UNIVERSITY OF LIVERPOOL

The Effect of Cytomegalovirus infection on Follicular Lymphoma biology

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy

By

Moses Dabah LUGOS

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Abstract

Follicular lymphoma (FL) is an incurable human B-cell neoplasm that follows a chronic relapsing course requiring multiple treatment episodes and culminating in therapy resistance and/or large-cell transformation. The disease is notable for its clinical variability but the biological basis for this variability is poorly understood. In addition to intrinsic genetic and epigenetic alterations that drive tumour growth, an intricate cross-talk between malignant B cells and the immune microenvironment have been shown to play a significant role in the pathogenesis of FL. This thesis tests the hypothesis that some of the heterogeneity of FL might result from chronic infection with cytomegalovirus (CMV). The latter affects ~50% of the adult population and has been linked to premature immune senescence and expedited age-related alterations in the T cell repertoire. I reasoned that CMV infection might have two distinct effects in FL: (1) by accelerating immune senescence, it might increase the risk of treated-related infection; and (2) by altering the lymphoma microenvironment, it might modulate the effect of chemo-immunotherapy.

To address these questions, I was able to access stored PMBCs, serum and tissue samples from patients of known CMV serostatus who were recruited into the PACIFICO trial. The trial compared two different chemo-immunotherapy regimens in older patients with FL and recruited 360 patients between 2009 and 2015. My experimental approach was to compare CMV-positive and CMV-negative patients for blood lymphocyte subsets by flow cytometry (Chapter 3), serum cytokines by Luminex profiling (Chapter 4), accessory cells in the lymphoma microenvironment by immunofluorescence of biopsy material (Chapter 5), and treatment effectiveness and toxicity using follow-up information obtained as part of the trial (Chapter 6). Chapters 3 and 4 employed a cohort of 42 cases (21 of which were CMV- positive) that were carefully selected to be well balanced in other respects. Chapter 5 employed a similar cohort of 42 cases (20 of which were CMV- positive) including 6 that were also in the cohort used for Chapter 4 (it was not possible to use exactly the same cases for Chapters 3, 4 and 5 owing to the limited availability of biopsy material). Chapter 6 related CMV serostatus to clinical outcome in the two cohorts employed for Chapters 3,4 and 5, and also in a third cohort consisting of all 269 patients of known CMV serostatus who were recruited into PACIFICO (109 of which were CMV- positive). Finally, Chapter 7 examined the relationship between CMV-dependent biological variables and treatment efficacy with the aim of shedding light on the mechanisms through which CMV might modulate therapy response in FL.

Flow cytometric analysis of PBMCs revealed significant accumulation of end-stage differentiated (TEMRA) T cell subtypes including pE2-CD4 (P=0.046), E-CD4 (P=0.029), EM3-CD4 (P=0.018), E-CD8 (P=0.033) and EM3-CD8 (P=0.005) phenotypes as well as increased NKT cells (P=0.031) in CMV-positive patients. Luminex data showed significantly reduced levels of multiple serum cytokines including IL-9 (P=0.006), IL-17A (P=0.020), FGF-basic (P=0.028), MIP-1α

(P=0.039), MIP-1β (P=0.029), IL-6 (P=0.029) and IL-8 (P=0.037) in the CMV-positive group. Taken together, these findings support the hypotheses that CMV infection in FL is associated with accelerated immune senescence with the accumulation of exhausted T cells and reduced cytokine production. Immunofluorescence staining of biopsy material revealed that the CMV-positive group expressed significantly higher levels of CD21 (expressed by follicular dendritic cells; P=0.043) and tended to express higher levels of FOXP3 (expressed by Tregs; P= 0.069) and PD-1 (expressed by exhausted T cells; P=0.097).

Analysis of therapy response in the two cohorts used for lymphocyte/cytokine profiling and tissue staining showed an overall trend for higher complete remission (CR) rates in the CMV-positive groups with a significant difference observed in the first cohort after 4 cycles of therapy (P=0.020). This finding could not be confirmed in the overall trial cohort. However, the CMV-positive group in this cohort contained more patients with adverse prognostic features, potentially biasing it towards inferior therapy response. Interestingly, a significant association was observed between the achievement of CR and the number of circulating E-CD4+ T cells but not serum cytokine levels or the tissue accessory cells expressing CD21, FOXP3 or PD-1. Analysis of toxicity data by CMV status showed a significant association between CMV positivity and heterologous infection of any grade. This association was observed in the overall trial cohort (P<0.001) as well as in the cohort used for tissue staining (P=0.017).

Taken together, my findings support the hypothesis underlying this thesis, namely that the biology of FL is influenced by chronic CMV infection. Specifically, CMV-positive patients with FL undergo accelerated immune senescence and experience more infections following rituximab-containing chemo-immunotherapy. CMV-positive patients may also achieve better responses, possibly due to the higher numbers of cells expressing Fcγ receptors required for antibody-dependent cell-mediated cytotoxicity (ADCC).

Based on my findings, a case could be argued for the routine application of CMV screening in FL prior to treatment with chemo-immunotherapy with the implementation of enhanced infection surveillance in CMV-positive patients. If properly harnessed, these understandings can ultimately improve on the therapeutic strategies in FL management toward a combinatorial approach for direct cytotoxic and indirect immunomodulatory perspectives.

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I declare that the entire work presented in this thesis belongs to me. However, Dr Victoria Shaw assisted with cytokine Luminex assays. Dr Carlos Rubbi wrote the ImageJ programme for the analysis of immunofluorescence images. Dr Richard Jackson, the PACIFICO trial statistician and Miss Silvia Cicconi helped with the explorative analysis of the effect of CMV on clinical outcome. Dr Brian Lane for the heat maps in chapter 3.

Presentations arising from this work

1. Poster presentation at the University of Liverpool Research (poster) day, Central Teaching Laboratories – April 10, 2014 (9:00 to 3:00) – POSTER 66

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3. Oral presentation at the Department of Molecular & Clinical Cancer Medicine (DMCCM) PGR Annual Progress Report Talks: June 3, 2013, June 12, 2014

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Glossary of Abbreviations

^{51}Cr	Chromium 51
ADCC	Antibody-dependent cell-mediated cytotoxicity
AEs	Adverse Events
AF488	Alexa Fluor® 488
AF647	Alexa Fluor® 647
APC	Allophycocyanin (dye)
APCs	Antigen-presenting cells (cells)
BCR	B-cell receptor
BV	Brilliant Violet
CA	Captured antibodies
CCL3	Chemokine (C-C motif) ligand 3
CCL4	Chemokine (C-C motif) ligand 4
CCR7	Chemokine (C-C Motif) Receptor 7
CD	Cluster of differentiation
CDC	Complement-dependent cytotoxicity
CHOP	Cyclophosphamide, doxorubicin (hydroxydaunomycin), vincristine (Oncovin ®) and prednisolone (a steroid)
CIRS	Cumulative Illness Rating Scale
CLL	Chronic lymphocytic leukaemia
CMI	Cell-mediated immunity
CMV	Cytomegalovirus
CNS	Central nervous system
CR	Complete remission
CRP	C-reactive protein
CS & T	Cytometer Setup and Tracking
CTLA4	Cytotoxic T-lymphocyte-associated protein 4
CTLs	Cytotoxic T lymphocytes
DAs	Detection antibodies
DAPI	4', 6-diamidino-2-phenylindole [fluorescent stain]
DCs	Dendritic cells
DLBCL	Diffuse large B-cell lymphoma
EBV	Epstein–Barr virus
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
ELISpot	Enzyme-Linked ImmunoSpot
EPHA7	Ephrin type-A receptor 7
FACS	Fluorescence-activated cell sorting
FCS	Foetal calf serum
FcγR III	Fc γ Receptor –III
FDCs	Follicular dendritic cells
FFPE	Formalin-fixed, paraffin-embedded

FGF-basic	Basic fibroblast growth factor
FITC	Fluorescein isothiocyanate
FL	Follicular lymphoma
FLIPI	Follicular Lymphoma International Prognostic Index
FOXP3	Forkhead box protein-3
GCBs	Germinal-centre B cells
GCLP	Good Clinical Laboratory Practice
GFP	Green Fluorescent Protein
GM-CSF	Granulocyte-macrophage colony-stimulating factor
hABs	human AB serum
Hb	Haemoglobin
HCV	Hepatitis C virus
HEV	High endothelial venules
HGF	Hepatocyte growth factor
HHV	Human herpes virus
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HLA-DR	Human Leukocyte Antigen - antigen D Related
HNSCC	Head and neck squamous cell carcinoma
HRP	The enzyme horseradish peroxidase
hs-CRP	High-sensitivity C-reactive protein
HSCT	Hematopoietic stem cell transplantation
IF	Immunofluorescence
IFN-γ	Interferon γ
IHC	Immunohistochemistry
IP-10	Interferon γ-induced protein 10
IQR	Interquartile range
iTreg cells	Induced Regulatory T cells
KLRG1	co-inhibitory receptor killer-cell lectin like receptor G1
LAG-3	Lymphocyte-activation gene 3
LDH	Lactate dehydrogenase
LNs	Lymph Nodes
MCL	Mantle cell lymphoma
MCP-1	Monocyte chemoattractant protein-1
MHC	Major Histocompatibility Complex
MIP-1α	Macrophage inflammatory protein -1α
MIP-1β	Macrophage Inflammatory Protein -1 β
MSC	Myeloid suppressor cells
NCRI	National Cancer Research Institute
NDS	Normal Donkey Serum
NHL	Non-Hodgkin lymphoma
NK cells	Natural Killer cells
NKR	NK cell receptor

NKT cells	Natural killer T cells
Non CR	Non-complete remission
OOR	Out of Range
OS	Overall survival
PACIFICO	Purine-Alkylator Combination In Follicular lymphoma Immuno-Chemotherapy for Older patients
PALS	Periarteriolar lymphoid sheaths
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PCa	Prostate cancer
PD	Progressive disease
PD1	Programmed cell death protein 1
PD-ECGF	Platelet-derived endothelial cell growth factor
PDGF	Platelet-derived growth factor
PDGF-C	Platelet-derived growth factor-C
PE	Phycoerythrin
pE-1, 2	pre-Effector -1, 2
PerCp	Peridinin-chlorophyll proteins
PFS	Progression-free survival
PP65	Phosphoprotein 65
PR	Partial remission
PTC	Papillary thyroid cancer or papillary thyroid carcinoma
Qdots	Quantum dots
QNAT	Quantitative nucleic acid testing
R-CVP	Rituximab – cyclophosphamide, vincristine and prednisolone
R-FC	Rituximab - Fludarabine and chlorambucil
RPMI	Roswell Park Memorial Institute (medium)
SAEs	Severe Adverse Events
SD	Stable disease
sELISA	sandwich ELISA
SPSS	Statistical Package for the Social Science
t(14; 18)	Translocation of the *BCL2* gene (18q21.3) to the *IGH* transcriptional enhancer, as a result of the t(14;18)(q32;q21)
TAMs	Tumour-associated macrophages
TBS/T	Tris-Buffered Saline and Tween 20
Tc	Cytotoxic T cell
T_{CM}	Central memory T cells
TCR	T cell receptor
T_{EM}	Effector memory T cells
TEMRA	T effector memory re-expressing CD45RA cells
Tfh	Follicular helper T cells
TGF-β	Transforming growth factor β
T_H cells	T-helper cells

TILs	Tumour-infiltrating lymphocytes
TIM3	T-cell immunoglobulin domain and mucin domain 3
TM7s	Seven-transmembrane spanning receptors
TME	Tumour microenvironment
TNFRSF14	Tumour Necrosis Factor Receptor Superfamily, Member 14
TNF-α	Tumour necrosis factor α
Tregs	Regulatory T cells
VDJ	Variable, diversity and joining genes
VEGF	Vascular endothelial growth factor

Chapter 1 : General Introduction

1.1 Introduction

This section offers an overview of the thesis with a particular emphasis on the main components of the study. In this chapter, a detailed review of related literature of key areas linked to this study is presented. Such areas include follicular lymphoma (FL) and cytomegalovirus (CMV) infection, amongst other pertinent subthemes, with a direct relation to the rationale, goals and objectives of the study. Furthermore, this section offers a concise description of the depth and scope of study in addition to the research content and niche.

1.2 Follicular Lymphoma

Follicular lymphoma (FL) is part of a group of cancers called Non-Hodgkin lymphomas (NHL) which are a highly prevalent group of haematological cancers (Song et al., 2013). It is comprised of some closely linked lymphoid proliferative disorders emanating from the lymphoid tissues at various phases of differentiation; comprising of heterogeneously diverse immunophenotypic, morphologic, clinical and genetic properties (Gibson et al., 2013, Morton et al., 2008, Song et al., 2013). NHL is characterised by malignant transformation of the lymphoid cells, either mature or precursor cells, and it is one of the leading causes of cancer death (Morton et al., 2008, Nagai et al., 2008). World Health Organization (WHO) has categorised NHL into two main groups, based on cells of origin,: those of B-cell origin and those of T-cell/natural killer (NK)–cell origin (Norris and Stone, 2008). According to the 15th Cochrane Haematological malignancies biannual report, about 85 percent to 90 percent of NHL cases emanate from B-cells, such as, mantle cell lymphoma, FL, Burkitt lymphoma and diffuse large B-cell lymphoma (DLBCL); while the T-cell

involved diseases include anaplastic large cell lymphoma and precursor T-lymphoblastic (Rancea et al., 2013). Although the aetiology of NHL is not clear, a growing body of evidence has listed certain viral and bacterial infections such as Epstein-Barr (EB), altered immune function and a considerable number of immune-related gene polymorphisms and alterations as predisposing risk factors (Charbonneau et al., 2012, Gibson et al., 2013).

The second most prevalent NHL is FL, after Diffuse large B-cell lymphoma (DLBCL or DLBL) (Taskinen et al., 2007). Literature confirmed the clinical progression of FL as being highly indolent but characterised by incredibly diverse clinical outcomes (Kiaii et al., 2013). Despite the fact that FL is incurable, it can be managed with about 11.1 years being the average documented median survival (Staudt, 2007). Interestingly, many FL patients live for decades after the initial diagnosis without treatment; but in contrast, a proportion develops rapid disease progression with a short survival rate (Kiaii et al., 2013). Additionally, it has been estimated that more than 30 percent of patients suffering from follicular lymphoma undergo histologic transformation to DLBCL and elucidating the cause of the heterogeneity in FL would better inform the clinical management and treatment approaches thus improving the outlook for FL patients.

1.2.1 The pathology of FL

From a morphological understanding, FL is described as the proliferation of malignant germinal centre B cells (GCBs) that are intermixed with non-malignant cells. The common non-malignant cells in the GC include the follicular dendritic cells (FDCs), T cells, and macrophages as well as centroblasts and centrocytes that represent the predominant cell types of the germinal centre reaction (Campo et al., 2011). The traditional grading method for FL utilises the relative proportion of

centrocytes to centroblasts, as its limits. Thus grade 1 FL has minimal centroblast numbers of 0–5 per high-power field while grade 3B FL is comprised of thick sheets of these cells (Kridel et al., 2012). However, the FL grading does not offer precise prognostic implications of disease as seen in several other malignancies. There is no satisfactory consensuson the characteristics of grade 3B FL, considering its similarity to de novo DLBCL and to some extent, the expression of different molecular features compared to grades 1–3A FL. There is also the case that, Grade 3B FL is frequently negative for t(14; 18) while inconsistently expressing CD10 (Salaverria and Siebert, 2011). The characteristic follicular growth pattern of FL can be revealed by staining for FDC markers, for instance, CD21 or CD23 (Kridel et al., 2012). Moreover, the disease's molecular hallmark is in the overexpression of anti-apoptotic BCL2. (Kridel et al., 2012) due to the translocation of t(14; 18) (q32; q21) identified by fluorescence in situ hybridization (FISH) in 85 percentage of all patients. This translocation, therefore, is not critical or adequate for diagnosis, since it is missing in 15 percent of FLs and also present in approximately 20 to 30 percent of GCB-type DLBCLs (Salaverria and Siebert, 2011, Weiss et al., 1987).

1.2.2 Cell of Origin of FL

The perceptions that the neoplastic cells are arranged in follicles, display germinal centre surface markers, for instance CD10 and BCL6, and have a characteristic gene expression profile of centroblasts or centroblasts (Alizadeh et al., 2000) provide that FL B-cells are capable of attaining a differentiation phase indicative of GCB cells (Küppers, 2005). The initial oncogenic cascade's hit resulting in FL is commonly associated with the t(14; 18), founded on the consistency of the genetic irregularity as well as its intended mode of action (Kridel et al., 2012). The occurrence of translocation takes place subsequent to a double-strand break within the

Immunoglobulin heavy (IGH) locus on chromosome 14 associated with faulty recombination-activating gene (RAG) -mediated VDJ recombination and a break at the BCL2 locus on chromosome 18 perceived to be connected to an intrinsic frailty at CpG sites (Tsai et al., 2008, Tsujimoto et al., 1985). The VDJ recombination takes place at an initial B cell growing phase in the bone marrow, and as a primary genetic hit, the t(14; 18) is equally likely to happen in the bone marrow but does not hinder additional differentiation. Colonization of secondary lymphoid tissue is undertaken by the naïve B cells expressing the t(14;18) upon exiting bone marrow, hence undertaking the germinal centre reaction although they hold survival benefit because of their BCL2 constitutive expression, which is typically not in the germinal centre (Roulland et al., 2011). BCL2 is equally capable of rescuing these cells from apoptosis because of weak BCR affinity (Roulland et al., 2011). Nevertheless, the appearance of a structurally and probably functionally conserved BCR appears critical as, regardless of the progressive somatic hypermutation, a discerning selection against adverse transformations in BCR is apparent (Bahler and Levy, 1992, Zuckerman et al., 2010). BCR signalling may be additionally activated by the interface of mannosylated immunoglobulin variable regions with lectins present on non-neoplastic immune cells' surface (Coelho et al., 2010). The primary FL progenitors subsequently attain secondary genetic modifications under the impact of activation-induced cytidine deaminase (AID), the value of which has been demonstrated in a mouse model whereby genetic deletion of AID prevented BCL6-driven germinal centre–derived lymphomas (Pasqualucci et al., 2008).

The present hypothesis that identifies naïve t(14;18)-positive peripheral B cells as likely tumour-triggering cells for FL pathogenesis is questioned by the findings that such cells have also been reported in the blood of 50 to 70 percent of healthy people

who are not prone to acquiring FL (Dölken et al., 1996, Limpens et al., 1995, Schüler et al., 2009). The prevalence of FL plus the occurrence of t(14;18)-positive cells in healthy people rises with age (Schüler et al., 2009). Literature confirms that some of these cells are not naïve B cells, but rather germinal centre-experienced IgD+CD27+ (or IgM+CD27+) memory cells (Hirt et al., 2007, Roulland et al., 2006). Identical to true FL tumour cells, these alleged FL-like cells in healthy people demonstrate class-switch recombination of the translocated IGH allele, while the functional allele encodes a surface IgD (or IgM) (Kridel et al., 2012). The contribution of these FL-like cells to the pathogenesis of FL remains obscure. Nevertheless, originating lymphoma-initiating cells ought to exist, as depicted by two FL reports on allogeneic hematopoietic stem cell transplantation (Carlotti et al., 2009, Weigert et al., 2012). In a retrospective study, the t(14;18) was detected from the donor lymphocyte infusion as well as a small number of mutated alleles that correspond to 14 of the 15 gene mutations common between the recipient's and donor's tumours (Weigert et al., 2012). This study report suggests proof that the necessary lymphoma progenitor holds more intricate genetic variations than initially thought. The understanding of the origin of FL is additionally made complicated by the identification of a loosely defined component described as FL in situ, which has been suggested as a valid precursor cell on the grounds of relatively rare progression to overt FL (Cong et al., 2002, Jegalian et al., 2011).

1.2.3 Genetic Landscape of FL

The understanding that BCL2 has anti-proliferative activities on top of its anti-apoptotic characteristics (Zinkel et al., 2006) and that the t(14;18) is also present in healthy people (Dölken et al., 1996, Limpens et al., 1995, Schüler et al., 2009), suggests that BCL2 over-expression alone is not enough to activate

lymphomagenesis. Furthermore, over-expression of BCL2 in transgenic mouse models does not recapitulate FL pathogenesis. In the inventive Eμ-*BCL2* reproduction, mice established follicular hyperplasia and, upon prolonged latencies, elevated-grade lymphomas that were evidently not indicative of FL (McDonnell and Korsmeyer, 1991). Egle et al. established a VavP-*BCL2* mouse model whereby BCL2 was overexpressed and controlled by pan-haematopoietic VavP promoter (Egle et al., 2004). The mice developed lymphomas that had common morphological characteristics with FL, however, the high *BCL2* transgene expression present in the T cell region illustrated a condition absent in FL (Egle et al., 2004). The consideration that clonal cytogenetic eccentricities, copy number variations, as well as uniparental disomy, are identified in FL and offers extra support to the idea that further genetic alterations add to FL pathogenesis (Kridel et al., 2012).

In addition to the t(14;18) translocation, other chromosomal abnormalities comprise of non-random 1p36 and 6q losses in addition to 7, 18, and X gains (Cheung et al., 2009, Horsman et al., 2001, Johnson et al., 2008, Schwaenen et al., 2009). Of late, there has been a positive outcome of insight regarding dominant players after a thorough assessment of candidate genes within such areas. For instance, considering the 1p36 region that is highly targeted by copy-neutral loss of heterozygosity as well as heterozygous deletions, the *TNFRSF14* gene was found being repeatedly mutated (Cheung et al., 2010, Launay et al., 2012). Two studies found that mutations in the *TNFRSF14* gene (Cheung et al., 2010, Launay et al., 2012) lead to a truncated protein in 57% of *TNFRSF14* mutations regardless of its transmembrane domain, causing a reduction in surface expression. TNFRSF14 mutations offered the initial evidence of the connection between FL genetics and the associated microenvironment. Although a collection of ligands bind TNFRSF14, the receptor can carry both stimulatory and

inhibitory signals to T cells irrespective of ligand, which warrants further investigation to illuminate the functions of these mutations in FL pathogenesis.

