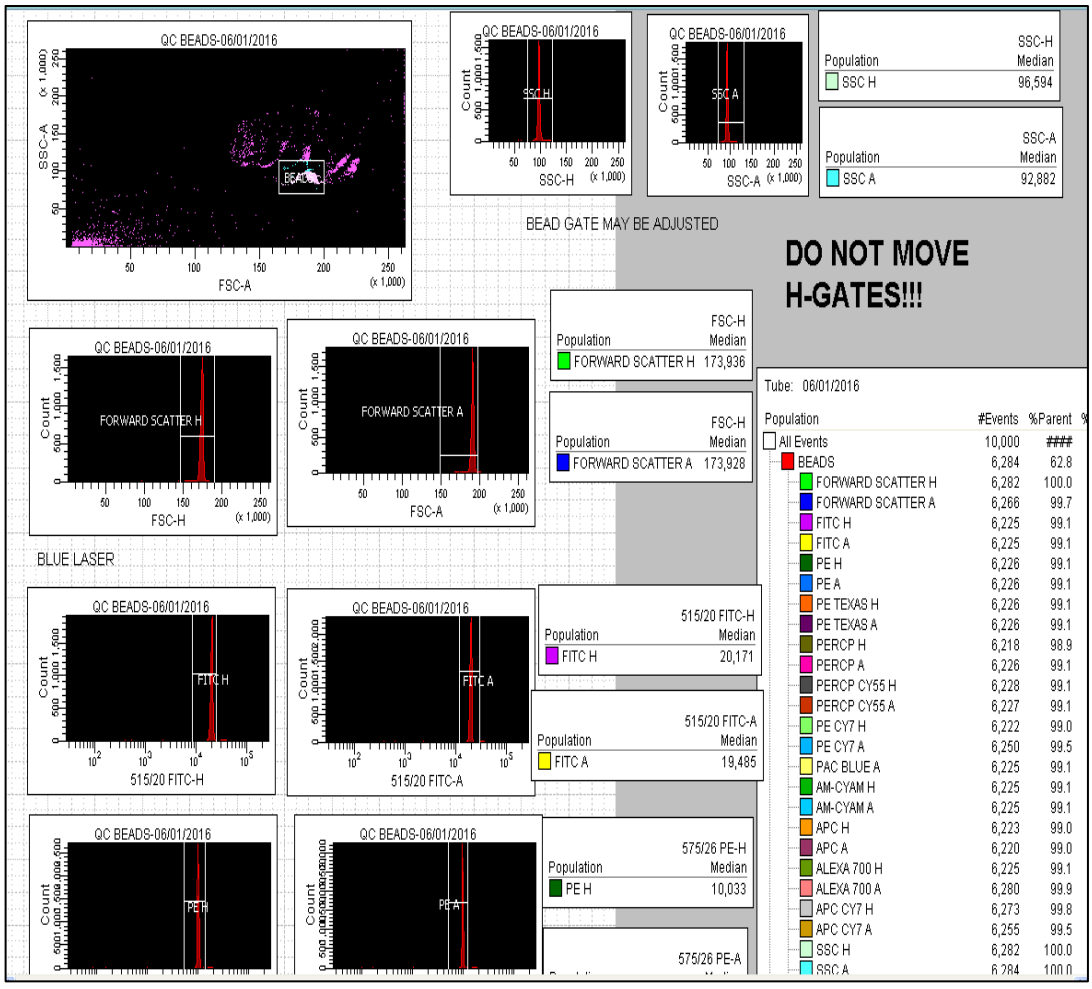

Figure 8: Snapshot of the daily QC SPHERO Rainbow Single Peak calibration beads.

2. Colour compensation setup on the LSRII flow cytometer

Multi-colour compensation was carried out to remove and correct for any background signal and spill over between different fluorochomes [194]. Colour compensation was performed using BD™ CompBeads negative control beads and BD™ CompBeads Anti-Mouse Igκ positive capture beads (BD-Biosciences, USA).The latter binds to any Ig bearing the mouse kappa light chain, while the negative control has no binding capacity, providing distinct positive and negative stained populations [195, 196]. An unstained control was used to adjust the forward scatter (FSC) and side scatter (SSC), and six single stained monoclonal antibody (MAb) tubes were used to adjust fluorophore photomultiplier tubes PMT voltages. A minimum of 5000 events were recorded for each MAb. Compensation was calculated and applied to all experimental tubes.