Frequently deleted regions on the long arm of chromosome 6 (6q) (Oricchio et al., 2011), are increased in FL which is associated with the poor prognosis (Cheung et al., 2009). It was evident that a negative NF-κB signalling regulator identified as *TNFAIP3/A20*, and a receptor tyrosine kinase *EPHA7*, were potential tumour suppressors in FL (Oricchio et al., 2011). Additionally, the studies illustrated that silencing EPHA7 resulted in an increase in lymphomagenesis in VavP-BCL2 transgenic mice while restoring its suppressor function of the tumour via exogenous EPHA7 administration hindered the formation of tumour in xenograft models. A model whereby EPHA2 is bound by EPHA7 on the cell surface of lymphoma is proposed by these authors, hence avoiding any signalling activation via extracellular signal-regulated kinases (ERK) in addition to other proto-oncogene tyrosine-protein (SRC) kinases. Furthermore, the researchers revealed that expression of EPHA7 was lacking by immunohistochemistry in 72% of FL cases for a tissue microarray and hypothesised that any lack of protein expression transpired via genomic deletions plus promoter methylation, harmonising with earlier results from Dawson et al. (Dawson et al., 2007).

High-throughput sequencing approaches have elevated the rapidity of cancer research to an exceptional level. The mutational landscape of FL has been recently revealed using whole-genome or whole-exome sequencing, in a combination of targeted deep sequencing (Okosun et al., 2014). Recurrent mutations in genes involving in linker histone, JAK-STAT signalling, NF-κB signalling and B cell developmental, are unequivocal evidence for a reservoir ancestral population, enriched in early mutations

in driver genes, including CREBBP, EZH2 and KMT2D (MLL2). Other mutations, such as EBF1, MYD88 and TNFAIP3, are acquired subsequently and propagate successive disease events. Although no single compelling genetic event has been identified to be responsible for FL transformation, the acquisition of distinct genetic alterations in ancestral common progenitor clone may prompt the onset of aggressive disease and resistance to therapies.

The mutational environment among nodal lymphomas has hitherto been precisely stipulated and described in DLBCL and, to a minor level, in FL (Lohr et al., 2012, Morin et al., 2011, Pasqualucci et al., 2011a, Pasqualucci et al., 2011b). Unpredictably, it was revealed that germinal centre–derived lymphomas are defined by recurrent alterations of histone-modifying genes. Specifically, in FL repeated mutations have been revealed in *EZH2* (7.2%), histone methyltransferases *MLL2* (89%) , *EP300* (8.7%), the histone acetylases *CREBBP* (32.6%) and *MEF2B* (15.3%) (Morin et al., 2010, Morin et al., 2011, Pasqualucci et al., 2011a). The increased repetitive occurrence of such mutations demonstrates how FL is possibly epigenomic in addition to the genomic disorder (Baylin and Jones, 2011). Arguably, the best-characterised lymphoma histone-modifying gene, EZH2, is the catalytic component of the polycomb suppressive complex 2. Tyr641 mutations within EZH2 SET domain have been found in 22 percent of GCB-like DLBCLs and 7 percent of FLs, resulting to enhanced histone H3 Lys27 trimethylation (H3K27me3) (Morin et al., 2011, Sneeringer et al., 2010, Yap et al., 2011). The result regarding the upregulation of EZH2 in B cells within germinal centre reaction (van Galen et al., 2004) provides that any activation in *EZH2* mutations results in a transcriptional outcome supporting proliferation. Anti-proliferative genes, EZH2-mediated silencing, was consequently highlighted in typical centroblasts (Velichutina et al., 2010), offering additional back

up to the concept that FL can subvert the GCB cells' normal functional gene expression program. Regardless of our increased comprehension of genetic aberrations in FL within the last few years, there is an urgent need for future studies to facilitate a wide mutational database of all genetic variations resulting in tumourigenesis.

1.3 The tumour microenvironment

The tumour microenvironment (TME) undoubtedly acts as a survival factor, with a close and steady relationship between the tumour and its immediate surroundings usually infiltrated by inflammatory cells (Balkwill and Mantovani, 2001, Mihm Jr et al., 1996, Whiteside and Parmiani, 1994). Paradoxically, tumours can affect the microenvironment through the release of extracellular signals that promote tumour angiogenesis that compels peripheral immune tolerance; while conversely, the accessory immune cells are capable of impacting the development and exacerbation of cancer cells. Regardless of the condition, such inflammatory cell infiltrates are capable of varying in amount as well as composition from one tumour to another. Hitherto, the existence of cellular infiltrates in the cancer environment is indicative of endeavours to interfere with the progression of the tumour in a process identified as immune surveillance (Zitvogel et al., 2006). Tumour inflammatory infiltrates are deemed as the host's effort to identify the growing tumour cells, attack and remove them (Zitvogel et al., 2006). Several reports have associated the existence of inflammatory infiltrates in human tumours to enhanced prognosis or improved patient survival (Baxevanis et al., 1994, Whiteside, 2007). Multiple analyses of immune markers, inclusive of microarrays and heat maps; provide that type, location and density of tumour's immune cells can confer prognostic assessment (Galon et al., 2006, Galon et al., 2007, Pagès et al., 2005). In contrast, several reports have justified

the absence of considerable connections between the intensity of lymphocytic infiltrates and enhanced prognosis or associated immune cell infiltrates to an inferior prognosis (Nakano et al., 2001, Sheu et al., 1999, Stewart and Tsai, 1993). These contradictory reports remained unexplained for years until recently it became possible to explore functional properties of tumour-infiltrating lymphocytes (TIL), which often represents the major component of immune infiltrates in tumours (Mihm Jr et al., 1996). In general, TIL in humans demonstrate reduced proliferation following antigens or mitogens stimulation, diminished signalling via T cell receptor and declined capacity to arbitrate cytotoxicity of tumour targets or to generate Th1-type cytokines after tumour antigens simulation (Kiessling et al., 1996, Reichert et al., 2002, Uzzo et al., 1999), as illustrated in Figure 3-11. TIL's functional impairments occur more frequently in patients that have chronic cancer than in early disease, while they seem to differ in the frequency as well as magnitude, depending on the kind of tumour or its originating tissue. The functional status of TIL has been argued to be an independent and significant correlate of good prognosis and long-term survival among cancer patients (Reichert et al., 1998). With the rapid improvements in the techniques of studying functions and characteristics of tissue infiltrating immune cells, plus the understanding of their local interactions with other cells, the part played by the microenvironment in health and disease is better understood. A thorough multivariate examination of cellular interrelations in the tumour microenvironment founded on the function, nature, location, and density of the immune cells present in human colorectal cancers revealed how clinical outcome can be affected by immune responses within the tumour (Galon et al., 2006, Galon et al., 2007, Pagès et al., 2005). From the present understanding, tumour microenvironment

is perceived in a manner that it exerts an effecton tumour development and growth and that adjusting this attribute could likely present exceptional therapeutic gains.

1.3.1 Cellular components of the tumour microenvironment

For a growing tumour, the microenvironment is composed largely of the tumour stroma, proliferating tumour cells, infiltrating inflammatory cells, blood vessels and a number of related tissue cells (Whiteside, 2008). It is a distinctive environment that develops during tumour growth resulting from its relations with the host (Whiteside, 2008). It is generated by and constantly shaped and overlooked by the tumour, which coordinates cellular and molecular events transpiring within the nearby tissues (Whiteside, 2008). A range of effector immune cells from the innate arm of the immunity; polymorphonuclear leukocytes and macrophages have been identified in the tumour, as well as mediators of the adaptive arm including T lymphocytes and dendritic cells (DC) and infrequently B cells, (Whiteside, 2007). Natural killer (NK) cells are rarely seen in tumours, acting as a critical first line of defence against a wide range of pathogens eliciting innate immune responses (Whiteside et al., 1998, Lanier, 2003, Wang et al., 2013). Tumour cells downregulate the expression of HLA antigens in addition to being enriched in MHC class I chain-related genes (MICB and MICA molecules) (Chang et al., 2005) which make them vulnerable to NK cell-mediated cytotoxicity (Lee et al., 2004); their scarcity in tumour infiltrates may be an example of the circumvention technique to avoid NK-cell attraction to the tumour location.

Normally, TILs comprise of some CD3+CD4+and CD3+CD8+T cell populations and are an essential component of the tumour microenvironment (Whiteside, 2007). Several of these T cells are particular for tumour-associated antigens, as evident from clonal evaluation (Miescher et al., 1987) as well examination of the CD8+T cells tetramer staining isolated from human tumours (Albers et al., 2005). In a few types of

tumours, e.g. medullary breast carcinomas, lymph node-like structures are formed by the infiltrating lymphocytes, implying that there is an immune response *in situ* (Coronella et al., 2002). Additionally, TILs are the basis for specificity of tumour lymphocytes employed in adoptive transfers upon expansion in IL-2-containing cultures (Zhou et al., 2004). Clones of TILs with the specific characteristics to a high multiplicity of the tumour-associated antigen can be expanded from human tumours, affirming that immune reactions are not simply directed at 'specific' antigens expressed by the tumour, but equally at a variety of representative or tissue-specific antigens (Romero et al., 2006). Even though the increase in effector T cells in the tumour microenvironment would be seen as an indication of immune surveillance by the host, they are mostly futile in controling tumour growth. Of the CD4+T cells in the tumour microenvironment, a subpopulation of CD4+CD25high Foxp3+cells is increased to about 5–15% of CD3+CD4+T cells of tumour-infiltrating lymphocytes compared to the small numbers reported in the circulating peripheral blood of cancer patients (Woo et al., 2001). These regulatory T cells (Tregs) can suppress the proliferation of other T cells within the microenvironment by contact-dependent mechanisms or TGF-β and IL-10 secretion. There are different subtypes of Tregs, for instance, the natural Treg (nTreg) and induced Treg (iTreg), and characterised microenvironment in human tumours (Bergmann et al., 2007). Macrophages present in tumours are known as tumour associated macrophages (TAMs). They are re-programmed to inhibit lymphocyte functions through release of inhibitory cytokines such as IL-10, prostaglandins or reactive oxygen species (ROS) (Mantovani et al., 2002, Mosser and Edwards, 2008). The myeloid suppressor cells accumulating in human tumours are CD34+CD33+CD13+CD15(-) bone marrow-derived undeveloped dendritic cells (Serafini et al., 2006). Mesenchymal stromal stem cells (MSCs)

promote tumour development and restrains functions of immune cells via abundant generation of an enzyme arginase 1 involved in L-arginine metabolism. Arginase 1 synergizes with inducible nitric oxide synthase (iNOS) with the intent of enhancing the production of superoxide and NO, thus dampening the lymphocyte responses (Ochoa et al., 2007). This mechanism will also enhance the induction of inducible oxygen species (iNOS) in neighbouring cells (Tsai et al., 2007). A study illustrates the expansion of CD14+HLA-DR-/low myeloid-generated cells adopting immune inhibition via TGF-β secretion within the peripheral circulation of patients undergoing treatment for metastatic melanoma using GM-CSF-based vaccines (Filipazzi et al., 2007). The tumour coordinates MSC's recruitment to the tumour location. Some factors produced by tumours, which support MSC accumulation as well as preventing DC maturation plus lymphocyte functions include VEGF, IL-10, and GM-CSF (Serafini et al., 2006).

Granulocytes are seldom seen in infiltrates of human tumours, with the exception of eosinophils that may exist in association with tumour cells in different squamous cell tumour (Loukinova et al., 2000). Evasion of the tumour from the host occurs through the capacity of human tumours to destabilise antitumour immunity by down-regulating or entirely supressing local and systemic innate immunity as well as adaptive antitumour immunity via a range of methods (Whiteside, 2008).

1.3.2 The FL microenvironment

In most instances, the microenvironment in FL is the lymph node (Yang and Ansell, 2012b). Structurally, the lymph node is compartmentalised into the outer region (the cortex), the paracortex, and the inner region (the medulla). It is within the cortex that follicles containing germinal centres (GCs) are found. The various immune accessory cells in the FL microenvironment live strategically in the different zones within the

compartments (Katakai et al., 2004). For instance, T cells are primarily located in the medulla and paracortex compartments whereas B cells occupy the follicles of the cortex (Yang and Ansell, 2012b).

Following antigenic challenge, B and T cells migrate to follicles in lymph nodes and the T-cell zone where they interact with antigen presenting cells (APCs) and experience clonal expansion (von Andrian and Mempel, 2003). To initiate primary immune responses, FDCs present antigen to B cells and are only present in follicles of primary and secondary lymph organs (von Andrian and Mempel, 2003). However, before antigen encounter, the follicle is comprised mainly of naïve B cells with no GC for B cell maturation. Following antigenic stimulation, GCs are developed in the follicles and drive the differentiation of B cells into plasma cells that secrete immunoglobulins (Calame, 2001). Despite the understanding that most T cells live outside of the follicle, the follicular T helper (T_{FH}) cells live in the GCs and arean essential subcategory of CD4+ T cells for antigen-dependent activation of B cells.

In FL, the structure and the cellular content of the follicles are different from the normal lymph node as a result of the enlargement of GC caused by the expanding quantities of B lymphoma cells (Amé-Thomas et al., 2007). An alteration in the cellular distribution can be studied in tissue biopsies from FL lymph nodes and relate to those of normal lymph nodes. For instance, an elevated number of Treg cells and reduced effector T cells, such as TH17 and TH1 cells characterise follicular content in FL. A lot of the effector T cells present in the tumour microenvironment of FL have an exhausted phenotype and display reduced functional potential, as well as the presence of elevated numbers of naïve immune suppressive macrophages. These changes have all been shown to possibly influence antitumoral immunity and support lymphoma cell survival and development in FL. Intratumoral T cells in Follicular

lymphoma is portrayed by the presence of a high number of T cells, up to half of the total number of cell content in the tumour microenvironment. These intratumoral lymphocytes substantially affect antitumor resistance and patient outcome (Dave et al., 2004). T cells are heterogeneous and impact tumour immunity in both a negative and positive manner, which is contingent upon the mix of the different T-cell types inside the microenvironment. For the most part, high quantities of intratumoral T cells are connected with positive prognosis in patients with FL, (Wahlin et al., 2011).

1.4 T-lymphocytes

1.4.1 Major histocompatibility complex (MHC)

MHC molecules are surface peptide receptors of the immune system that permit T lymphocytes to recognise antigens (Kindt et al., 2007, Savill and Fadok, 2000, Murphy, 2011). Their crystal structures contain a groove which is occupied by a short peptide specific to an antigen that allows for immune recognition (Falk et al., 1991). For example, after a macrophage engulfs bacteria, it partially digests it and presents peptide particles of the microorganism on its surface, attached to MHC molecules (Chaplin, 2010, Abbas et al., 2014). The T lymphocytes recognise the foreign particle attached to the MHC molecule and binds to it, activating an immune response (Benacerraf, 1978). Within healthy cells, the MHC molecule displays peptides from its cell (self-peptides) and the T cells do not usually respond to them (Matzinger, 1994).

There are two major categories of MHC molecules: The MHC class I molecules are ligands for inhibiting or stimulating natural killer (NK) cell receptors displayed on T cells and NK cells (Bauer et al., 1999, Karabekian et al., 2015). They are carried on the majority of nucleated cells, including cardiac myocytes and fibroblasts. This class I molecule contain a heterodimer of a highly polymorphic α heavy chain, non-

covalently associated with a conserved light chain called β-2 microglobulin. On the other hand, MHC class II molecules are a family of molecules mainly expressed by professional APCs, such as dendritic cells (DC), mononuclear phagocytes, some endothelial cells, thymic epithelial cells, and B cells and composed of two polypeptide chains an α and a β chain of approximately equal length (Matzinger, 1994, Rock et al., 2016).

To efficiently attack and eradicate foreign bodies including those contained within or on bacteria, viruses, other microorganisms, or cancer cells; T-cells have to recognise the antigenic peptides presented on the surface of the cells (Larsson et al., 2001, Höftberger et al., 2004). Antigens from endogenously manufactured proteins within the cytosol are attached to MHC class I molecules and are identified by CD8 positive T-cells, while peptides internalised through the process of endocytosis and produced within vesicles are attached to MHC class II molecules and identified by CD4 positive T-cells (Larsson et al., 2001).

1.4.2 Activation of Naïve T cells

The naïve T cells are programmed to recognise antigens only in the T cell zones of the secondary lymphoid tissues, and for this reason, initiation of the immune response depends upon transport of antigen to these areas from the site of infection (Cyster, 2005, von Andrian and Mempel, 2003). Naïve T cells leave the thymus and continually migrate between secondary lymphoid organs (spleen, lymph nodes, and Peyer's patches) (Gowans and Knight, 1964, Picker and Butcher, 1992, Butcher and Picker, 1996) entering the circulation as resting cells in the G_0 stage of the cell cycle (Tough and Sprent, 1994). However, naïve T cells can only make contact with antigen within the secondary lymphoid organs. After the antigen has been taken up by cells it is processed into immunogenic peptides as the cells migrate to the secondary

lymphoid tissues. En route the cells may differentiate into mature dendritic cells (DC), and localise in the T cell zones expressing antigen peptides on the MHC molecules (Sprent and Surh, 2002) for the naïve T-cells to recognise.

The first admission of T cells to the spleen is nonspecific; along the splenic artery. The cells (and antigens) travel through the splenic trabeculae artery into the trabeculae and empties into the marginal zone at the margin of the white and red pulp (Steiniger et al., 2001). After that, the T (and B) cells move selectively to the white pulp in which they accumulate around central arterioles; these regions known as the periarteriolar lymphocyte sheaths (PALS) are the key T cell area within the spleen (Kraal, 1992, Van Ewijk and Nieuwenhuis, 1985, Gunn et al., 1998a). Even though the process through which T cells move into PALS from the marginal zone is not indistinct, it is noteworthy that activated T cells without the chemokine receptor CCR7, enter the red pulp but are barred from the white pulp (Potsch et al., 1999). Therefore, T cell entry into the white pulp might be directed by CCR7 recognition of distinct chemokines expressed on stromal cells (Cyster, 2005). After around 12 h, immature T cells migrate from the PALS into the red pulp and depart the spleen through venous blood (Sprent and Surh, 2002). Quite the opposite to the spleen, admission of T cells into Peyer's patches and lymph nodes is extremely precise and happens once cells go into high endothelial venules (HEV) (Miyasaka and Tanaka, 2004, von Andrian and Mempel, 2003, Sprent and Surh, 2002). With respect to lymph nodes, the luminal surface of HEV expresses peripheral node addressin (PNAd) (Berg et al., 1991) and CCL21 (SLC), a chemokine (Gunn et al., 1998b). These ligands are identified by two lymph node homing receptors on T cells, CD62L and CCR7 correspondingly (Rosen, 2004, Weninger et al., 2001, Cavanagh and Von Andrian, 2002). The lymph node homing receptors facilitate T cells to attach to and enter the

walls of HEV and arrive at the T cell zone, the paracortex. The T cells then migrate out of the paracortex and depart lymph nodes through efferent lymphatic vessels, followed by re-admission into the bloodstream through the thoracic vessel (Girard et al., 2012, Förster et al., 2012). This course of blood-to-lymph recirculation via lymph nodes takes around 12–18 hours (Sprent, 1973).

The constant migration of T cells via the secondary lymphoid organs is extremely significant for permitting T cells to create an active contact with antigens discharged from pathogens (Sprent and Tough, 2001). The antigens released are presented to T cells in the form of peptide fragments bound to MHC molecules (Berczi and Szentivanyi, 2003). For naïve T cells, these immunogenic peptide/MHC complexes have to be presented by specialised APCs, primarily by dendritic cells (Steinman, 1991, Inaba et al., 1990). These cells are strategically located as a compact system in the T cell zones and are constantly scrutinized by recirculating T cells for expression of foreign peptides (Banchereau and Steinman, 1998). Because naïve T cells are mainly tolerant to self-components, they overlook the self-peptide/MHC complexes on normal dendritic cells. As a result, T cells are permitted to permeate gradually through the T cell area and then re-enter the bloodstream for further recirculation. Throughout their usual course of blood-to-lymph recirculation, immature T cells are metabolically dormant and have a long life span (von Boehmer and Hafen, 1993, Weng et al., 1995). However, the durability of immature T cells is dependent on constant contact with at minimum two outer ligands, known as self-peptide/MHC compounds on dendritic (Ernst et al., 1999) and IL-7 (Martínez et al., 2015). The recognition of these ligands most probably delivers low-level signals that maintain T cells to prevent apoptosis.

It is well accepted that cessation of the primary response is followed by a broad-scale loss of effector cells. The elimination of effector cells following the clearance of pathogens makes sense since leaving these cells to remain would overload the immune system with non-functional cells, resulting in lymphoid hypertrophy and traffic congestion and thus compromise the response of naïve T cells to novel pathogens (Sprent and Surh, 2002).

1.4.3 Memory T cells

Following exposure to infectious agents or cancerous cells, a state of immunological memory is attained. Thus the memory acquired by antigen-specific T and B cells is frequently enduring (Sprent and Surh, 2002). Primarily, immune responses to antigenic challenge might be categorised into primary or secondary reactions. The primary immune response to antigen happens on the initial encounter with a foreign body and results in the formation of memory cells with extremely high avidity and specificity for the stimulating antigen. After this first experience, antigen-specific CD4 T-cells and CD8 T-cells display an improved performance in providing support to B cells by producing cytokines and other stimulatory factors and the production of a vast number of antigen-specific cells that can directly kill infected cells accordingly. This phase of response can be said to be a secondary response which moreover are more robust compared to primary responses (Ahmed and Gray, 1996).

Signals from the T cell receptor (TCR) due to large doses of peptide antigen attached to MHC class II molecules on APCs are critical for the activation of naïve CD4 T cells and their transition to memory and effector cells (Swain et al., 1999, Rogers et al., 1998). It has been reported that transport of effectors, produced in vitro from immature CD4 T cells to adoptive hosts leads to the growth of lifelong memory CD4 although no additional antigen is added (Swain, 1994). Diverse strengths of TCR

interplay with peptide-MHC are required for T cell response at different steps of T cell differentiation and can influence different outcomes. It has been demonstrated that immature CD8 T-cells within class I– deficient mice (Tanchot et al., 1997) and immature CD4 T cells in class II– deficient mice (Takeda et al., 1996, Murali-Krishna et al., 1999, Rooke et al., 1997) have abridged life-spans that suggest that interactions between MHC and TCR increase the life span of naïve T cell. Immature CD4 T cells need high avidity or intense TCR interactions for the induction of cytokine synthesis while effector and memory cells react effectively at lesser avidity/ intensity (Dubey et al., 1996). Therefore, memory and effector cells might be expected to overcome this MHC dependence.

Memory CD8 T cells are a significant constituent of long-standing immunity due to their prolonged persistence and their unique capability to quickly enlarge their numbers and generate cytotoxic molecules and antiviral cytokines following second antigen encounter (Kaech et al., 2002). Throughout the majority of infections, most of the antigen-specific CD8 T cells produced by clonal unidentified, and particularly the process that decides which of the antigen-specific CD8 T cells will remain and convert into memory cells and which will die has yet to be clarified (Kaech et al., 2003). This quest has been stalled mostly due to lack of authoritative markers that differentiate among the two cellular fates of survival or death. A classic CD8 T cell response comprises of three key developmental phases: increase in effector cell and differentiation; reduction in effector cell; and stabilisation and preservation of the memory cell numbers (Kaech et al., 2003).

Memory T cells are quite suitable to fight pathogens since they exist in greater numbers compared to naïve cells, they persevere for long periods because of the antigen-independent homeostatic turnover, and they react speedily on a reencounter

with a pathogen (Kaech et al., 2002). By their anatomical location, two subgroups of memory T cells were defined, the composition of cell surface markers and consequence of expansion die following antigen removal, and the surviving cells turn into the long-lasting memory cell population (Busch et al., 1998, Murali-Krishna et al., 1998). Central memory cells (T_{CM}) refer to a class of T cells that express molecules such like CCR7 and CD62L, which facilitate effective homing to lymph nodes (LNs). On the other hand, memory T-cells that do not express the LN homing receptors resident within non-lymphoid organs are called effector memory cells (T_{EM}). Nevertheless, both T cell subtypes are available in the spleen and blood. Some studies have as well demonstrated that T_{EM} obtains effector roles such like cytokine secretion and killing, more rapidly compared to T_{CM} (Sallusto et al., 1999, Masopust et al., 2001, Reinhardt et al., 2001). Wherry et al. have been able to demonstrate that T_{CM} has a greater capacity to persevere in Vivo than T_{EM} and are more proficient in mediating defensive immunity due to their enhanced proliferative potential (Wherry et al., 2003b).

1.4.4 The T cell surface markers

Cell surface markers are proteins on the surface of cells and act as markers for recognition. TLymphocytes separate into numerous cell subtypes, essential for biological functions. Throughout lymphocyte differentiation diverse surface receptors are expressed that can recognise cellular subtypes and ligands. Improper cellular proportions of differentiated leukocytes, such like the comparative quantities of T_H1 and T_H2 cells, take place in pathophysiological circumstances such like autoimmunity (Pitkänen, 2005). The presence of cell surface markers can likewise figure out whether a cell type expresses the particular receptor essential for a biological response. Testing for surface marker expression is likewise fundamental in

determining whether a trial medication or ligand will be recognised by the cell type of interest (Wingender and Kronenberg, 2015). The expression of diverse combinations of chemokine receptors can be employed to name specific T helper subsets (Zhu et al., 2010), and through the secretion of a particular combination of cytokines, the subsets of T helper can define the ongoing immunological state (Wingender and Kronenberg, 2015).

Identification of antigen by the immune mechanism elicits a coordinate number of variations in lymphocyte and lymphocyte subtypes that enable the system (a) to eradicate or neutralise likely dangerous pathogens and (b) to react faster and suitably following repeated antigen encounter in a process known as immunological memory. Inside the T cell compartment of the lymph node, immature T cells, memory T cells, and effector T cells can be discerned. Practically, memory T cells have various qualities that differentiate them from naïve T cells. Not just do they react effectively to recall antigens; memory T cells likewise have less stringent prerequisites for activation and can possibly discharge a broad range of cytokines (Merkenschlager et al., 1988, Byrne et al., 1988, Picker et al., 1995). Also, naïve and memory T cells vary in the expression of numerous cell surface antigens (Sanders et al., 1988) though it is not clear if these markers can be regarded as valid memory markers or if they indicate cellular stimulation (Michie et al., 1992).

Studies have reported phenotypic differences between effector, naïve, and memory T cells that showed that with relation to immature T cells, cytotoxic T lymphocyte effectors have downregulated the lymph node homing receptor, CD62L (L-selectin) whereas expression of CD44, CD11a/CD18, CD11b, and CD49d are increased in expression (Hamann et al., 1997). In contrast to effector T cells, memory T cells have

varied levels of CD62L expression and do not possess CD11b on their surface; they nevertheless display an intermediate CD49d expression. Studies have demonstrated that memory, naïve and effector kind of cells can be differentiated with the combination of functional and phenotypic characteristics (Hamann et al., 1997).

Models have been used to assist in defining populations and subpopulations of CD4 as well as CD8 T cells sequential progression from naïve to terminally differentiated effector memory (TEMRA) populations. For instance, this study adopts a classic differentiation system showing cell types: Naïve >> Central Memory >> Effector Memory-1 >> Effector Memory- 2 >> pre-Effector -1 >> pre-Effector -2 >> Effector Memory-4 >> Effector Memory-3 >> Effector end-stage non-proliferative effector cells. These phases of cell development have been identified using relevant markers and in combination with proliferation and cytokine secretion assays (Koch et al., 2008b, Mojumdar et al., 2012, Alves et al., 2007). Consequently, the presence of specific T cell types and expressions of cell surface markers have been useful as prognostic tools, for example, an elevated number of peripheral T cells has often been reported in CLL patients, which is largely as a result of increased frequency of CD8+ T cells and lowering the CD4/CD8 ratio (Zaknoen and Kay, 1990). Also, the capacity to elicit an effective immune response against novel pathogens depends on the availability of circulating naïve T cells, hence shortage of naïve T cells is blamed for the increased susceptibility to infectious and malignant diseases, as well as impaired vaccine efficacy among the older persons (Briceño et al., 2016).

Within the peripheral blood, a subpopulation of CD4 T_{CM} cells expressing CXC chemokine receptor 5 (CXCR5) is essential for antibody production (Chevalier et al., 2011, Morita et al., 2011, Vinuesa and Cook, 2011). These cells have been referred to as peripheral T follicular helper cells (pTfh) because they share functional features

with germinal centre-Tfh and can help B cells to generate antibodies through promotion of somatic hypermutation and class-switch recombination (Crotty, 2011, Chevalier et al., 2011, Morita et al., 2011, Vinuesa and Cook, 2011). As a standard method in numerous laboratories, flow cytometry plays a pivotal role in identifying T-cell populations in comparison with single-parametric analysis (Chen and Cherian, 2013). Immunohistochemistry (IHC) and immunofluorescence (IF) give outstanding immunophenotyping methods employed to identify the comparative abundance and location of protein biomarkers within fixed cells.

1.4.5 T cell exhaustion

T cell exhaustion has for a long time been defined as a dysfunction and subsequent physical elimination of antigen-specific T cells throughout chronic viral infection in mice (Zajac et al., 1998, Gallimore et al., 1998, Jiang et al., 2015). Accordingly, exhausted T cells may be described as a condition of T-cell dysfunction in a chronic setting leading to reduced cytokine expression and effector function (Jiang et al., 2015). Typically, exhausted T cells demonstrate high degrees of inhibitory receptors such as programmed cell death protein 1 (PD-1), T-cell immunoglobulin domain and mucin domain protein 3 (TIM-3), lymphocyte activation gene 3 protein (LAG-3). Other markers of exhaustion include T-cell immunoglobulin and immunoreceptor tyrosine-founded inhibitory motif domain (TIGIT), band T lymphocyte attenuator (BTLA) and cytotoxic T lymphocyte antigen-4 (CTLA-4) (Barber et al., 2006, Jin et al., 2010, Crawford and Wherry, 2009, Blackburn et al., 2009, Fourcade et al., 2012, Joller et al., 2011). T cell exhaustion has been shown in a broad assortment of animal models and humans having chronic bacterial, viral, and parasitic infections, as well as in human tumours (Virgin et al., 2009, Baitsch et al., 2011). Exhausted T cells have been demonstrated to represent a discrete phase of T cell differentiation. . Two points

are considered to help understand the T cell exhaustion phenomenon. The foremost is the perception that both extrinsic negative regulatory pathways, for instance, immunoregulatory cytokines and cell-intrinsic negative regulatory courses, such as PD-1 play essential functions in exhaustion.

1.4.6 Effector function during T cell exhaustion

The exhausted CD8+ T cells were initially discovered during chronic Lymphocytic Choriomeningitis Virus (LCMV) infection of virus-specific, tetramer-positive CD8+ T cells that do not generate cytokines (Zajac et al., 1998). Functional impairment in the course of the T cell exhaustion has been portrayed to adopt a stepwise pattern (Virgin et al., 2009, Wherry et al., 2003a, Wherry and Ahmed, 2004, Fuller and Zajac, 2003), for instance IL-2 generation, high proliferative capability, and ex vivo killing aspects of memory T cells are the initial set of functions that are impaired. Other qualities, including the capacity to generate Tumour Necrosis Factor (TNF), are frequently lost at more intermediate phases of dysfunction (Wherry, 2011). Severe exhaustion ultimately results in virus-specific cells that partly or, in some situations, fully lacks the capacity to generate good quantities of interferon-γ (IFN-γ), β-chemokines or the capacity to degranulate. The last phase of exhaustion is physical elimination of virus-specific T cells (Moskophldis et al., 1993, Wherry et al., 2003a, Zajac et al., 1998). Severe CD8+ T cell exhaustion corresponds with a high viral load that implies that the potency of activation has some correlation to T cell exhaustion (Jameson and Masopust, 2009). On top of the antigen load, lengthy time of infection or lack of support from CD4+ T cells drives to severe exhaustion (Virgin et al., 2009, Wherry et al., 2003a). Likewise, virus-specific CD4+ T cells have as well demonstrated loss of effector function during chronic viral infection (Brooks et al., 2005, Oxenius et al., 1998, Kaufmann et al., 2007, Urbani et al., 2006). Most likely,

the persistent viral infection might signify a diverse impact on virus-specific CD4+ T cells compared to an effect on CD8+ T cells (Fröhlich et al., 2009, John et al., 2009, Elsaesser et al., 2009, Blackburn and Wherry, 2007).

1.4.7 T cell exhaustion and T cell senescence

Senescence can simply be described as conditions of terminal differentiation and nonresponsiveness, which are significant qualities of numerous biological processes like T cell responses. T cells expressing diminished proliferative potentials can be detected by the expression of the co-inhibitory receptor killer-cell lectin-like receptor G1 (KLRG1) in the mouse and CD57 in humans (Joshi and Kaech, 2008, Brenchley et al., 2003). Several latent infections, like EBV or CMV infections, produce virus-specific T cells that express markers of senescence and terminal differentiation (Van Leeuwen et al., 2006, Moss and Khan, 2004). Diverse biological systems might have differing reasons for cellular senescence, replicative senescence, and terminal differentiation. During the process of T cell reaction to infection, several T cells get terminally differentiated and lose the proliferative ability, in line with a simplistic description of senescence (Wherry, 2011). However, exhausted CD8+ T cells also exhibit defective proliferative capability. Nevertheless, gravely exhausted CD8+ T cells have reduced expression of immunological markers of senescence-like KLRG1 (Wherry et al., 2007). The expression of CD57 is not significantly related to that of PD-1 in HIV infection (Day et al., 2006), even though there is a link between PD-1 and the length of telomere in HIV-infected individuals (Lichterfeld et al., 2008). In most cases, CD8+ T cells expressing markers of senescence such as KLRG1 or CD57 can still actively undertake effector functions, but not exhausted CD8+ T cells (Akbar and Henson, 2011). The transcriptional profiles of repeatedly stimulated CD8+ T cells that have some features of senescence or terminal differentiation also experience

some alterations indicative of T cell exhaustion (Wirth et al., 2010, Hertoghs et al., 2010). Up to date, most findings infer that exhaustion and senescence are discrete mechanistic processes. However, the molecular relationships between exhaustion and senescence has still be definitively elusidated (Akbar and Henson, 2011).

1.5 Cellular components of innate immunity

Innate immunity prevents the admission of microorganisms and swiftly eradicate them if they incidentally gain entry into the host system (Abbas et al., 2012). The key constituents of which are soluble mediators competent of instantly inhibiting alien microbes and specialised cells enriched with detection receptors and the capacity to phagocytize microbes (Coico and Sunshine, 2015). The central cellular mechanisms of innate immunity are the phagocytosis of foreign bodies followed by the intracellular killing of the ingested microbes. Phagocytes discharge lytic substances within the surroundings to mediate intracellular destruction of pathogens (Thompson and Wilton, 1992, Lollini et al., 2006).

Basophils and neutrophils are two cell types termed granulocytes containing intracellular granules (Amulic et al., 2012, King and Wills, 2005). Neutrophils serve a significant role in providing protection against bacterial infections (Conlan and North, 1991), whereas basophils possess an antiparasitic function. Basophils express the αβγ2 type of the high-affinity receptor for IgE (FcεRI) on their surface and can be stimulated to produce different preformed mediators and lipid mediators in addition to cytokines, following crosslinking of FcεRI-bound IgE with bivalent or multivalent antigen (Wedemeyer et al., 2000).

1.5.1 Natural Killer (NK) Cells

Natural killer (NK) cells are nonphagocytic components that destroy virus-infected cells. NK cells constitute part of the innate immune system and possess the capacity to destroy target cells and offer an immediate supply of immunoregulatory cytokines (Cooper et al., 2001a, Robertson and Ritz, 1990).

Human NK cells consist of about 10% - 15% of the entire circulating peripheral blood lymphocytes (Cooper et al., 2001a, Cooper et al., 2001b) and are phenotypically described by the expression of CD56 and absence of CD3 expression (Robertson and Ritz, 1990). Over the past three decades, and with the introduction of monoclonal antibodies (Abs) for particular NK-cell markers, it was reported that two discrete populations of human NK cells could be distinguished by their specific cell-surface density of CD56 (Lanier et al., 1986b, Cooper et al., 2001b). Around 90% of human NK cells show low-density expression of CD56 (CD56dim) in combination with raised levels of CD16 (Fcγ receptor III), expression, whereas about 10% of NK cells express $CD56^{bright}CD16^{dim}$ or CD56brightCD16^{-} (Cooper et al., 2001a). At first, NK cells were functionally identified by their capability to recognise and destroy foreign cells in the host, without needing previous exposure (Campbell et al., 2001, Ribas et al., 2003). The majority of NK cells express the FcR CD16 that could decide Ab-dependent cytotoxicity (Eischen et al., 1996), an additionally recognised function of NK cells (Campbell et al., 2001). Nevertheless, a tiny subset of NK cells do not express CD16 but express higher levels of CD56 and CD94, an attribute that made this group distinct from the majority of NK cells (Campbell et al., 2001). There is evidence to suggest that discrete immunoregulatory functions can be attributed to the CD56bright and CD56dim NK-cell subsets (De Maria et al., 2011). Studies have demonstrated that CD56bright NK cells are the main population accountable for NK

cell cytokine production in response to monokines (Cooper et al., 2001b). These findings endorse a model in which CD56bright and CD56dim NK cells symbolise functionally different subsets of fully developed human NK cells. NK cells are inborn immune effectors that generate immunoregulatory cytokines, for instance, interferon γ (IFN-γ) and granulocyte macrophage–colony-activating factor (GMCSF), crucial to immediate host protection against a diversity of bacterial, viral, and parasitic pathogens (Robertson and Ritz, 1990, Biron et al., 1999, Bancroft, 1993, Scharton-Kersten and Sher, 1997). CD56bright NK cells constitutively express the high to intermediate affinity IL-2 receptors and enlarge in vitro and in vivo in response to picomolar doses of IL-2 (Caligiuri et al., 1990, Baume et al., 1992, Caligiuri et al., 1993). This category of NK cells as well displays the c-kit receptor tyrosine kinase whose ligand improves IL-2–induced proliferation (Matos et al., 1993, Carson et al., 1997). On the contrary, resting CD56dim NK cells convey simply the intermediate affinity IL-2 receptor, and proliferate feebly in response to elevated doses of IL-2 in vitro, still after the introduction of the high-affinity IL-2 receptor (Baume et al., 1992). Resting CD56dim NK cells are severely cytotoxic towards NK-sensitive targets compared to CD56bright NK cells (Nagler et al., 1989). Nevertheless, after stimulation with IL-2 or IL-12, CD56bright cells show comparable or improved cytotoxicity toward NK targets matched with CD56dim cells (Nagler et al., 1989, Ellis and Fisher, 1989, Robertson et al., 1992). NK cell subtypes have distinct natural killer receptor (NKR) repertory. The entire resting CD56bright NK cells display high expression of CD94/NKG2 C-type lectin receptors (Voss et al., 1998). A minor proportion expresses killer cell immunoglobulin-like receptors (André et al., 2000), whereas the majority, accounting for above 85% resting CD56dim NK cells are KIR1 and express low CD94/NKG2. CD56bright NK cells as well express CD62L, an

adhesion molecule L-selectin that mediates first interplays with vascular endothelium (Frey et al., 1998). CD56dim NK cells do not have this receptor but lately have been found to express an NK cell-restricted sulphated lactosamine epitope (PEN5,) that partly mediates the binding of L-selectin (André et al., 2000), therefore signifying the potential for differential transportation of human NK cell subtypes in vivo. Accordingly, CD56bright and CD56dim NK cells vary in their proliferative response to IL-2, adhesion molecule expression, inherent cytotoxic potential, and NKR repertoire (Cooper et al., 2001b).

1.5.2 Macrophages

Macrophages originate from circulating monocytes that migrate the entire organs and tissues of the body bearing organ-specific names, for example, Kupffer cells resident in the liver and tumour associated macrophages (TAMS) in the tumour microenvironment. Other than immunological roles, macrophages play a significant role in the turnover of ageing cell constituents such as erythrocytes that are constantly phagocytosed by splenic macrophages. Identification in natural immunity is expressed by interactions with pathogen-associated molecular pattern expressed by microbes (Gordon, 2002, Takeuchi and Akira, 2010). Pattern recognition receptors refer to the cellular receptors and comprise of mannose receptors (MRs), toll-like receptors (TLRs), and seven-transmembrane spanning receptors (TM7s) (Kokate, 2011, Kawai and Akira, 2010, Akira et al., 2001). TLR recognise bacterial and viral nucleic acids, bacterial peptides, flagellin, lipopolysaccharide (LPS) and other bacterial elements (Akira et al., 2006, Takeda et al., 2003). MRs have the ability to bind carbohydrate moieties on various pathogens including fungi, bacteria, viruses and parasites (Kawai and Akira, 2009, Delves et al., 2011), while TM7 receptors are typically stimulated by

endogenous chemokines or by bacterial peptides (Suresh and Mosser, 2013, Taylor et al., 2005).

1.6 Cytokines

Cytokines are small soluble cell-signalling protein molecules that are produced by cells, particularly the cells of the immune system and are used in the process of cell-to-cell communication (O'Garra and Murphy, 1994, Weaver et al., 2007). These kinds of proteins are capable of changing and modifying the behaviour and features of diverse categories of cells. Even though functions are multifaceted, cytokine expression profiles are extremely applicable parameters of an immune reaction. Diverse cytokines have biological overlapping roles, and they have the capability of regulating the secretion of further cytokines (O'Garra and Murphy, 1994, De Jager et al., 2003). Cytokines encompass lymphokines, chemokines, interleukins (IL), interferons (IFNs), tumour necrosis factor (TNF) but commonly may not comprise the growth factors or hormones (Watford et al., 2003). These cell-signalling molecules are secreted by various types of cells and contribute significantly to several physiological reactions, including immune reactions, inflammatory responses, angiogenesis, apoptosis and cell production (Singh et al., 2014, Kantola et al., 2012, Balkwill and Burke, 1989, Watford et al., 2003). Cytokines perform their roles through receptors, and are particularly critical in the general immune system as they control a state of synergy between humoral as well as cell-based immune responses. They are also responsible for regulating the growth, maturation and receptiveness of specific cell types. Activities of some cytokines can enhance or inhibit the action of other cytokines in complex ways (Zhang et al., 2013). Certain cytokines are biochemical regulators that turn some immune cell categories on and off. They are significant in disease and health, particularly in responding to infectious agents,

immune reactions, inflammation, , sepsis, tumour and in reproduction (Schulte et al., 2013). An example is, Interleukin 2 (IL-2) activates the immune system of the body to produce T cells. This immunity-boosting feature of IL-2 has conventionally made it a potential treatment for numerous types of illnesses (Kierszenbaum and Sztein, 1994).