3. Calibration of the Bio-Rad Luminex200

The Bio-Rad 200 Luminex xMAP™ (Bio-Rad Laboratories Inc., USA) was appropriately calibrated and maintenance was done to monitor the laser and instrument performance before use. The lasers were warmed up automatically using the xPONENT 3.1 software (Luminex Corporation, Austin, Texas**)** before calibration was performed.

An initialization step ran which included a prime to remove any trapped bubbles in the system and two alcohol flushes. The instrument was then calibrated by running the following calibration control beads in a 96 well plate: CAL1, MagCAL1, CAL2, CON1, MagCON1 and CON2 (Luminex Corporation, Austin, Texas).

APPENDIX G

Standard Curves

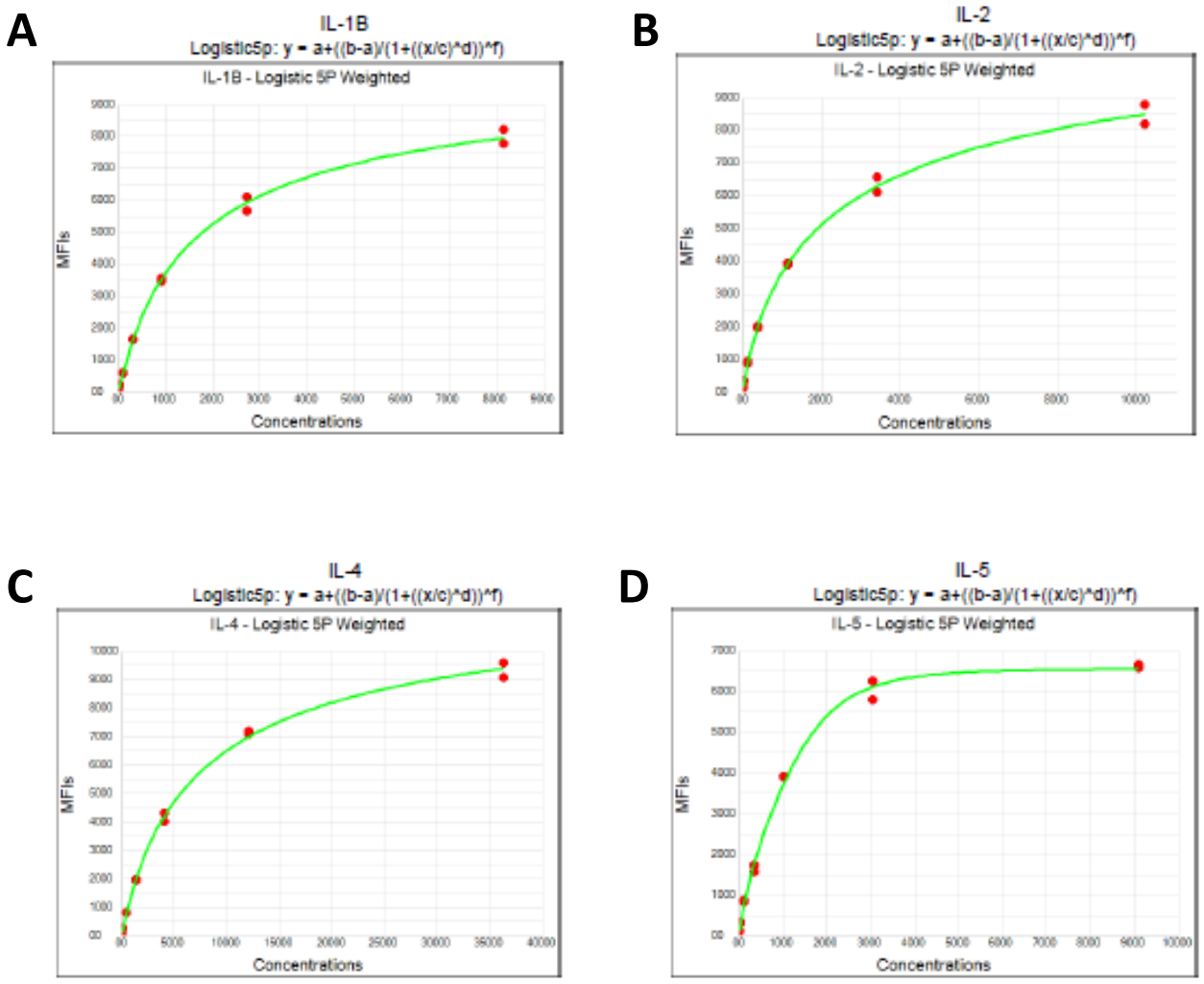

Figure 9: Human 10-plex cytokine panel 5-parameter logistic standard curves. A) IL-1β, **B)** IL-2, **C)** IL-4 and **D)** IL-5. Concentrations (pg/ml) were plotted against the mean fluorescence intensity (mfi) using Luminex Technology.