Recently, cytokine receptors have taken centre stage of more researcher than the cytokines themselves, partially due to their attributes, and because a deficiency of cytokine receptors has been reliably associated with some incapacitating immunodeficiency conditions (Spangler et al., 2015). Since the redundancy, as well as pleiomorphism of cytokines, are, a result of their homologous receptors, it is contemplated that categorising cytokine receptors is likely more clinically valuable. Research indicates that antagonising effects of cytokines are associated with several disease conditions from depression and Alzheimer's disease to cancer with either raised or altered expressions (Herbert and Lucassen, 2015, Fillman et al., 2015). Serum levels of various cytokines may give information on the presence, or even predictive value of inflammatory processes involved in autoimmune diseases (Miller et al., 2009). The expression levels of serum cytokines are progressively being applied in population studies, however, there are insufficient studies that describe the epidemiology of cytokine levels in healthy cohorts (Tsunoda et al., 2003). Cytokines are hormonal passengers responsible for most of the biological effects in the immune system, such as in regulation of cell function in the innate and adaptive immune responses. Measurement of these immune mediators has become increasingly important as a means of understanding immune responses during disease or infections, or in response to therapy and interventions (Biancotto et al., 2013). In a nutshell, cytokines play a prominent part in intercellular communications in inflammatory responses as well as haematopoiesis (Gu et al., 2009b, Biancotto et al.,

2015) while chemokines play a significant part in chemotaxis in conditions such as angiogenesis.

1.6.1 Sources of cytokines

Cytokines are produced by an extensive variety of cells, comprising mainly of immune cells such as macrophages, T lymphocytes, B lymphocytes, as well as mast cells, and not forgetting endothelial cells, fibroblasts, also numerous stromal cells. Individual cells can produce more than one kind of cytokine (Lackie, 2010, Zhang et al., 2013). Nevertheless, T lymphocytes have been recognised as principal producers of cytokines. These cells carry certain antigen receptors on their cell surface to permit identification of external pathogens. Similarly, these can identify normal tissues in episodes of autoimmune diseases. There exist two primary subsets of T lymphocytes, characterised by the appearance of cell surface molecules recognised by CD4 and CD8. Also, T lymphocytes expressing CD4 are identified as helper T (T_H) cells and are considered with the potential of being the most prolific cytokine producers. Furthermore, the TH cells can be classified into T_H1 and T_H2, and the cytokines that they produce are recognised as T_H1-type cytokines as well as T_H2-type cytokines (Dumortier et al., 2013).

1.6.2 Classifications of cytokines

Cytokines are categorised based on their source or according to their function, their range of actions, the group of actions they influence, the cell types they target, or on particular characteristics of their ligand-receptor synergy (Cohen and Cohen, 1996). By cell type as well as a more valuable utility in clinical and experimental practice, immunological cytokines are categorised into those that heighten cellular immune responses, as type 1 as well as ones that support antibody reactions as type 2 cytokines (Ellyard et al., 2007). The T_H1-type cytokines (for example, interleukin

(IL)-12, IL-15, interferon γ (IFN-γ) plus transforming or tumour growth factor-β (TGF-β)) support cellular immune responses and deemed crucial for an efficient response against tumour cells. On the other hand, T_H2-type cytokines (comprising IL-4, IL-5, IL-10, and IL-13) may contain the tumour-specific immune response (Ellyard et al., 2007, Cui and Florholmen, 2008, Kantola et al., 2012). Besides, the T_H1-type cytokines incline to secrete the proinflammatory responses accountable for killing intracellular organisms and for the role of perpetuating autoimmune reactions. The principal T_H1 cytokine is interferon γ (IFNγ). Excessive pro-inflammatory reactions can cause unrestrained tissue harm. Therefore, there is the need to have a counterbalancing mechanism. On the other hand, the T_H2-type cytokines are linked to the elevation of immunoglobulin-E (IgE) and eosinophilic reactions in atopy, and besides IL-10 has more of an anti-inflammatory response. In surplus, T_H2 responses will counter the T_H1 arbitrated microbicidal action. The optimal situation would thus appear to be that persons ought to produce a well-balanced T_H1 and T_H2 response, suitable for the immunologic provocation. Upholding metabolic homoeostasis necessitates a balanced immune response, which is achievable by extracellular signals, as well as cytokines (Dumortier et al., 2013)

By chemical compositions, cytokines can be categorised as peptides, proteins, or glycoproteins. Despite they are plentiful; cytokines can be broadly grouped into proinflammatory and anti-inflammatory cytokines. The proinflammatory cytokines may comprise, for instance, IL-1α, IL-6, IL-8, GM-CSF, GRO-α, MCP-1, TNF-α; and mediators that include IL-1β, IL-33, IFNγ, TGFβ, IL-11, IL-12 and IL-17 as well as IL-20 family. On the other hand, the anti-inflammatory group, for example, IL-4, IL-10, IL-13, IL-35 and other mediators including IL-16, IL-1ra, G-CSF that encourage allergic responses (Li et al., 1998, Mamet et al., 2002). Furthermore, cytokines have

been categorised as lymphokines, interleukins, as well as chemokines, by their supposed role, the cell of secretion, or target of their action. The word interleukin was applied initially by scholars for those cytokines whose alleged targets are mainly leukocytes (Hegazi and Abdel-Rahman).

1.6.3 Expression levels and profiles of cytokine in a variety of human diseases

Measuring cytokine types and levels in a range of human illnesses has been useful in providing understanding of the pathogenesis of infections and disorders, immune responses in the course of disease or response to treatment and interventions (Whiteside, 1994). The elevation and decrease levels of a cytokines can be used as valuable biomarkers to evaluate disease condition, particularly to compare variations in the biomarker to disease situation or treatment (Gu et al., 2009a, Bastarache et al., 2011). For instance, the elevated levels of TNF and IL-6 in serum have been reported in septic compared to nonseptic shock (Pinsky et al., 1993). Besides, the level of IL-6 in serum was observed to correspond to the severity of the disease in septic shock (Damas et al., 1992). Also, raised levels of serum TNF-α and IL-6 have been associated with severe dysfunctions of body organ in human Plasmodium falciparum (P. falciparum) malaria. Therefore, disparities in the cytokine system in untreated P. falciparum infection serve as markers of the severity of disease (Kern et al., 1989).

In colorectal cancer, higher levels of serum IL-6 have been reported in patients whose tumours were more than 5.0 cm in width or that had metastasized in a circumferential manner. Higher levels of Serum IL-6 and IL-8 were described in patients who had liver metastasis as compared to ones with no liver metastasis. Similarly, higher serum levels of both cytokines were seen in patients with lung metastasis than in patients with no lung metastasis. These findings suggest that IL-6 and IL-8 might have a

significant role in the haematogenous metastasis of colorectal cancer (Ueda et al., 1994).

A comprehensive analysis of serum cytokine expression to help in the development of predictive panels for diagnosis, staging and the process of observing cancer vaccine trials has associated serum IL -6 with poor survival rate in patients that have hormone-refractory metastatic breast cancer (Bachelot et al., 2003).

Colorectal cancer (CRC) has been linked to extensive modifications in serum cytokine expression, stressing the significance of studying comparative cytokine level alterations (Kantola et al., 2012). The expression of serum cytokine levels provides a promise in splitting CRC patients from healthy controls but then again its clinical importance is yet to be established. Likewise, a study proposes that high serum concentrations of IL-6, IL-10, and IL-12 in people who are of African Americans origin and IL-6 and TNF-α in Caucasians are linked with a prognosis of lung cancer. It established that IL-6 associated with good life in both populations that reinforces the indication for the role of IL-6 in the prognosis of lung cancer (Enewold et al., 2009). Certainly, the profile of serum cytokine has been valuable as an analytical and predictive tool in ovarian cancer. This research group established that a mixture of IL-7 and CA-125 serum levels was likely to predict 69% of the ovarian cancer patients in an accurate manner, with no false classification of patients with the help of benign pelvic mass. Besides, IL-6, IL-7, IL-8, IL-10, monocyte chemotactic protein-1 (MCP-1), and IP-10 and CA-125 were related with disease-free as well as overall survival in univariate analysis. On the other hand, in multivariate analysis, IL-7 and IP-10 were autonomous predictors of overall survival, even though after including the clinicopathological features, merely stage as well as the residual disease continued to be the independent forecasters of the rates of survival. This study demonstrated that

IL-7 levels were established to be intensely connected with ovarian cancer and might be applied in combination with CA-125 to differentiate amid malignant and benign ovarian tumours (Lambeck et al., 2007).

In a research study via the use of human pancreatic cancer cell lines, pro-inflammatory cytokines, IL-6 and IL-8 concentrations in serum were raised in comparison with negative controls that could hypothetically facilitate systemic inflammatory response (Wigmore et al., 2002). Unfortunately, the overall survival of patients with pancreatic ductal adenocarcinoma remains remarkably inferior. Therefore, the need to identify prognostic markers is urgent. To this effect, Torres, Carolina; at al undertook an extensive analysis of serum prognosis biomarkers using an antibody array comprising 507 human cytokines, and a panel of serum cytokines including B7-1/CD80, EG-VEGF/PK1, IL-29, NRG1-β1/HRG1-β1, and PD-ECGF showed correlation to poor prognosis for patients with pancreatic cancer (Torres et al., 2015). Moreover, it has been shown that serum cytokine profiles were potentially valuable biomarkers in the differentiation of pancreatic ductal adenocarcinoma (PDAC) from patients suffering from a benign pancreatic disease (Shaw et al., 2014).

The majority of the cytokines long-established to date are to some extent thought to relate to cancer biology including cancer immunity and cancer progression (Tazaki et al., 2011). It has been reported that abnormally unbalanced production of cytokines was related to the pathophysiology of prostate cancer (Pca), and cytokine profiles in Pca patients were distinct according to the stage of the disease. IL-1β and IL-12 have indicated a promise as early diagnostic pointers of Pca (Tazaki et al., 2011). Increased levels of serum IL-4, IL-10 and high-sensitivity C-reactive protein (hs-CRP) have been found to associate with persistent or recurrent disease in papillary

thyroid carcinoma (PTC) and PTC with Hashimoto's thyroiditis (HT) patients. This information can be helpful in improving patient stratification according to the risk of recurrence, especially in patients with PTC plus HT, where thyroglobulin (Tg) levels are not reliable due to the presence of antithyroglobulin antibodies (TgAb) (Stanciu et al., 2015). Cytokine expression levels have also been studied in head and neck cancer to help understand the pathogenicity and prove useful as biomarkers or targets for therapy for head and neck squamous cell carcinoma (HNSCC). The serum concentrations of IL-8 and vascular endothelial growth factor (VEGF) were found to correlate weakly with large primary tumour volume in HNSCC. Raised IL-1- and IL-6-inducible acute-phase reactions were also noticed in cancer patients but this was not the case in patients that were suffering from papilloma or control individuals (at $P < 0.05$). This research demonstrated that cytokines that are significant in proinflammatory, as well as proangiogenic reactions, are noticeable in cell lines, tissue specimens, as well as serum from HNSCC patients (Chen et al., 1999).

Gupta et al. undertook a case-control study using pretreatment serum samples from 68 newly diagnosed T-cell neoplasms patients and 14 healthy controls. He analysed the expression pattern of thirty cytokines and compared findings with clinical end points (Gupta et al., 2015). Epidermal growth factor (EGF), IL-6, IL-12, interferon γ-induced protein (IP)-10, soluble interleukin (sIL)-2Rα, monokine induced by γ interferon (MIG), and IL-1RA were significantly elevated in all T-cell neoplasms compared to the controls (Gupta et al., 2015). Raised levels of epidermal growth factor were predictive of a poor event-free survival; IL-1RA, sIL-2Rα, and monokine induced by γ interferon (MIG predicted a poor overall survival (Gupta et al., 2015).

1.6.4 Serum Cytokines in Follicular lymphoma

The impact of cytokines in lymphoma biology appears to be complex (Kimby, 2015). A subtype of B lymphoma cells express the IL-2R, and IL-2 is a precocious homeostatic cytokine necessary for growth, development, as well as the activity of regulatory T cells (Kimby, 2015). IL-2 is likewise essential for the growth of cytotoxic T-cell function and has been applied for the systemic immune treatment to increase immunity against tumour (Kimby, 2015). However, a variety of cytokines and growth factors, such as TNF-α, transforming growth factor-β (TGF-β), hepatocyte growth factor (HGF), epidermal growth factor (EGF), etc., have been implicated in tumour–stroma cross-talk (Singh et al., 2014).

Due to high variability in the progression of follicular lymphoma (FL) that resulted in several treatment options, identifying reliable markers that can help streamline cancer type and for predicting outcome are undoubtedly demanding. Happily, serum cytokines and chemokines have been shown to reflect tumour biology and host response in FL, demonstrating the prognostic value of cytokines (Mir et al., 2015, Kimby, 2015). The action of the primary cells in the tumour microenvironment, as well as tumour-reactive T cells, follicular dendritic cells (FDCs), also macrophages, are likely to be reflected by the state of serum cytokines (Mir et al., 2015, Labidi et al., 2010). The overall "cytokine milieu" can be of significance for T cell–mediated antitumour protection.

Recently, an enzyme-linked immunosorbent evaluation (Multiplex) was used by the group of Mir et al., and they established that raised levels of IL-2R, IL-1R1, and CXCL9 are related with shorter event-free survival (EFS) in FL patients on chemotherapy or chemoimmunotherapy. Meanwhile, IL-1R1 and IL-12 were linked

with a shorter EFS in patients in a wait-and-watch cohort, and a similar outcome in patients who were treated with rituximab single-therapy (Mir et al., 2015). In this research, serum levels of IL-1R1, IL-6, IL-7, IL-10, IL-13, TNF-α, vascular endothelial growth factor (VEGF), and platelet-derived growth factor (PDGF) were raised in 60 FL patients in comparison with controls (Mir et al., 2015). Early stage and high TGF-β levels have been identified as independent predictors of improved overall survival in FL, while high lactate dehydrogenase (LDH) and VEGF levels were independently associated with poorer progression-free survival (Labidi et al., 2010). These serum factors seem independent of the FL international prognostic index (FLIPI), a validated prognosticator, and might be of great clinical impact, especially as they are readily available in the blood (Kimby, 2015). Furthermore, it has been shown that elevated soluble IL-2R levels before treatment were associated with reduced survival in FL patients (Yang et al., 2011). It has also been reported that IL-12 and IL-1R1 are predictive for poor outcome in FL patients who are initially observed (or getting rituximab monotherapy) (Yang et al., 2011).

Herewith, evaluating cytokine expression levels and profiles in FL, and understanding how they relate to disease biology and other clinicopathological features of patients will undoubtedly impact positively on the treatment and management of FL.

1.7 Cytomegalovirus (CMV)

Herpes viruses are big, sophisticated viruses that comprise of double-stranded deoxyribonucleic acid (DNA). More than 100 herpesviruses are identified, but then again only eight passes on a disease to humans (Berman, 2011). The human herpesvirus (HHV) family is classified into three subfamilies grounded in the period of viral reproduction cycle, capacity for cell culture growth, as well as the site of viral

dormancy in the human body. The subfamilies are α-herpes virinae encompasses the simplex viruses (HSV-1 and HSV-2) as well as varicella virus (VZV), whereas β-herpesvirinae comprises of cytomegalovirus (CMV) in and Roseolovirus (HHV-6 and HHV-7). The γ-herpesvirinae comprises of lymphocryptovirus(Epstein-Barr virus, or EBV) plus Kaposi's sarcoma-associated herpesvirus (HHV-8) (Berman, 2011).

CMV is a species of β-herpesvirus (Harari et al., 2004, Emery, 2001, Chiche et al., 2012). It infects nearly all human beings and typically leads an asymptomatic chronic course in a large set of an estimated 40 -100% of the global human population (Papadakis et al., 2001, Khan et al., 2002, Gandhi and Khanna, 2004, Ludwig and Hengel, 2009, Mustakangas et al., 2000). In immunocompetent persons, CMV infection is frequently chronically asymptomatic and infrequently leads to a light mononucleosis-like set of symptoms, it continues to be an essential source of deadly diseases of different organs like the liver, the lung, the retina, and the CNS (central nervous system) among immune-deficient hosts(Almanzar et al., 2005). Beneficiaries of allogeneic stem cell and bone marrow transplantation (Mori et al., 2004, Kotton, 2010), HIV positive individuals, new-borns that are infected by birth (Khan et al., 2002); cancer patients, and patients on the course of chemotherapy and corticosteroids (Vega et al., 1999) are listed in the high risk group.

A wide-ranging meta-analytical assessment of severe CMV infection in immunocompetent grown-ups established that CMV infection usually involves gastrointestinal tract (mainly colitis), then the CNS (as well as meningitis, encephalitis plus myelitis) and at that point haematological abnormalities (Lancini et al., 2014). CMV illness of the eye, liver, lung and vasculature were also recognised, in the middle of other diseases. Research Studies have discovered that the occurrence of severe exhibitions of CMV infection in immunocompetent persons seemed to be

meaningfully more prevalent than previously understood (Lancini et al., 2014). Besides, a higher risk of CMV-related disease and death rates in “immunocompetent” patients that are critically ill has been documented (Lancini et al., 2014). It is consequently highly possible that incomplete immune dysfunction might signify a presently ignored risk factor for severe CMV disease.

While clinical indicators of CMV infection in immunosuppressed patients might include asymptomatic viraemia, CMV syndrome (viraemia with symptoms that comprise of fever and malaise) as well as tissue-invasive sickness like, colitis, retinitis, pneumonitis, hepatitis, and disease in other locations (Kotton, 2010); the nonexistence of clinical manifestation, mainly among immunocompetent persons credited to the fact that CMV-specific CD8+ T cells, most of which possess specificity for the immediate-early protein 1 (IE-1) and the structural Tegument Protein pp65 (ppUL83), can regulate the virus by stopping its replication as well as eliminating cells that harbour the virus (Almanzar et al., 2005).

After the first infection, this βherpesvirus is not eliminated but starts a life-long viral perseverance in its host (Chiche et al., 2012, Libard et al., 2014). CMV starts to spread and eventually grows dormant in manifold end organs; this situation is known as “latency", nevertheless, endogenous resurgence can take place at in the course of time in circumstances that undermine immune function like in inflammation and immunosuppression (Cook, 2007, Goodman et al., 2015, Chiche et al., 2012). For example, it has been established that during a severe illness, and precisely in sepsis, CMV is reenergized in approximately 30% of latently infected persons (Cook, 2007). Information also proposes that immunocompromised in the type of NK cell dysfunction has the possibility of setting the platform for viral recurrence. NK cell

action is essential to viral restraint following acute infection and control for a resurgence (Amadei et al., 2010).

1.7.1 Pathophysiology of CMV

The pathological trademark of CMV infection in an individual is the presence of a giant cell full of viral bodies (Varani and Landini, 2011). Also, cytomegaly is observed in infections due to other β-herpesvirinae superfamily viruses. The microscopic characteristics of these enlarged cells are commonly described as the" owl eyes". Though these may be regarded as diagnostic, such histologic findings may be insignificant or even nonexistent in infected organs. During CMV infection, the polymerase chain reaction (PCR) is employed to detect CMV DNA in various cells of the body organs (A Ross et al., 2011). At the first stage of infection, the epithelial cells of the salivary glands are infected leading to the viral persistence in the body and viral exuviating (Gerna et al., 2004). However, when the infection goes to the genitourinary system, it causes insignificant viruria. Nonetheless, the virus can resist treatment and still continue multiplying to various parts of the body of the host. Nevertheless, this can lead to other complications such as kidney failure and the renal dysfunction, though it is not common unless for the patients who have had a renal and kidney transplant. Such patients show few cases of glomerulopathy as well as chances of graft rejection (De Souza and Olsburgh, 2008).

1.7.2 The immunology of CMV Infection

The primary CMV infection is commonly referred to patients who before CMV infection had been CMV seronegative (Ljungman et al., 2002). Such patients express detectable CMV immunoglobulin M (IgM) antibodies as early as four to seven weeks following the first infection. The IgM viral antibodies produced can persist for some

time such as 16 to 20 weeks. The neutralising antibodies neutralise the enclosed glycoprotein B (gB). It has been reported that over 50% of this neutralising process in patient serum is directed towards the glycoprotein B (gB). The viral tegument proteins including pp150, pp28, as well as pp65 elicit powerful and long-lasting antibody responses. CMV is a virus which weakens the immune system of individual exacerbating hidden immune diseases. The CMV-seropositive healthy people express CMV DNAenemia as well as viruria. During this point, the natural immunity is not able to stop reinfection (Walter et al., 1995). Cell-mediated immunity (CMI) is considered the most important factor in controlling CMV infection. Patients deficient in CMI are at greatest risk for CMV disease and reactivation (Kumar et al., 2009). Essentially, the immune protection can be handled by the help of CMV-specific CD4+ and CD8+ lymphocytes following primary infection. It has been documented in bone marrow transplantation experiments that individuals who did not develop CMV-specific CD4+ or CD8+ cells following transplantation of bone marrow are at greater risk of contracting CMV pneumonitis. However, patients that received allogeneic marrow transplant with CMV-specific CD8+ cells infusion did not present with CMV pneumonia (Bonkowsky et al., 1975).