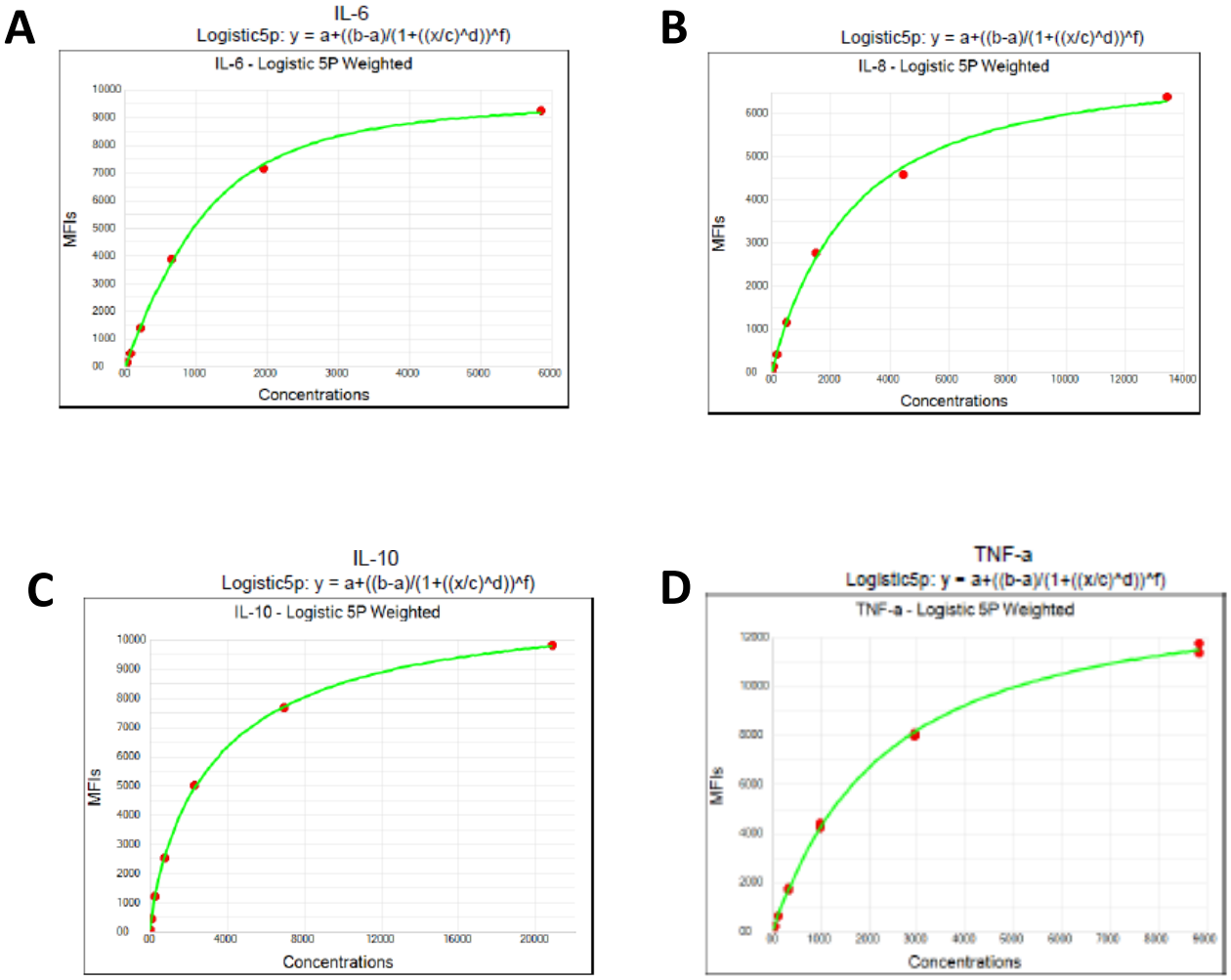

Figure 10: Human 10-plex cytokine panel 5-parameter logistic standard curves. A) IL-6, **B)** IL-8, **C)** IL-10 and **D)** TNFα.