1.7.3 The Influence of CMV infection on Normal Immunity

Various chronic illnesses and impaired immune responses are linked to the CMV infection (Alonso Arias et al., 2013). The latent CMV infection expedites age-associated alterations in the T cell repertory in the aged people, particularly those who are using drugs that suppress the immunity (Welzl et al., 2014). The CMV-infected HSCT patients have high chances of contracting bacterial and fungal infections due to the additional suppressive adverse effects of drug leading to tremendous immune impairment. Similarly, CMV infection continues to exert significant problem in

beneficiaries of haematopoietic stem cell transplantation (HSCT) (Holtappels et al., 2008). Also, CMV infection is implicated in the pathogenesis of the cardiovascular diseases, immunosenescence, as well as cancer (Ohlin and Söderberg-Nauclér, 2015). Because CMV evokes a powerful antigenic response in immunologically unimpaired persons, which increases with increasing age (Freeman Jr, 2009), the virus has developed many ways to suppress as well as escape this response to remain latent. Various sources of evidence show that the mechanisms deployed by CMV to evade the host's immune response are by downregulating the inherent and acquired parts of the immune system (Freeman Jr, 2009). For example, CMV reduces the expression of the HLA classes I and II antigens on the APCs (Söderberg-Nauclér, 2006) and on T cells (Fletcher et al., 1998). It later yields an HLA class I homologue which impedes the process of antigen presentation by the infected cells to prevent it from being recognised by the NK as well as the T cells (Reyburn et al., 1997). Moreover, the virus also produces various proteins which particularly antagonise the delivery of peptides by APCs hence reducing the activation of the CMV-specific T-cell reactions (Ahn et al., 1996). Various studies have revealed that CMV may directly repress proliferation T-cells that are active (Schrier et al., 1986) that increases their presentation PD-1, which influences the apoptosis of the T-cells that are stimulated. Consequently, this minimises their capacity to produce the IL-2 as well as INF-c (Sester et al., 2008). Another growing body of evidence suggests that the CMV does the suppression of the immunity of the host through stimulation of the Fc receptors on infected cell surfaces through the production of their Fc receptor homolog (Rahman et al., 1976, Atalay et al., 2002). The receptors then attract the circulating immunoglobulin G (IgG) to attach to the infected cells, causing the virally infected cells to evade binding to the immunoglobulins directed against other viral proteins

carried on the infected cell surface. This can potentially decrease the binding process along with the CMV's induction of the patient's complement inhibitors (Spiller et al., 1997). Also, CMV expresses various chemokine receptors homologues which bind the local MCP-1 as well as MIP-1α chemokines that reduce the concentration of the chemoattractant in the microenvironment. As a result, it affects the attraction of inflammatory cells to CMV vicinity (Neote et al., 1993). Moreover, CMV infection interrupts the macrophage cytoskeletal structures and increases the production of the macrophage migration inhibitory factor (MMIF) that consequently reduces the trafficking of the macrophages to blunt the natural immune reaction (Frascaroli et al., 2009).

The combination of all these CMV-arbitrated reactions will potentially lead to nonspecific inhibition of the cell-mediated and antibody-mediated immune responses. Data from in vitro studies indicate that CMV may engage some other mechanisms which make the host vulnerable to other diseases. The reduced expression of HLA impedes antigen presentation to decrease the host cellular response to pathogenic challenges. On the other hand, increased Fc receptors and homologues bind circulating immunoglobulin, which reduce the ability of the humoral immune systems to identify and attack other pathogenic antigens. The ability to repress complement as well as chemotaxis and macrophage migration implies that CMV is likely to intervene with far-broad aspects of the humoral immune system. Previous studies have provided possible reasons for increased chances of fungal and bacterial infection among patients with CMV viraemia and as well as in kidney and liver transplant patients. Another study indicates that the immunosuppressive feature of CMV occurs due to offensive episodes of HCV among HCV-infected liver transplant beneficiaries who had records of CMV infections (Falagas et al., 1996). By implication, patients with

CMV infections have had abnormal antiviral responses to other viruses different from the CMV. Also, the study states that the active CMV infection can exercise an immunosuppressive influence which makes the patients highly vulnerable to other infectious agents. A meta-analysis shows that prophylaxis on the primary CMV infection leads to a lasting survival (Hodson et al., 2013) as well as reducing the incidence of secondary infections (Snydman, 2006). The is assumed to be achieved by limiting CMV primary infection and minimise the consequences of CMV on the immune system as well as reducing the expedition of inflammatory responses by dividing CMV (Freeman Jr, 2009).

1.7.4 Human cytomegalovirus (HCMV) and tumours

The pathophysiology of quite many tumours including colorectal cancer, primary intracerebral tumours, non-melanoma skin carcinomas, intracerebral tumours, prostate cancer, and neuroblastoma have been linked to HCMV (Cobbs et al., 2002, Harkins et al., 2002, Samanta et al., 2003, Zafiropoulos et al., 2003, Sehic et al., 2013, Wolmer-Solberg et al., 2013). The relationship between HCMV protein expression and primary non-curable brain tumours like glioblastoma (GBM) has been demonstrated (Baryawno et al., 2011, Scheurer et al., 2008, Cobbs et al., 2002). Despite this growing body of evidence, however, the effects of HCMV protein expression have been investigated only in a few tumour types such as glioblastoma (Scheurer et al., 2008, Cobbs et al., 2002, Lau et al., 2005, Sabatier et al., 2005).

Recent studies have reported that by treating for HCMV infection results in reduced glioblastoma, although it is not statistically significant (Stragliotto et al., 2013). Consequent to the findings and the increased rate of the occurrence of the HCMV protein expression in glioblastoma, HCMV is considered a therapeutic target despite only a progress associated impact is accomplished. Therefore, the modern studies are

including the antiviral therapies as a paired treatment for glioblastoma (Stragliotto et al., 2013, Rahbar et al., 2013, Hadaczek et al., 2013).

The HCMV virion consists of the three main structural units just like other herpes viruses. The three units include the envelope, capsid, and tegument. The tegument unit connects the envelope to capsid and contains regulatory proteins, pp65 and pp71 that passed into the host cell in the course of infection by the virus (Libard et al., 2014). It has been shown that pp65 is the most common virion protein and plays immunomodulatory functions (Libard et al., 2014). Also, it contains the dense bodies which are non-infectious viral particles which accumulate during the infection (Becke et al., 2010, Landolfo et al., 2003). However, pp71 is known as a transcriptional transactivator. HCMV resides in the monocytes during the latent stage of an infection, and the virions are not yet produced. At this point, the viral DNA divides in proximity to the host DNA through host-to-cell replication machinery. Sensitive techniques like polymerase chain reaction (PCR) can be applied to detect the HCMV DNA at this stage. However, during the active stage of infection, the virions are released leading to the presence of viral elements as intranuclear inclusions in the infected cells, known as the owl eyes (Trgovcich et al., 2006).

Immunohistochemical techniques (IHC) are efficient for detecting the HCMV proteins in the various human cellular compartments (Landolfo et al., 2003). The components of tegument and envelope of the virion are some of the HCMV viral proteins that have been studied. These proteins are exhibited at various stages of the HCMV cycle. The cycle and proteins expressed may include the immediate-early 1 (IE1), immediate-early (IE), early - envelope glycoprotein GP48, early/late-protein polymerase processivity factor (p52) and finally the late - tegument protein (pp65) (Cobbs et al., 2002, Scheurer et al., 2008, Mitchell et al., 2008, Slinger et al., 2010).

The HCMV proteins are reported to be connected to many activities which are essential for tumour advancement (Libard et al., 2014). They are said to regulate immunosuppression, the telomerase activation, chronic inflammatory environment, angiogenesis, cellular motility and invasion, anti-apoptotic, as well as cell-cycle modulation (Dziurzynski et al., 2012, Soroceanu et al., 2011, Michelson, 1999, Michaelis et al., 2004, Michaelis et al., 2009). Evidently, there have been no cases of the low levels of HCMV proteins in regions of necrosis in tumours, healthy brain tissues next to tumour; brain tissues collected from controls or people suffering from several neurodegenerative diseases (Landolfo et al., 2003, Soroceanu et al., 2011). By implication, the HMVC proteins may be essential to the tumour induction or growth.

1.7.5 Diagnosis of CMV

Based on the pathogenesis of CMV and the need for effective control of the patients, it is important to implement a diagnosis protocol which can differentiate between the CMV invasive disease as well as latent CMV infection. There are various methods of diagnostic tests for CMV. However, there have been various limitations regarding these tests. The serological method gives the impression of the pre-exposure to the CMV, and testing for the active disease may require the quantitative nucleic acid testing (QNAT), antigenaemia, histopathological examinations, as well as culture. The immunological analysis they are meant to reveal the cellular immune response to the CMV (Lancini et al., 2014).

CMV IgG status and IgG avidity can be determined serologically to assist in differentiating between the primary and secondary CMV infections. The IgM antibody will only be positive for approximately 12 months following the initial infection (Goodman et al., 2015, Ross et al., 2011). hence the suggested use of the anti-CMV IgG as well as improved specificity . Appraised immunological methods

that can be employed to diagnose CMV IgG antibodies may include radioimmunoassay (RIA), indirect haemagglutination (IHA), complement fixation (CF), enzyme-linked immunosorbent evaluation (ELISA), and immunofluorescence (IF) (Ross et al., 2011). False negative results of CMV serological assay such as low immunoglobulin status and plasmapheresis, and transfusion of blood products can lead to the false positive. Therefore prior transfusion samples can be more reliable. Nevertheless, active HCMV disease tends to elude both IgG and IgM serology and are not as such effective in the detecting the active CMV disease (Lancini et al., 2014). The antigenaemia test or the QNAT have been employed to determine CMV viraemia. By principle, this test is based on the use of monoclonal antibodies which detect for the viral pp65 antigen which is structurally late protein that is expressed in the leukocytes in the first phase of the CMV replication cycle (Ross et al., 2011). Testing for CMV pp65 antigen gives a semiquantitative assessment which has been instrumental in the commencement of preventive treatment, disease diagnosis and monitoring of the patient's response to treatment (Lancini et al., 2014). This method is relatively easier to perform as it does not need expensive tools though it lacks test standardisation.

Therefore, the CMV QNAT or CMV viral load testing can be an option for testing the antigenaemia. This type of test needs expensive tools as well as skilled professionals. The viral load changes profusely between plasma and the whole blood samples and CMV DNA is detectable earlier and ordinarily higher quantities in the blood. It is, therefore, advisable that one type of specimen used when monitoring treatment progress in follow-up patients. The QNAT data can differ in various settings because of the variation in the methods of extracting nucleic acid and test designs.

The choice of assay category can be affected by the availability of resources, technical speciality, and the number of patients, turnabout time, and the number of samples as well as the expenses to be incurred. Antigenaemia and QNAT viral load tests have both given great results that are applicable in clinical practice. Nevertheless, the relationship between QNAT viral load and CMV antigenaemia levels has not been compatible. A study shows that even other tissues, as well as body fluids such as biopsies, bronchoalveolar lavage and CSF specimens, can be tested for CMV by the QNAT since it enhances the sensitivity and possibly with quicker results compared to the traditional culture method.

The conventional technique for the detection of CMV infection is via conventional cell culture (Ross et al., 2011). The process requires clinical specimen inoculation onto human fibroblast, which is then incubated and observed for 2 to 21 days. This method may seem slow, costly and associated with low sensitivity. Also, seropositive individuals may pass CMV via their bodily secretions, particularly during periods of stress, thus yielding positive cultures, do not, in reality, translate to an active disease phase. Blood viral culture for CMV is known to have inferior sensitivity, whilst cultures of CMV sputum, urine and stool cultures are associated with reduced specificity. The culture of a specimen from tissues remains the appropriate options for the diagnosis of diseases that are tissue invasive, especially for samples from the gastrointestinal tract, where PCR or antigenaemia testing on blood may fail to be positive, even in the presence of invasive disease. To boost assay sensitivity, Immunohistochemistry should typically be conducted on every biopsy specimen during cases where CMV infection is suspected. Detection of viral antigens or inclusion bodies in bronchoalveolar lavage or biopsy tissues is highly specific for CMV infection, particularly with a positive culture. CMV infections that are tissue

invasive, for instance, hepatitis or colitis is supposed to be confirmed via immunohistochemistry or through in situ DNA hybridization. Immunologic techniques represent an emerging technology, which will potentially offer personalised prophylaxis and treatment of CMV.

1.7.6 Treatment Options for CMV infection

Therapy for CMV infection may involve antiviral treatment, which can be followed by prophylaxis or monitoring; occasional application of CMV immunoglobulin and reducing immunosuppression.

1.7.6.1 Antiviral therapy

Typically, ganciclovir forms the first-line anti-CMV therapy. Nonetheless, its associated low oral bioavailability restricts its application and thus intravenous treatment has been made compulsory. Valganciclovir represents a prodrug of ganciclovir with over 60% bioavailability and is now a substitute to intravenous ganciclovir. Valganciclovir has increasingly continued to be the drug of choice for treating recipients of solid organ transplant (SOT) having a mild-to-moderate CMV infection. Studies have shown that CMV oral valganciclovir is no inferior to intravenous ganciclovir for the therapy of CMV infection. Regarding patients suffering from severe CMV infections, intravenous ganciclovir remains the drug of choice since there is no documentation of oral treatment as an equivalent therapeutic intervention. It is supposed to be used in patients who fail to tolerate oral therapy or have demonstrated suboptimal valganciclovir absorption. Secondary prophylaxis using antivirals for one to three months following treatment differs significantly with respect to transplant centres. Nonetheless, it is often used particularly when there is a high relapse risk. Close clinical as well as microbiologic check-ups within the initial few months following therapy is still an intervention that is often adopted.

1.7.6.2 Reduction of the immunosuppression

Immunosuppression reduction can be adopted in cases of severe CMV infections, with increased viral loads in diseases that are not responding to treatment and with leukopenia. It may also be appropriate in cases of CMV infections that are less severe. Data on the impact of the intensity of immunosuppressive therapy on the result of CMV infection treatment in recipients of organ transplant tends to show that better early elimination of viral load took place with dual versus triple immunosuppressive treatment, reduce levels of calcineurin inhibitors in blood, and a longer time after transplantation.

1.7.6.3 CMV immunoglobulin

The activity of CMV immunoglobulin in the therapy of CMV infections has not yet fully elucidated. Rather, at times, it is applied as a concomitant therapy for serious CMV infections like pneumonitis and with a virus that is resistant. Adoptive infusions of T-cells that are specific for CMV, even though it is still being tested, may seem promising in some circumstances.

The primary methods of preventing CMV infection, which involve preemptive therapy and universal prophylaxis, are known to lower the CMV infection risk, even though each method has its merits and demerits. Late CMV is mainly detected in individuals undertaking universal prophylaxis. This is suggestive of the notion that CMV may be delayed efficiently than prevented, particularly during cases of primary infection. Research findings reveal that NHL including FL patients under rituximab therapy within one year prior to autologous HSCT have an elevated risk of CMV infections following HSCT (Lee et al., 2008). Anti-CMV drugs like ganciclovir and immunoglobulins that are specific for CMV may serve as efficient therapy for CMV

infections in FL patients under rituximab therapy and autologous HSCT (Lee et al., 2008).

1.8 Study Aims and Objective

The overall aim of this study is to appraise the impact of CMV infection on FL biology. As a result, four primary objectives are set out to achieve the aim of the project: 1. Assess the impact of CMV infection on the quantities of lymphocyte and NK cell subsets in circulating blood of FL patients by flow cytometry.

2. Evaluate the effect of chronic CMV infection on serum cytokine levels and profiles of the study cohort using Luminex assay

3. Examine the intensities of accessory infiltrating cells in the FL microenvironment using dual IF staining of tissue sections from FFPE tissue biopsies.

4. Undertake an explorative analysis relating lab data to clinical data and evaluating the impact of CMV infection on drug toxicity, response and infection status.

1.9 Study Rationale

CMV infection has been implicated in impairing the immune response, expedited immune senescence as well as in different chronic ailments (Almanzar et al., 2005, Alonso Arias et al., 2013). Nevertheless, latent infection with CMV leads to an accelerated age-associated variations in the repertoire of T-cells in immunosuppressed elderly individuals (Welzl et al., 2014). Additionally, it has also been demonstrated that some high grade chronic viral infections such as lymphatic choriomeningitis virus (LCMV), adenovirus, polyomavirus, HIV, HBV, HCV, Friend leukaemia virus as well as malignancies lead to exhaustion of T-cells. T cell exhaustion is characterised by T-cell dysfunction demonstrated by the inability of T cells to display the typical

array of effector functions (robust, protective) and memory T-cell populations (Wherry, 2011, Yi et al., 2010). As a cohort of FL patients will be CMV-positive elucidating the effect of CMV infection on T-cells and the subsequent effect on FL may offer insight into the disease, which will consequently lead to enhanced patient management.

1.10 Study Hypothesis

The hypothesis being tested is that CMV infection alters immune function in follicular lymphoma in two ways:

1. CMV infection induces premature immune senescence, that would increase the risk of infection following immunosuppressive chemo-immunotherapy; and

2. CMV infection alters the composition of the tumour microenvironment and would modulate the behaviour of the lymphoma and/or its response to therapy.

Chapter 2 : Research Design & Methodologies

2.1 The Study Design

This chapter provides a general overview of the study design, approach, materials and laboratory methodologies employed to test the study hypotheses as well as to answer the research questions. It also presents highlights on the patient recruitment and selections of the study cohort, sample types, research consent and ethical considerations. Furthermore, it includes the optimisation of protocols and statistical approaches.

The study design as depicted in Figure 2-1 comprised of five complementary research strands. Chapter 3 of the thesis, which is the first result chapter, explored the quantities of T- and NK- cell populations and subpopulations using stored peripheral blood mononuclear cells (PBMCs) from a small cohort of PACIFICO trial patients. A multicolour flow cytometric analysis was employed to undertake immunophenotyping of circulating peripheral T and NK cells. The second result chapter presented in Chapter 4 looked at the levels and patterns of serum cytokines using pre-treatment stored serum samples.

The third result chapter (Chapter 5) used immunofluorescence (IF) to analyse cells within the tissue microenvironment of FL. Immunohistochemistry (IHC) was used to optimise for the working dilutions of the primary antibodies. The fourth result chapter (Chapter 6) presents an explorative statistical analysis of the effect of CMV infection on treatment efficacy and toxicity in patients. The last result chapter (Chapter 7) explores the relationship between laboratory variables and the treatment response data.

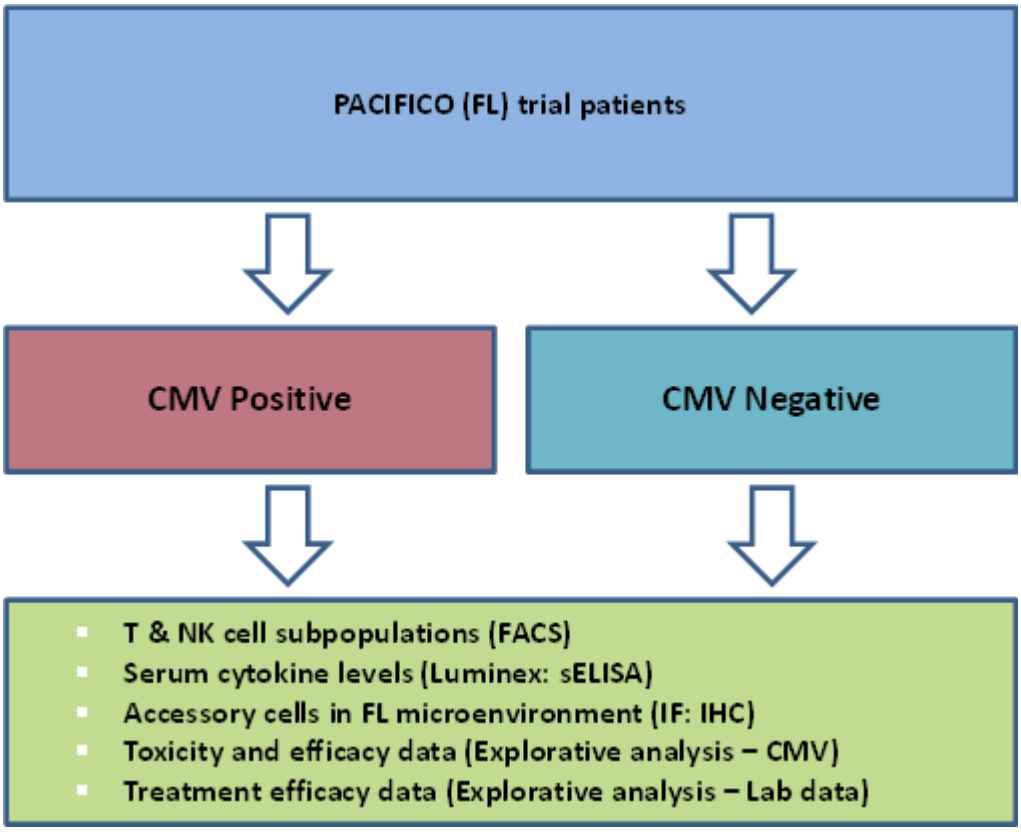


Figure 2-1: Study design.

The design was set to evaluate the impact of CMV infection on the biology of FL. The cohort of patients, source of samples and clinical data used in this study is represented on the upper bar. The study evaluated CMV-positive and CMV-negative FL patients illustrated by the two arrows pointing to the separate two blocks below the study patients. The bottom box shows the laboratory methods and clinical analysis employed to test the hypothesis. These include flow cytometric analysis (FACS), Luminex assay (correlated with sandwich ELISA (sELISA) and immunofluorescence (IF) using immunohistochemistry (IHC) to determine working volumes of monoclonal antibodies (mAbs). Furthermore, the relationships between clinical data and CMV infection status or laboratory data were explored.

2.2 The study Cohorts and patient samples

The project benefited from stored pre-treatment samples from patients recruited into the National Cancer Research Institute (NCRI) PACIFICO trial (Purine-Alkylator Combination In Follicular lymphoma Immuno-Chemotherapy for Older patients).

This is a two-arm, open label, multi-centre, parallel group phase 3 randomised clinical trial. The trial targeted patients with previously untreated follicular lymphoma aged 60 years or more and compared two alternative chemo-immunotherapy induction regimens: R-CVP (rituximab, cyclophosphamide, vincristine and prednisolone) versus R-FC (rituximab, fludarabine and cyclophosphamide). Patients who achieved a complete or partial remission then received 2 years of maintenance treatment with rituximab. The co-primary endpoints were progression-free survival (PFS) and infection ≥grade3. Secondary endpoints included response, survival and toxicity which was defined and quantified in accordance with the Common Terminology Criteria for Adverse Events (CTCAE) version 4.0. The study was approved by the National Cancer Research Institute (NCRI) Lymphoma Clinical Studies Group (CSG), Cancer Research UK, the Medicines and Healthcare Regulatory Agency (MHRA), Liverpool Adult Research Ethics Committee (LREC), and local NHS Research and Development departments. Central running costs were covered by Cancer Research UK with contributions from Roche and Chugai, while local research costs were covered by the National Institute for Health Research (NIHR). The trial was jointly sponsored by the University of Liverpool and Royal Liverpool and Broadgreen University Hospitals NHS Trust and centrally co-ordinated by the Cancer Research UK Liverpool Cancer Trials Unit (LCTU). The trial opened on 9th September, 2009 and closed on 30th April, 2016 after recruiting 360 patients. It is anticipated that analysis of the primary endpoint will take place in 2018 once a sufficient number of events have accumulated. Until then, only limited data is available for correlative translational studies, and it is not possible to compare data across the two treatment arms. Toxicity and efficacy data from individual patients

were obtained from local sites via Case Report Forms (CRFs), collated by the Cancer Research UK Liverpool Cancer Trials Unit and made available by the trial statistician.

The trial protocol included the collection of baseline samples from all patients. The PACIFICO Clinical Trial Sample Collection Kits (each with their own unique kit identification code) are prepared to contain all components to collect samples from patients, with documentation. All kits sent out to the sites to obtain patients samples for the PACIFICO clinical trial were prepared according to the GCP Lab Facility Protocols. Deviations from the protocols were logged as quality incidents. Blood and formalin-fixed paraffin-embedded (FFPE) lymph node biopsies were taken at baseline for all consenting patients. It is expected that the blood samples arrive within 24 hours of being taken from the patient. Whilst being transported a data logger within the package records the temperature the samples are exposed to. Upper and lower limits are set to alert if the sample has been outside of the range defined e.g. a lower limit of 2°C would alert that blood maybe compromised. The data from the loggers is then down loaded once the sample is received in the lab. As part of the Quality Assurance for the lab any deviations from protocols are recorded as quality incidents against the sample, eg if the sample is delayed in the post, or if another tube was used to that supplied. The samples stored for the trial are tissue blocks (to be returned), germline DNA, plasma and peripheral blood mononuclear cells (PBMCs, from anti-coagulated blood) and serum from coagulated blood. On arrival after logging receipt and coding of the samples the anti-coagulated blood samples had an aliquot removed and stored in the -80^{o}C freezers before processing into DNA. The remaining anti-coagulated blood was processed using density centrifugation (see Section 2.6.3 for PBMC Isolation) to plasma and PBMCs, which were stored in the -80^{o}C and -150 ^{o}C freezers, respectively, in the GCP Lab Facility freezer room. The clotted blood samples were

centrifuged at 2500g for 10 minutes before preparing 1ml aliquots of serum and stored in the -80^{o}C freezers in the GCP Lab Facility freezer room. The Formalin-fixed, paraffin-embedded (FFPE) tissue blocks were stored in tissue cabinet at room temperature (RT).

As a nested case-control study, sample types were selected from patients that tested positive for CMV-IgG antibodies and those that were negative for the CMV-IgG antibodies. The CMV -IgG antibodies statuses were determined by the local (trial) participating recruiting sites. For FACS and Luminex analysis, 42 sample types were selected from patients within relatively similar clinicopathological characteristics as in Table 3-1 (Chapter 3). The clinical features of patients included Follicular lymphoma prognostic index (FLIPI), Ann Arbor stage, haemoglobin value, Lactate dehydrogenase (LDH), cumulative illness rating scale (CIRS), Gender, Age and number of lymph nodes involved. Patient samples were carefully chosen to reduce the impact of confounders in the analysis and to enhance the chances of accurate evaluation of the biological effect of CMV infection status on FL.

The data generated from this design will offer an opportunity for a comparative analysis of the relationship between circulating peripheral lymphocytes and levels of serum cytokine in follicular lymphoma.

For the FACS and cytokine analyses in Chapters 3 & 4, PBMC and corresponding serum samples from 42 patients with 21 being CMV positive & 21 CMV negative were used. The study on cellular infiltrates in the FL microenvironment used FFPE tissue blocks from 42 patients (including 20 CMV positive & 22 CMV negative) with only 6 patients included in the FACS/cytokine cohort. The explorative analysis of clinical data in Chapters 6 and 7 included patients from the entire trial cohort whose

clinical data had been verified and available. A summary of study cohorts regarding sample size and overlap is displayed in Figure 6-1. The cohort of patients used for the study of cellular infiltrates in the FL microenvironment was selected based on the availability of FFPE tissue material for the immunofluorescence. Only a small proportion of the cases were available for use. Therefore no particular consideration was made regarding the demographic features of patients on both arms of the CMV status (Table 5-1; Chapter 5). The cohort used for the explorative analysis of the overall trial according to CMV infection status, were 360 patients. Of these, 269 (75%) had information on CMV status, of which, 59% were CMV negative, and 41% were CMV positive. Table 6-1 in Chapter 6 gives patient demographics broken down by CMV status as well as some initial investigation of response rate and toxicities.

2.3 Inclusion and Exclusion Criteria

The summary of the inclusion criteria for recruiting patients into the NCRI PACIFICO phase III clinical trial includes established grades 1, 2 and 3a patients of mainly 60 years or above, at Ann Arbor stages II to IV without prior treatment, as well as minimal haematological and other health complications. The exclusion criteria can be summarised to include Grade 3b FL and over, cases of transformed FL to DLBCL and other health complications that may not withstand the adverse effects of chemotherapy.

2.4 Ethical Approval & Informed Consent

As part of the PACIFICO trial ethics application, this project was included in the context of the translational work. This trial has approvals of the European Union Drug Regulating Authorities Clinical Trials (EudraCT) on a unique number 2008-004759-31 and the International Standard Randomised Controlled Trial (ISRCTN) number

ISRCTN99217456. Written informed consent to participate in the NCRI PACIFICO trial was obtained before recruitment to the study as per the International Council for Harmonisation (ICH)-Good Clinical Practice (GCP) regulations

2.5 Study Sample Size Estimation

The aim of the project is to compare CMV status from patients taking part in the PACIFICO trial. The interest lies in correlating CMV status to biological samples of interest. Sample size calculations are carried out based on pilot data collected prior to the start of the project.

The previous investigation found that 8 out of the 27 cytokines to be studied were shown to be significantly different between CMV-positive and CMV–negative patients with FL.

Based on replicating these results, sample size calculations are based on a Bonferroni adjusted α level of 0.00625 to control the overall type I error rate at 0.05. As cytokines are measured on different levels, sample size calculations assume the data are measured on the standardised normal distribution. From this, it was determined that difference would be interesting if the difference between CMV-positive and negative cytokines were at least 1 standard deviation. Given each cytokine is measured on a different scale; each cytokine will have its standard deviation. Therefore, the parameters used for the sample size calculation for comparison between two groups with equal number of patients include the statistical power at 80%, α level at 0.00625, standard deviation of 1 and clinically relevant difference of 1. This gives an overall sample size of 42 patients.

Further to this, patients for FACS and cytokine analyses were selected from the PACIFICO trial by the trial statistician who also ensured that 21 CMV-positive and

21 CMV-negative patients were selected and that these groups were balanced in terms of the CIR score, FLIPI score and treatment allocation to help account for any confounding that may occur due to these covariates.

2.6 Laboratory and experimental methodologies

2.6.1 Instrument used flow cytometric analysis - BD LSRFortessa™

The flow cytometer used for data acquisition is the BD LSRFortessa™ Special Order Research Product (BD Biosciences, Oxford, UK), an air-cooled multi-laser benchtop flow cytometer, equipped with Red, Yellow/Green, Blue, Violet and UV lasers and capable of supporting the analysis of up to 18 parameters

2.6.2 Cytometer Setup and Tracking (CS&T)

The CS&T is a fully automated BD FACSDiva software and reagent research system, unique to BD digital cytometers, designed to provide Characterization, Setup and Tracking for baseline settings. This system optimises and standardises cytometer setup and tracks cytometer performance ensuring consistency and reproducibility of FACS data by offsetting the routine instrument variability. The BD FACSDiva CS&T Research Bead set (Cat #: 655051, BD Biosciences, Oxford UK) consists of uniform beads of different intensities (bright, mid and dim beads) designed to fully characterise the flow cytometer.

For daily performance checks, a CS&T passed result was considered essential for running FACS samples for the day.

2.6.3 PBMC isolation

In a biological class II safety Saftey Cabinet, Lymphoprep (Product #: 1114740, Axis-Shield, Alere Ltd., Stockport-UK) was pipetted into prelabeled tubes in a 2:1 ratio of blood to Lymphoprep. Blood was gently layered on top of the Lymphoprep using a

serological pipette or sterile pastette. The tubes were centrifuged at 800 g for 30 minutes at RT with the brake setting at low. Following centrifugation, mononuclear cells in the buffy coat layer were carefully removed into a tube, which was filled up with Roswell Park Memorial Institute (RPMI)-1640 medium (LM-R1641/500, Labtech International Ltd, East Sussex, UK) and centrifuged at 550 × g for 10 minutes to wash and pellet the cells with the brake on. The supernatant was discarded, and cells were resuspended in appropriate volume of freezing solution A (10% foetal calf serum (FCS) in RPMI 1640 media). Cells were counted to determine live and dead cells using ChemoMetec Nuclear Counter in the GCP Lab facility and an equal volume of medium B (10% FCS, 20% Dimethyl sulfoxide (DMSO in RPMI) was added slowly. Cells were aliquoted in vials and placed in Nalgene cryofreezing containers to -80 oC freezer for a minimum of 12 hours before moving to -150^{o}C freezer in the GCP Lab Facility freezer room.

2.6.4 The PBMC Samples: Thawing and recovery of cells

Frozen specimens were thawed by being held in a closed fist before transferring the cells to pre-label Universals on ice. Each 1mL of thawed cell suspension was slowly diluted with 10mL of RPMI- 1640 medium supplemented with 10% foetal calf serum (FCS). The RPMI 1640 solution was added to cells gradually in a drop-wise manner with constant and careful agitation throughout the process. The Universals were centrifuged at 500 × g for 5 minutes, and the supernatants were discarded. Then the cell pellets were resuspended, counted and assessed for viability using ChemoMetec NucleoCounter (ChemoMetec A/S, Denmark), by detecting total and dead cell counts. Cells were resuspended in BD FACSFlow Sheath Fluid (Cat #: 342003, BD Biosciences, Oxford UK) for lymphocyte immunophenotyping.

2.6.5 The Compensation Set up

After vortexing the BD™ CompBeads(Cat #: 552843, BD Biosciences, Oxford UK) had one drop each of the negative control and anti-Mouse Ig, κ was added to each of 12 prelabelled compensation tubes representing each fluorochrome. The optimal working volume of each conjugated primary antibody was added to the respective tube. The tubes were mixed gently and incubated for 30 minutes in the dark at RT. Subsequently, 2ml of CellWash (Cat# 349524; BD Biosciences, Oxford UK) was added to each tube and centrifuged for 5 minutes at 300 × g with a low brake setting to wash beads. Supernatants were discarded, and the bead pellets were resuspended in 500µL BD FACSFlowTM Sheath Fluid and vortexed thoroughly before analysis. Five thousand events were acquired from each of the 12 compensation tubes. Using BD FACSDiva software version 7, the bead population is carefully gated, and the positive and negative populations were identified before the compensation values were calculated.

2.6.6 Determination of working volumes of conjugated mAbs

As part of the optimisation protocol, optimal working volumes of all the 26 fluorochrome-conjugated monoclonal antibodies in the panel were determined by single fluorescent staining of washed PBMCs from healthy volunteers (HV) using three different concentrations of the antibodies. The recommended volume by the manufacturers was used as the starting point for the working volumes, and a value above and one below for each was also used. Table 2-4 shows all the antibodies, and the dilutions tested.

Table 2-1: Protocol for determination of working volume experiments

S/N	mAbs	Channel	Tube #1 (µl)	Tube #2 (µl)	Tube #3 (µl)
1	CD3-FITC	AF488	10	20	30
2	CD3-PERCP-CY5.5	PerCp	2.5	5.0	10
3	CD4-APC-H7	APC-Cy7	2.5	5.0	10
4	CD8-AF700	AF700	2.5	5.0	10
5	CD11a-PE-CY7	PE-Cy7	2.5	5.0	10
6	CD14-PE-Cy7	PE-Cy7	2.5	5.0	10
7	CD16-PE-CF594	PE-Texas Red	2.5	5.0	10
8	CD19-BV650	Qdot655	2.5	5.0	10
9	CD25-PE-CF594	PE-Texas-Red	2.5	5.0	10
10	CD27-PerCP-Cy5.5	PerCP	2.5	5.0	10
11	CD28-PE	PE	10	20	30
12	CD43-BV421	Pacific Blue	2.5	5.0	10
13	CD45-BV510	Amcyan	2.5	5.0	10
14	CD45-BV650	Qdot655	2.5	5.0	10
15	CD45RA-BV510	Amcyan	2.5	5.0	10
16	CD45RO-BV605	Qdot605	2.5	5.0	10
17	CD56-PerCP-Cy5.5	PerCp	2.5	5.0	10
18	CD57-APC	APC	2.5	5.0	10
19	CD62L-APC	APC	10	20	30
20	CD62L-BV605		2.5	5.0	10
21	CD69-PE-Cy7	PE-Cy7	2.5	5.0	10
22	CD127-BV510	Amcyan	2.5	5.0	10
23	CD197 (CCR7)-BV421	Pacific Blue	2.5	5.0	10
24	CD279 (PD1)-PE	PE	10	20	30
25	LAG3-FITC	AF488	5.0	10	20
26	TIM3-APC	APC	5	10	20
27	CD45-BV510 (ISOTYPE)	Amcyan	2.5	5.0	10
28	CD45-BV650 (ISOTYPE)	Qdot655	2.5	5.0	10

A total of between 10,000 and 50,000 events were acquired per FACS tube. A representative process used for the determination of working volumes of CD45-BV510 with CD45-BV510-Isotype control is illustrated in Figure 2.2. The dot plots of events for the three different volumes of antibodies were created, and the lymphocytes population gated as P1 on the FSC/SSC plot. A histogram of the lymphocytes population (P1) was drawn, and the amount of the respective fluorescent cells in P1 was determined on the AmCyan channel. An interval gate was drawn on the Isotype control for the lowest concentration so that approximately 0.5% of this population was included in P2. This interval (P2) was retained for all the antibody dilutions.

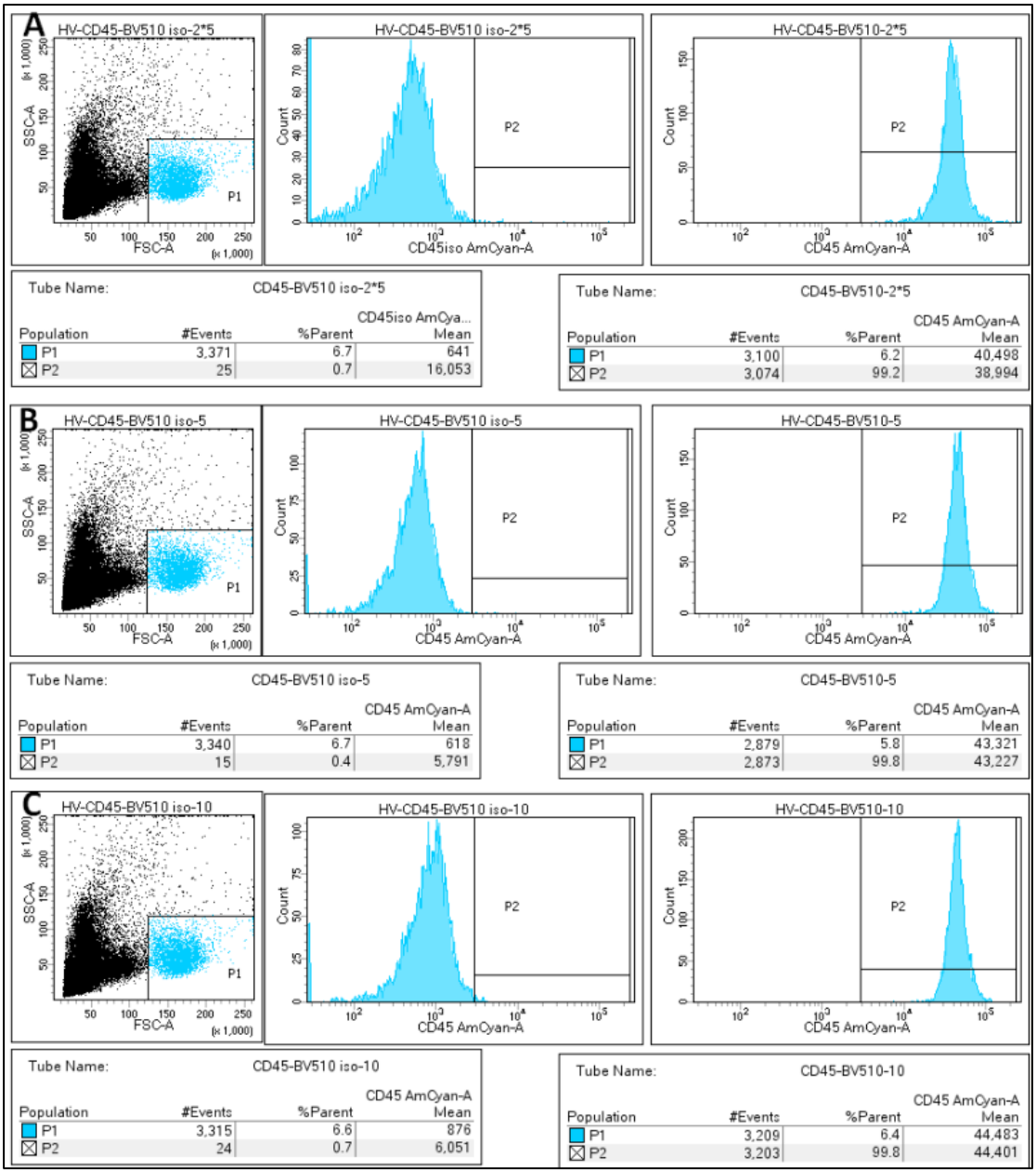


Figure 2-2: Determining the working volume of CD45-BV510.

Panel A represents the Isotype control and anti-CD45-BV510 antibodies at 2.5µl; panel B represents 5µl of antibodies, and panel C represent 10µl of antibodies incubated with. PBMCs from a healthy volunteer (HV). The statistics displayed underneath the histograms represent the number of events and the mean fluorescent intensity (MFI).

The criteria used for the choice of optimum working volumes of the fluorochrome-conjugated antibodies was a combination of the values of the MFI for P2, the number

of events in P2 as well as the clearance of the Isotype control histogram peak from P2. In Fig 2.2, panel A CD45-BV510 Isotype control had the highest MFI of 16,053 (P2) and the lowest MFI for CD45-BV510 compared to the other two tubes. In panel B the CD45-BV510 Isotype control had the least MFI of 5,791 with an MFI of 43,227 for CD45-BV510. Although panel C (10µl) had a slightly higher MFI for CD45-BV510 than the 5µl tube, the MFI for the Isotype control is higher. Therefore the 5µl volume of CD45-BV510 was used for all other staining work. This process was carried out for all the 26 monoclonal antibodies (mAbs).

2.6.7 The staining and acquisition of cells for FACS

Since more than two mAbs are conjugated with BD Horizon Brilliant fluorescent polymer dyes in each FACS tube, 50µL of the BD Horizon™ Brilliant Stain Buffer was added to each tube to minimise spectral overlaps. As illustrated in Figure 2-3, respective quantities of the conjugated mAbs shown in brackets were added to the respective tubes.

Following the addition of antibodies, 100µL of well-mixed washed PBMCs containing about 2×10^5 to 1×10^6 were placed in the prelabelled FACS tubes. Tubes were incubated for 30 minutes in the dark, at room temperature, after which 2ml of CellWASH (Cat #:349524, BD Bioscience Oxford, UK) was added and the tubes centrifuged at 300 × g for 5 minutes with low brake. The supernatants were discarded before repeating the washing process. Finally, the cells were resuspended in 500µL of BD FACSFlowTM Sheath Fluid before being gently vortexed. The aim was to count a minimum of 500,000 events per patient sample, however, in some cases there were less than 500,000 cells per tube. In a minority of cases more than 500,000 cells were acquired due to the availability of material.

Tube 1	Tube 2	Tube 3
		
CD3-FITC (20)	CD3-FITC (20)	LAG-3-FITC (10)
CD56-PERCP-CY5.5 (5)	CD27-PERCP-CY5.5 (5)	CD3-PERCP-CY5.5 (2.5)
CD57-APC (10)	CD62L-APC (20)	TIM-3-APC (5)
CD8-AF700 (2.5)	CD8-AF700 (2.5)	CD8-AF700 (2.5)
CD4-APC-H7 (10)	CD4-APC-H7 (10)	CD4-APC-H7 (10)
-	CD197 (CCR7)-BV421 (10)	CD43-BV421 (10)
CD45-BV510 (5)	CD45RA-BV510 (10)	CD127-BV510 (5)
-	CD45RO-BV605 (2.5)	CD62L-BV605 (10)
CD19-BV650 (5)	CD45-BV650 (10)	CD45-BV650 (10)
CD28-PE (20)	CD28-PE (20)	CD279 (PD-1)-PE (10)
CD16-PE-CF594 (5)	CD25-PE-CF594 (10)	-
CD14-PE-CY7 (5)	CD11a-PE-CY7 (2.5)	CD69-PE-CY7 (5)

Figure 2-3: Illustrating the 3-tube design and the respective panels of mAbs.