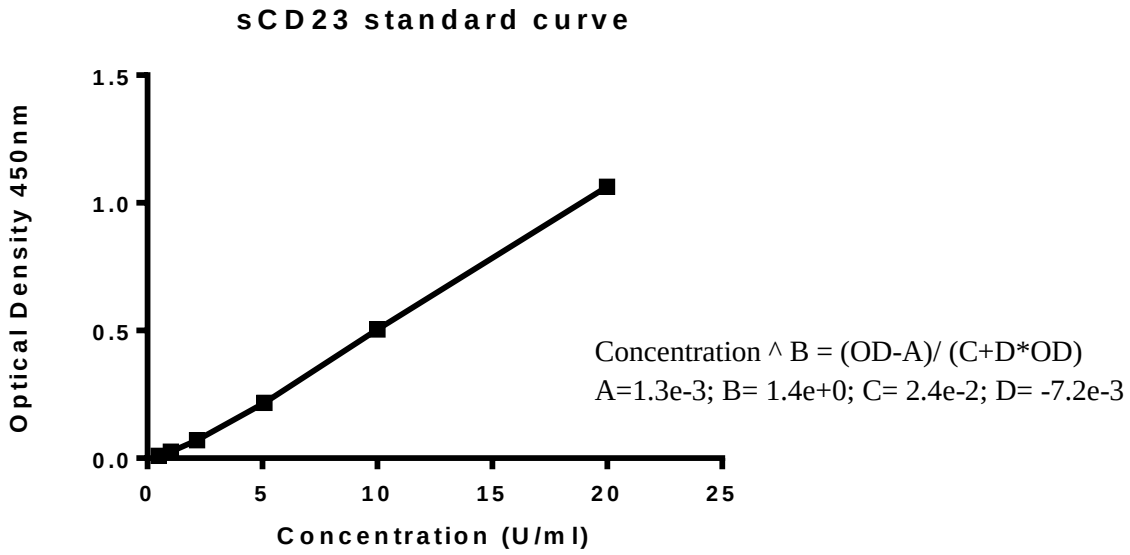

Figure 11: sCD23 4-parameter logistic standard curve. The standard curve was generated automatically using the Adaltis™ Easy Interface software. Concentrations (U/ml) were plotted against the optical density (absorbance 450nm).

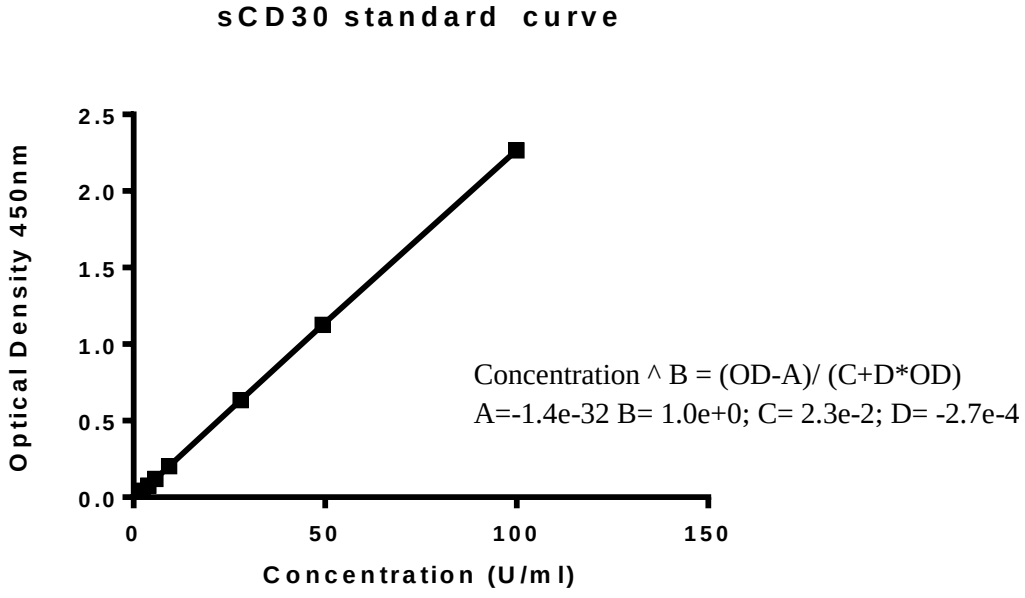

Figure 12: sCD30 4-parameter logistic standard curve.

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