All mAbs were purchased from BD Bioscience, Oxford UK with exception of LAG-3-FITC (Enzo Life Sciences, UK) and TIM-3-APC (R & D Systems, UK). The values in brackets represent the respective optimal working volumes of the mAbs in microlitres.

2.6.8 Multiplex (Luminex) Assay for Serum cytokines

The multiplex instrument, Bio-Rad Bio-Plex 200 system (Bio-Rad Laboratories, Hertfordshire, UK) powered by Luminex xMap Technology was used for the Bio-Plex Pro™ Human Cytokine 27-plex assay. Consequently, all reagents comprising cytokine standards, coupled beads, 96-well plates, detection antibodies, streptavidin-PE and diluents as well as the software used for the analysis of data were purchased from Bio-Rad Laboratories (Cat #: M500KCAF0Y: Bio-Rad Laboratories, Hertfordshire, UK).

As part of the validation protocol for Luminex assay and before data acquisition, each bead region number of the cytokine was entered against each cytokine in the Bio-plex

manager software. The Human Cytokine Standard was reconstituted following the manufacturer's instructions and incubated on ice for 30 minutes prior use.

2.6.8.1 Preparation of the Standard Curve

An 8-point standard curve design with a fourfold dilution between each point was used. The reconstituted standard was vortexed gently for 1-3 seconds before transferring the reconstituted standard to tube one (denoted as S1) containing standard diluent.Tube S1 content was thoroughly mixed, and 100µl of the standard diluent mixture in tube S1 was transferred to tube S2 containing 300µl of standard diluent. The dilution process was repeated through to the S8 tube. This preparation is for a standard triplicate run assay.

2.6.8.2 Preparation of Coupled Beads for 96 wells

A 1× working concentration of the 10 × Coupled Beads was prepared using Assay Buffer in a 15 ml tube. The diluted coupled beads were vortexed for 30 seconds at medium speed. A multichannel pipette and reagent reservoir were used to add 50µl of beads to each well of the 96-well microplate. The plate was sealed with a transparent sealant and wrapped in aluminium foil and allowed to equilibrate for 20 minutes at RT. The microplate was washed twice with Bio-Plex wash buffer (Catalogue #: 171-304500) using Fisher Scientific™ Wellwash 4 Mk2 Microplate Washer. Fifty microlitres (50µl) each of the diluted standards, internal controls, blank (diluent) and respective samples were added to the designated wells of the 96-well Bio-Plex Pro flat-bottom microplates (cat #: 171-025001) in triplicates as in the plate layout in Figure 2-4. The preparation was incubated at RT for 30 minutes whilst gently shaken on a microplate shaker (Cat #: 444-7094, VWR England, UK).

	1	2	3	4	5	6	7	8	9	10	11	12
A	S1	S1	S1	1	1	1	9	9	9	17	17	17
B	S2	S2	S2	2	2	2	10	10	10	18	18	18
C	S3	S3	S3	3	3	3	11	11	11	19	19	19
D	S4	S4	S4	4	4	4	12	12	12	20	20	20
E	S5	S5	S5	5	5	5	13	13	13	21	21	21
F	S6	S6	S6	6	6	6	14	14	14	IC	IC	IC
G	S7	S7	S7	7	7	7	15	15	15	IC	IC	IC
H	S8	S8	S8	8	8	8	16	16	16	B	B	B

Figure 2-4: Illustrating a 96-well plate layout for Cytokine Multiplex assay.

S1 – S8 for standards S1 to S8, internal controls (IC) for interassay quality control, blank (B) for background noise.

2.6.8.3 Addition of Detection Antibodies (DAs)

The 10 × DAs supplied by the manufacturer was diluted with Detection Antibody Diluent to make a 1 × working solution. Following the incubation period; the sealing tape was removed gently and slowly. The plate was washed three times with Bio-Plex wash buffer using Fisher Scientific™ Wellwash 4 Mk2 Microplate Washer, before adding 25µl of 1 × DAs. The microplate was covered with a new sheet of sealing tape and incubated at RT for 30 minutes with a gentle shake on a microplate shaker.

2.6.8.4 Addition of Streptavidin-PE

A 1:100 dilution of the stock streptavidin-PE was made in Assay Buffer, vortexed at medium speed for 3 – 5 seconds and 50µl added to each assay well. Again, the

multiplex microplate was carefully covered with a new sheet of sealing tape and incubated at RT for 10 minutes with gentle shaking on a microplate shaker. Following the incubation; the microplate was washed 3 × with 300µl of Bio-Plex wash buffer using Fisher Scientific™ Wellwash 4 Mk2 Microplate Washer, and 125µl of Assay Buffer was added to each well. This was covered with a new sealing tape and incubated at RT on a shaker at 1,100 rpm for 30 seconds prior plate reading. The Bio-Plex was calibrated with Bio-Plex Manager software before setting up the assay and following the start-up and calibration processes for CAL1 and CAL2 calibration. Before sample acquisition, the system must pass the calibration.

2.6.8.5 Data acquisition and analysis

The multiplex assay employs Luminex platform to detect targets using antibody-coupled bead systems. The unique content of two addressing colours recognised each bead in the set and a third dye is used to censure the coupling of the target analyte through a biotin-coupled antibody plus streptavidin conjugated secondary step indicator. A dedicated flow cytometer of the Bio-Plex® 200 system platform (Luminex MAP Technology, Texas, USA) was used to acquire the data.

Data was acquired in Bioplex reader after calibrating the Bioplex manager software. The values that fell way beyond the curves had an asterisk (*) and were removed before analysis. For the evaluation of assay reproducibility, the inter-assay % coefficient of variation of most of the cytokines appears within the acceptable limit of 20% and below. However, cytokines with low levels of abundance that fall below the limit of detection of the assay expressed zero scores, for instance, IL-1β, IL-2, IL-5, IL-15 had unacceptably high %CV of above 100%.Also, cytokines with elevated levels of abundance for example RANTES, the %CV appeared very high. This is because the %CV can usually be higher due to cytokine levels that were ‘out of range’

(OOR). The intra-assay precision of triplicate wells for the 27-plex assay was excellent, with %CV of ≤ 20% across most of the 27 analytes from the two Luminex plates, except IL-2, IL-15 and IL-17A that had %CV of 173.21% in plate-1, plate-2 and plate-1 respectively. Assay precision data was obtained using healthy serum control and test samples. The standard curves for the two plate assays appear comparable, an indication that raw MFI values for each point in the standard curve are highly reproducible from run to run.

2.6.9 Sandwich ELISA for Cytokines

The ELISA Development Kits contains essential components for the quantitative measurement of human cytokines in a sandwich ELISA format including capture antibodies (CAs), detection antibodies (DAs) Wash Buffer, Block Buffer and Diluent were supplied by PeproTech EC Ltd, London, UK. Additional materials and reagents required and not provided in the kit are ELISA microplates, ABTS Liquid Substrates solution (Sigma Cat. #: A3219), Bovine serum albumin (BSA; Sigma Cat. #: A-7030), Dulbecco's Phosphate buffered saline (PBS; [10 ×]) (Gibco BRL. Cat #: 14200-075), Wash Buffer, Block Buffer, Diluent and Tween-20 (Sigma Cat. #: P-7949). The sELISA assay targets included the human IL-4 (Cat. #: 900-K14), IL- 6 (Cat. #: 900-K16), IL-8 (Cat. #: 900-K18) and IL-9 (Cat. # 900-K20) (PeproTech EC Ltd, London, UK).

2.6.9.1 Reconstitution of Standards and Antibodies

Following manufacturer's instructions (PeproTech, USA), CAs, DAs and standard cytokine reagents were reconstituted to stock concentrations by the addition of the respective amounts of sterile distilled water (dH2O). This forms the stock working reagents.

2.6.9.2 Plate Coating with Capture Antibodies

Following manufacturer's instructions (PeproTech, USA), respective cytokine working CA and DA reagents were prepared by diluting the stock reagents in 1 × PBS thereby producing the recommended working concentrations as contained in the Table 2-2 for each cytokine. The working reagents were prepared for a single 96-well ELISA experiment and were not stored.

Table 2-2: Preparation of working capture and detection antibodies

Stock Capture Antibody (CA)	Working CA (µg/ml)	Stock Detection Antibody (DA)	Working DA (µg/ml)
100µg/ml of anti-hIL-4	0.5	100µg/ml of anti-hIL-4	0.5
100µg/ml of anti-hIL-6	1.0	100µg/ml of anti-hIL-6	0.5
100µg/ml of anti-hIL-8	0.5	100µg/ml of anti-hIL-8	0.5
200µg/ml of anti-hIL-9	2.0	100µg/ml of anti-hIL-9	1.0

Reagents are prepared by diluting the stocks in 1 × PBS sufficient for a 96-well ELISA plate.

The vials of CAs were gently vortexed avoiding air bubbles and100 µL of the working CA solution was added to each well of the ELISA plate. The microplate was sealed firmly with sealing film and incubated overnight at RT. The following day, the plate was washed four times using 1× Wash Buffer and blotted on clean, dry paper towel. To block for non-specific binding, 300 µL of ready-to-use Blocking Buffer was added to each ELISA well and incubated for 1 hour at RT.

2.6.9.3 Addition of Cytokine Standards & Samples – Specific Binding of Antigen

The 8-point concentrations of standard cytokines were prepared by diluting each cytokine in 1× Diluent. Briefly, 400µL of Diluent was added to standard tubes except for the first tube. Then 800µL of the reconstituted cytokine standard solutions were added to the first (highest) standard tube (S1) and 400µL was transferred to the

second tube (S2). This was mixed carefully, making a 1:2 dilution of S1. The serial dilution process was repeated through to the 8th concentration (S8) where 400µL of the standard diluent solution was discarded. Each standard curve tube contains a final volume of 400 µL for a triplicate of each point of the standard curve.

Table 2-3: 8-point Concentrations for the cytokine standards

Cytokine (pg/ml)	IL-4	IL-6	IL-8	IL-9
Tube	1000 - 0	1500 - 0	1000 - 0	3000 - 0
1	1000	1500	1000	3000
2	500	750	500	1500
3	250	375	250	750
4	125	187.5	125	375
5	62	93.75	62	187.5
6	31	46.88	31	93.75
7	15.5	23.44	15.5	46.88
8	7.8	11.72	7.8	23.44

Samples were either assayed neat or diluted depending on the concentration of the analyte as determined by ELISA optimisation experiments for optimal results. Then 100µl of each standard concentration, samples, internal control (IC) and blank (B) were added to assay wells in triplicates as shown on the plate layout on table 2-6. ELISA plates were sealed and incubated for 2 hours at RT. This plate layout allows for the inclusion of 8-point standards S1-S8 with a 2-fold serial dilution between each point, twenty-one test samples, one internal control (IC) or human AB serum (hABs) and two blank (B) rows in triplicates.The blanks are essential in determining the

efficacy and validity of the measurements of the samples. After the 2 hours incubation, the plate was removed and washed four times in 1 × Wash Buffer ready for the addition of DA reagent.

	1	2	3	4	5	6	7	8	9	10	11	12
A	S1	S1	S1	B	B	B	8	8	8	16	16	16
B	S2	S2	S2	1	1	1	9	9	9	17	17	17
C	S3	S3	S3	2	2	2	10	10	10	18	18	18
D	S4	S4	S4	3	3	3	11	11	11	19	19	19
E	S5	S5	S5	4	4	4	12	12	12	20	20	20
F	S6	S6	S6	5	5	5	13	13	13	21	21	21
G	S7	S7	S7	6	6	6	14	14	14	IC	IC	IC
H	S8	S8	S8	7	7	7	15	15	15	B	B	B

Figure 2-5: An illustration of a 96-well ELISA plate layout

S1 – S8 for standard points 1 to 8, internal controls (IC) for interassay quality control, blank (B) for background noise. All standards, controls and patient samples were tested in triplicates indicated by numbers and colours).

2.6.9.4 Detection

DAs were prepared fresh and enough for a single experiment. Each assay well had 100 µL added immediately before the plate was sealed and incubated for 2 hours at room temperature.

After incubation, plate content was discarded and washed four times in 1 × Diluent and a 1:2000 dilution of Avidin conjugated with horseradish peroxidase (HRP) was

prepared in 1 × Diluent. The solution was mixed gently, and 100 µL was added to each ELISA well immediately. The ELISA plate was sealed and incubated for 30 minutes at room temperature.

ELISA plate was aspirated and washed four times in 1 × Diluent. A multichannel pipette was used to add 100ul of ready-to-use ABTS substrate to each ELISA well. A SPECTRAmax PLUS 384 ELISA Plate Reader (Molecular Devices, California, USA) set at 405nm with wavelength correction at 650nm was used to monitor colour development at 5 minutes intervals at RT for up to 50 minutes. Colour development was recorded by reading of optical density (OD) of the wells and SOFTmax Pro software (Molecular Devices, California, USA) was used for endpoint reading. When readings fall between zero and the highest standard values; reliable standard curves are said to be obtained.

2.6.10 Immunofluorescence (IF) of FFPE tissue sections

Immunohistochemistry (IHC) was used to determine the working dilutions of the primary antibodies used for the immunofluorescence staining of FFPE tissue sections.

2.6.10.1 IHC protocol for working dilutions of primary antibodies

IHC technique was employed for the determination of optimal working dilutions of the respective primary antibodies by staining three microns sections of recommended positive FFPE tissue using three dilutions of the primary antibodies and an Isotype control (used at the highest concentration). To dewax, rehydrate, and retrieve antigens the Dako PT-Link water bath with high PH buffer system was used as described elsewhere (Shi and Taylor, Pirog, 2015). Briefly: The PT-Link system was preheated at 65°C, and well-labelled tissues sections on slides in staining racks were placed in the PT-Link tank and heated to 96°C for 20 minutes. It was allowed to cool back to

65°C before removing the rack from PT-Link. The dewaxed slides were immediately dipped into the Envision Flex-Wash buffer for 5-15 minutes. After circling tissue sections with PAP Pen (Cat # ab2601, Abcam, Cambridge, UK), Envision Flex-Wash buffer was used to wash sections on the staining tray. Then 100µl of Envision-Flex block was added to sections and incubated at RT for 5 minutes to block, and washed with Envision Flex-Wash buffer. The respective primary antibodies and Isotype controls were diluted in Envision-Flex antibody diluent accordingly, and 100µl of each dilution was added to sections, CD4 (Leica, Milton Keynes, UK), CD8 (Abcam, Cambridge, UK), CD16 (Abcam, Cambridge, UK), CD21 (Abcam, Cambridge, UK), CD56 (Leica, Milton Keynes, UK), CD68 (Dako, Cambridgeshire, UK), CD163 (Abcam, Cambridge, UK), FOXP3 (Abcam, Cambridge, UK) and PD1 (Abcam, Cambridge, UK). These were covered and incubated at RT for 30 minutes.

FLEX buffer was used to wash sections 2 - 3 times before 100µl of HRP was added to section and incubated at RT for 20 minutes. Sections were washed with FLEX buffer briefly and 100µl fresh 3, 3'-diaminobenzidine (DAB) - substrate solution was added to the sections and incubated at RT for 20 minutes. Sections were washed with FLEX buffer again for 2 - 3 times before being dipped into distilled water. The sections were stained with haematoxylin for 30 seconds, dipped in acid alcohol briefly (immediately, and in ammonia water for 30 seconds while placing the rack into the bath of running tap water in between. Sections were dehydrated through the ethanol series of 3x IMS (Industrial Methylated Spirits) and 2x xylene in the fume hood and agitating vigorously for 10 seconds at each stage. Finally, a drop of DPX mounting medium was added to a coverslip and applied to slide removing all air bubbles. Positive control tissue was cut at 3 microns to give sections for optimising concentrations (dilutions) of antibodies, as shown in Table 2-4.

Table 2-4: Details of the primary antibodies and the positive control tissues used in determining the working dilutions

S/#	Primary antibody (concentration)	Product #	Isotype control (Concentration)	Working dilution	Positive tissue type
1	M-anti CD4 (405µg/ml)	NCL-L-CD4-368	IgG1 (100µg/ml)	1:500	Tonsil
2	R-anti CD8 (439µg/ml)	ab93278	IgG (1858µg/ml)	1:500	Tonsil
3	R- anti CD21 (138µg/ml)	ab75985	IgG (1858µg/ml)	1:500	Tonsil
4	R-anti FOXP3 (24µg/ml)	ab99963	IgG (1858µg/ml)	1:100	Tonsil
5	M-anti PD1 (500µg/ml)	ab140950	IgG1 (100µg/ml)	1:25	Tonsil
6	M-anti CD56 (18µg/ml)	NCL-CD56-136	IgG2b (100µg/ml)	1:100	Neuroendocrine carcinoma
7	R-anti CD16 (34µg/ml)	ab183354	IgG (1858µg/ml)	1:100	Lung
8	M-anti CD68 (30µg/ml)	M0876	IgG3 (500µg/ml)	1:200	Tonsil
9	R-anti CD163 (16µg/ml)	ab100909	IgG (1858µg/ml)	1:100	Lung

2.6.10.2 IHC interpretation and working volumes of targets

The images of stained IHC slides were digitalized using Aperio ScanScope. Digital images of sections stained with the three different dilutions of the primary antibodies and the corresponding Isotype controls were reviewed in ImageScope by a Consultant Pathologist for positivity, intensity and histological distribution. Taking the case in Figure 2-6, the positivity and distribution of FOXP3 on the tonsil sections at three concentrations were assessed for application in the immunofluorescence experiments. A1:100 dilution of anti-FOXP3 on a positive tonsil section was chosen as the optimal concentration as the cells are not overstained and the brown is just clearly visible. The working dilution of all the remaining 8 antibodies in Table 2-4 were determined using similar procedure, and the optimal working dilutions of the antibodies are presented in pairs in Figure 2-7 to Figure 2-10.

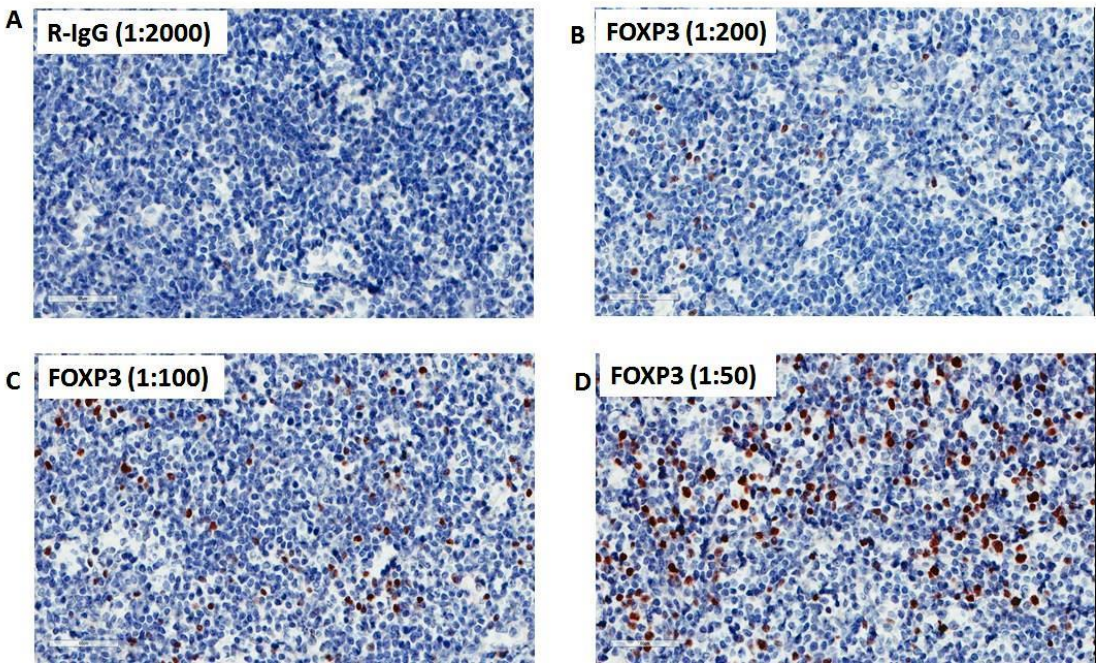


Figure 2-6: IHC staining of Rabbit anti- FOXP3

IHC stained images from sections stained with Rabbit immunoglobulin-G (R-IgG) and the three dilutions of Rabbit anti-human FOXP3 showing dilutions in brackets. (All images were taken at × 40 magnification).

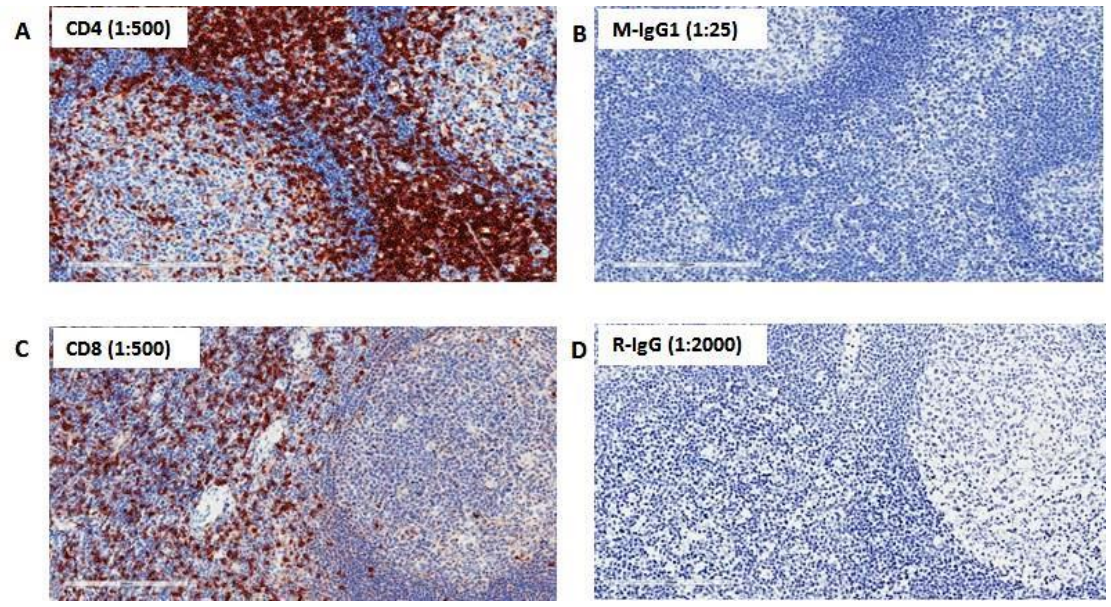


Figure 2-7: Working dilutions of Mouse anti human CD4 and Rabbit anti human CD8.

Images A and C are of sections stained with CD4 and CD8 respectively, while the corresponding Isotype controls are shown in images B and D. The dilutions of antibodies are shown in brackets. (All images were taken at × 20 magnification).

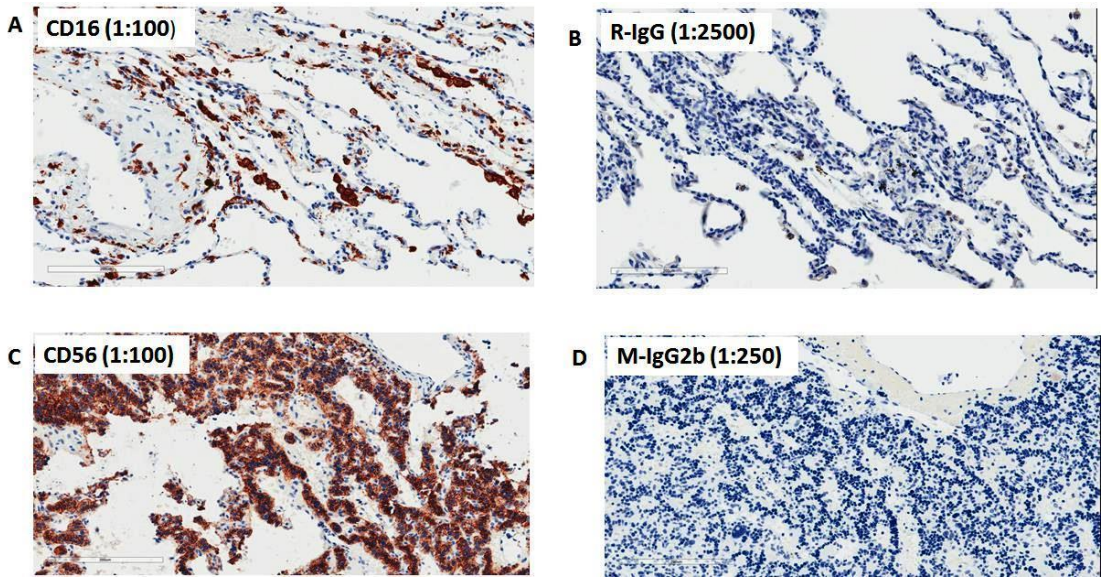


Figure 2-8: Working dilutions of Rabbit human anti human CD16 and Mouse anti human CD56.

Images A and C are of sections stained with CD16 and CD56 respectively, while the corresponding Isotype controls are shown in images B and D. The dilutions of antibodies are shown in brackets. (All images were taken at × 20 magnification).

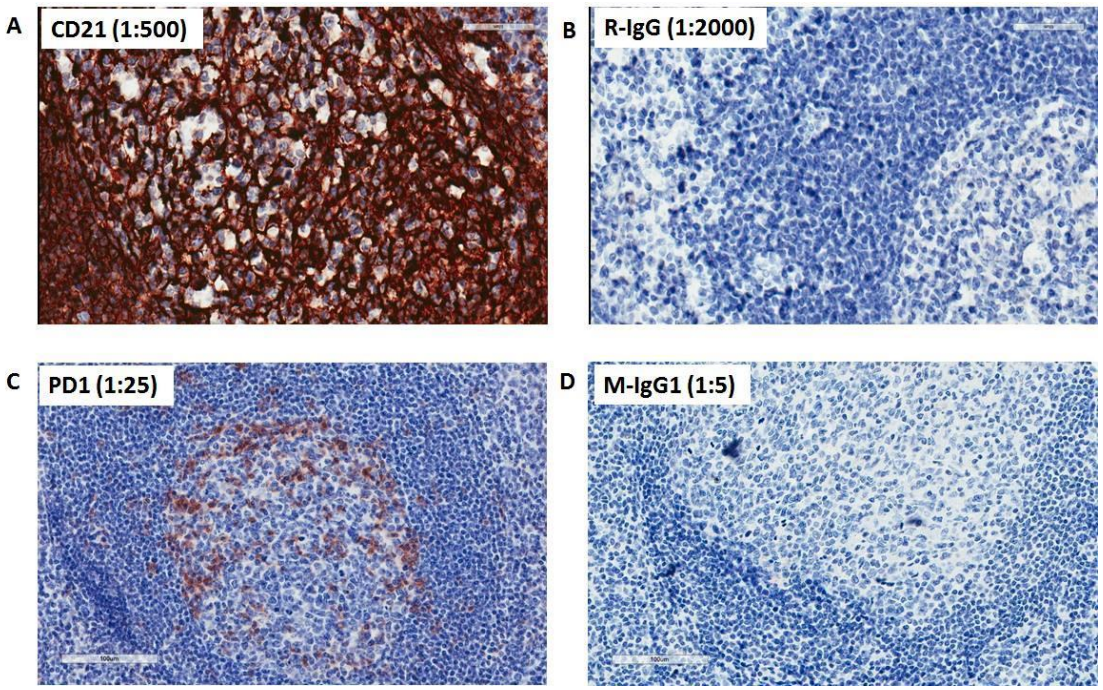


Figure 2-9: Working dilutions of Rabbit anti human CD21 and Mouse anti human PD1.

Images A and C are of sections stained with CD21 and PD1 respectively, while the corresponding Isotype controls are shown in images B and D. The dilutions of antibodies are shown in brackets. (Images of CD21 and corresponding Isotype control were taken at ×40 while those of PD1 and Isotype at × 20 magnification).

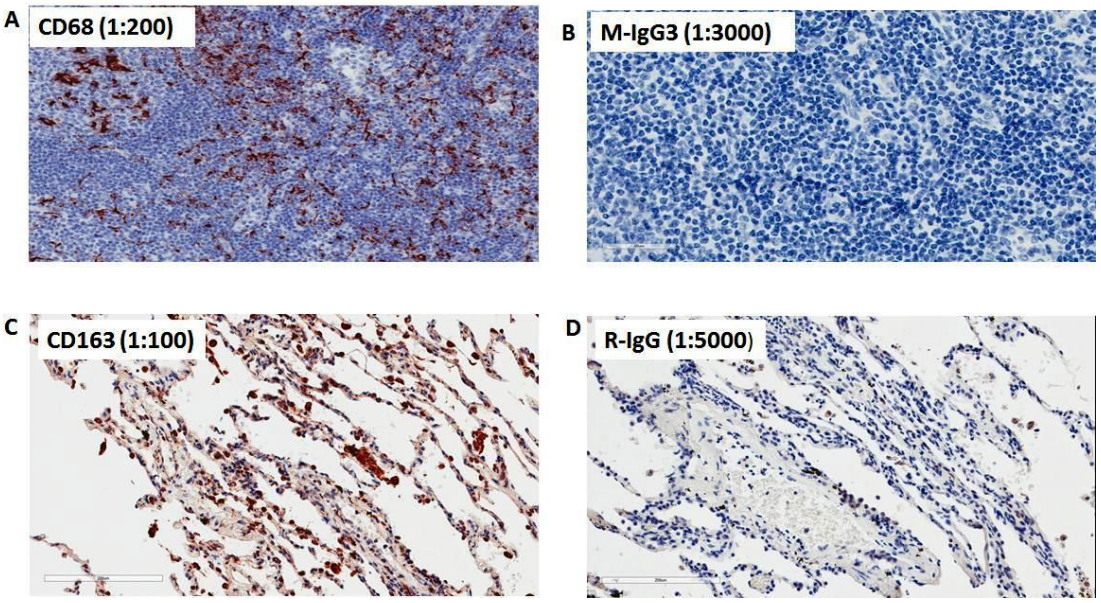


Figure 2-10: Working dilutions of Mouse anti human CD68 and Rabbit anti human CD163.

Images A and C are of sections stained with CD68 and CD163 respectively, while the corresponding Isotype controls are shown in images B and D. The dilutions of antibodies are shown in brackets. (All images were taken at × 20 magnification).

After determining the optimal working dilutions for the primary antibodies, the working dilutions of the fluorochrome-conjugated secondary antibodies were determined. Three different dilutions around the manufacturers' recommended working dilutions for AF488-Donkey anti-Mouse, and AF647-Donkey anti-Rabbit antibodies were used to stain for sections of defined dilutions of Mouse anti-human CD56 (1:100) and Rabbit anti-human CD8 (1:500). Following a thorough assessment of the staining characteristics, optimal working dilutions of 1:2000 for AF488-Donkey anti-Mouse antibody and 1:100 for AF647-Donkey anti-Rabbit antibody were determined for the immunofluorescence staining of the PACIFICO FFPE tissue sections.

2.6.10.3 The optimisation protocols for immunofluorescence (IF) Staining

After determining the working dilutions of all the antibodies and adoption of the simultaneous double staining technique, a trial autostaining experiment was performed to test the suitability of the Autostainer for IF. Following the satisfactory images and minimal cross-reactivity observed, autostaining was adopted for double staining of the test FFPE tissue sections.

2.6.10.4 Immunofluorescence staining of trial sections using Autostainer

In all, five tissue sections from a total of forty-two PACIFICO trial tissue blocks (patients) were processed and stained. Four of these were double-labelled: CD4/CD8, CD16/CD56, PD1/FOXP3 and CD68/CD163, then one section were stained for a single target, CD21.

The test sections were loaded onto the DAKO PT Link system for antigen retrieval. The Autostainer was programmed to stain for 42 test sections with a mix of diluted primary antibodies as in Table 2-4 and an isotope control section for 60 minutes. This was followed by blocking for non-specific binding with 10% normal donkey serum in PBS/1% bovine serum albumin for another 60 minutes, and finally, the addition of a mix of secondary antibodies for 30 minutes at room temperature. The required amounts of secondary antibody mix were prepared for each cycle of staining. A 1:2000 dilution of Alexa Fluor 488 conjugated to Donkey Anti-Mouse IgG (Cat #: A-21202, Life Technologies Ltd, Paisley, UK) and 1:800 dilution of Alexa Fluor 647 conjugated to AffiniPure Donkey Anti-Rabbit IgG (Cat#711-605-152, Stratech Scientific Ltd, Suffolk, UK) was prepared.

Envision FLEX wash buffer was used to rinse slides at each step of the process. During the staining, the Autostainer was covered with a piece of blackout material to protect the fluorochrome against the effect of light. At the end of staining, slides were

placed in Envision FLEX wash buffer for 10 minutes, and coverslips were immediately mounted using Vectashield Antifade Mounting Medium containing DAPI (Cat#: H-1200; Vector Laboratories Ltd, Peterborough, UK). The slides were stored in a dark cardboard overnight, and edges were sealed with nail varnish to protect from drying. Eight images were taken per section for analysis using an Olympus BX63 motorised upright microscope with a 40× objective, equipped with a Photometrics CoolSnap camera and MicroManager acquisition software (Edelstein et al., 2014). The filter sets used (Chroma Technology Corp) were as follows: DAPI: set 49000 ET DAPI; AF488: set 49002 - ET - EGFP (FITC/Cy2); AF647: set 49006 - ET - Cy5.

2.7 Statistical considerations and Data Analysis

Descriptive statistics were performed for baseline characteristics, both overall and by CMV Status. Categorical variables are described as frequencies and proportion percentages. Continuous variables normally distributed are summarised with their mean and 95% confidence interval, while continuous variables that did not reach normality are presented with their median and IQ range. P-values, obtained after performing the Chi-squared test (or Fisher's exact test, if any expected value was smaller than 5), t-test (or Mann-Whitney U test, if data was not normally distributed) using IBM SPSS Statistical software version 22.0 (SPSS, Inc., Chicago, IL, USA) are used to examine for any statistically significant differences in the outcomes between the groups.

All correlation assessments were determined using the Pearson correlation coefficient. A statistically significant difference was considered if the test P value was <0.05. The hierarchical clustering and heat maps were generated using Partek Genomic Suite Version 6.6 (Partek Inc., St. Louis, MO, USA) and all data were floored (i.e. 0

transformed to 0.01) and logged. ImageJ (Schneider et al., 2012) macros and Java plugins were written in-house and used to analyse image intensities and colocalisation. Output data from image analysis was further analysed using Microsoft Excel as required.

Chapter 3 : Effect of CMV on the circulating lymphocytes

3.1 Introduction

A variety of cell types and subtypes such as granulocytes, macrophages, dendritic cells, T cells, and B cells at different populations constitute an essential part of the immune system (Lu et al., 2015). In CMV infection, a large fraction of T cell subtypes has been reported to be committed to keeping the virus under checks thereby causing alterations in the dynamics of T-cell populations, which strongly influence T cell immunity (Gamadia et al., 2001). Although not much is known about the role of circulating T and NK cells in FL, but depending on the frequencies and location of the different intratumoral T cell populations in the GCs, the tumour immunity and patient outcome in FL is influenced both negatively and positively (Yang et al., 2015). Therefore, quantitation, defining and ascribing specific roles played by the diverse T cell populations, for instance in the immunity of different pathological conditions, will help in providing diagnostic and prognostic evidence for patient management in FL. Multicolour flow cytometry can be used to quantify different subtypes of T cells and other cell types. Different research evidence has explained the events leading to cellular differentiation, acquisition of effector potential and eventual death of T cells. Activation of immature CD4+ T cells has been reported to present a linear differentiation model, in which cells continuously acquire functional abilities by every additional differentiation step (Lanzavecchia and Sallusto, 2002). However, persistent antigenic provocation over time can result in a gradual decline of memory and cytokine secretion capacities, and consequently, accumulation of short-lived CD4+ T cells (TEMRA cells) with poor cytokine production ability. At this stage of differentiation, further antigenic assault on the CD4+ TEMRA cells can activate the CD4+ T cell causing apoptosis. On the other hand, two alternative patterns of

differentiation have been reported for the production of CD8+ T_{CM} cells and CD8+ T_{EM} cells: linear differentiation and fixed lineage (Seder et al., 2008, Kaech and Wherry, 2007). A continued antigenic challenge will lead to accumulation of CD8+ TEMRA cells and death similar to the CD4+ T cells pattern; however, CD8+ T_{EM} cells have the ability to regain IL-2 expression and become CD8+ T_{CM} cells unlike CD4+ T_{EM} cells (Seder et al., 2008). NK cells are a subcategory of lymphocytes of the innate immune system that form about 10% of the circulating blood mononuclear cells in humans (Milush et al., 2009), they are usually described as CD3−CD56+ cells (Lanier et al., 1986a), and can be subgrouped by the intensity of CD56 on the cell surface. The distinct subtypes of NK cells can be classified based on relative expression of CD56 and CD16 surface markers (Yang and Ansell, 2012a). The majority of circulating NK cells (about 90%) exhibit low expression for CD56 (CD56dim) with high levels of CD16 and perforin and are described as terminally differentiated NK cells (Cooper et al., 2001a). NK cell development is characterised by the reduction in the expression of CD56 and the addition of CD16 expression. However, the presence of activated CD56dimCD16+ has been shown to represent the highest level of NK cell activation, which implies that CD56dimCD16+ cells represent the most mature NK cells subtype (Senpuku et al., 2016, Dulphy et al., 2008, André et al., 2000).

In this Chapter I therefore set out to evaluate the impact of CMV infection on circulating peripheral T and NK cell populations in FL patients.

3.2 Patients and materials

As described in section 2.6.6, the optimal working volumes of the 26 conjugated monoclonal antibodies were determined by staining cryopreserved PBMC samples from healthy volunteers (HV) using three different experimental volumes. For the

FACS analysis, 42 stored pre-treatment PBMC samples from PACIFICO trial patients, comprising 21-CMV- positive and 21-CMV- negative were used. Aside the strict inclusion and exclusion criteria that applied in the recruitment of FL trial participants as stated in section 2.3, a careful selection of study patients based on balanced clinical and pathological baseline characteristics as in Table 3-1 between the CMV groups was adopted. This approach is aimed to minimise possible influence of confounders in the analysis and to enhance the chances of accurate evaluation of the biological effect of CMV infection status on FL.

A summary of demographic and baseline characteristics of this study cohort are described in Table 3-1. CMV- positive patients are marginally older compared to CMV- negative with median age of 73 years versus 69 years (p=0.055). Patients' risk factors and the prognosis are stratified according to the Follicular Lymphoma International Prognostic Index (FLIPI) score into three risk categories: good (0 or 1), Intermediate (2) and poor (3-4). The FLIPI score is determined using five adverse prognostic factors including Ann Arbor stage (III-IV vs. I-II), age (> 60 years vs. ≤ 60 years), haemoglobin level (< 120 g/L vs. ≥ 120 g/L), number of nodal areas (> 4 vs. ≤ 4), and serum LDH level (above normal vs normal or below) (Solal-Céligny et al., 2004). A slightly higher proportion of CMV- positive patients are on the high risk grade compared to the CMV- negative (71% vs.67%). The Cumulative Illness Rating Scale (CIRS) is used for the physical assessment of impairment of patients under 13 near independent areas. It is a 5-point rating of the degree of severity scale that ranges from none (1) to extremely severe (5) (Linn et al., 1968). There is no significant difference in the proportion of patients in Table 3-1 due to CIRS. Also, as can be seen in Table 3-1, there is no significant difference in the clinicopathological

variables of patients included in the two arms of study cohorts, the CMV- positive and CMV- negative.

Table 3-1: The comparison of baseline characteristics with CMV infection status of patients studied in this chapter and Chapter 4

Characteristics	Total (n=42) *	CMV Status (n=42)		
		Positive (n=21) *	Negative (n=21) *	P **
Age Median (IQR)	71.5 (68 – 75)	73.0 (70 – 76)	69 (66 – 73)	0.055
Gender N (%)				1.000
Male	17 (40.5)	9 (42.9)	8 (38.1)	
Female	25 (59.5)	12 (57.1)	13 (61.9)	
Haemoglobin (g/dL), Median (IQR)	13.2 (9.25 - 20.75)	13.2 (11.5-14.6)	13.2 (11.6 - 14.1)	0.812
Ann Arbor stage N (%)				1.000
2	6 (14.3)	3 (14.3)	3 (14.3)	
3	14 (33.3)	7 (33.3)	7 (33.3)	
4	22 (52.4)	11 (52.4)	11 (52.4)	
CIRS Score N (%)				1.000
≤5	34 (80.9)	17 (81.0)	17 (81.0)	
>5	8 (19.1)	4 (19.0)	4 (19.0)	
No. lymph nodes N (%)				0.666
1	1 (2.4)	0 (0.0	1 (4.8)	
2	1 (2.4)	1 (4.8)	0 (0.0)	
3	3 (7.1)	1 (4.8)	2 (9.5)	
4	4 (9.5)	2 (9.5)	2 (9.5)	
5	2 (4.8)	2 (9.5)	0 (0.0)	
6	31 (73.8)	15 (71.4)	16 (76.2)	
LDH Median (IQR)	385 (266-481)	388 (216-453)	382 (349-492)	0.584
FLIPI Score N (%)				1.000
0-1 (low risk)	3 (7.1)	1 (4.8)	2 (9.5)	
2 (intermediate risk)	10 (23.8)	5 (23.8)	5 (23.8)	
3-5 (high risk)	29 (69.1)	15 (71.4)	14 (66.7)	
Histology N (%)				0.541
1	12 (28.6)	5 (23.8)	7 (33.3)	
2	12 (28.6)	13 (61.9)	9 (42.9)	
3	8 (19.0)	3 (14.3)	5(23.8)	

*For continuous variables this refers to the Mean (SD), or Median (IQR) if indicated
**P value by x^2 test or Fisher's exact test for the difference between categorical variables or t test for the difference between two means.

3.3 The conjugated monoclonal antibodies

The list and description of the conjugated monoclonal antibodies (mAbs) used for the FACS analysis are found in Table 3-2.

Table 3-2: The description and sources of the mAbs used for the FACS

S/#	mAbs	Format	Clone	Isotype	Cat #	Supplier
1	CD3	FITC	UCHT1 (RUO)	Mouse IgG_1, κ	555916	BD Biosciences
2	CD3	PERCP-CY5.5	UCHT1	Mouse IgG_1, κ	560835	BD Biosciences
3	CD4	APC-H7	RPA-T4 (RUO)	Mouse IgG_1, κ	560158	BD Biosciences
4	CD8	AF700	RPA-T8 (RUO)	Mouse IgG_1, κ	557945	BD Biosciences
5	CD11a	PE-CY7	HI111 (RUO)	Mouse IgG_1, κ	561387	BD Biosciences
6	CD14	PE-CY7	M5E2 (RUO)	Mouse IgG_{2a}, κ	557742	BD Biosciences
7	CD16	PE-CF594	3G8 (RUO)	Mouse IgG_1, κ	562293	BD Biosciences
8	CD19	BV650	SJ25C1(SJ25-C1) (RUO)	Mouse IgG_1, κ	563226	BD Biosciences
9	CD25	PE-CF594	M-A251 (RUO)	Mouse IgG_1, κ	562403	BD Biosciences
10	CD27	PERCP-CY5.5	M-T271 (RUO)	Mouse IgG_1, κ	560612	BD Biosciences
11	CD28	PE	CD28.2 (RUO)	Mouse IgG_1, κ	555729	BD Biosciences
12	CD43	BV421	1G10 (RUO)	Mouse IgG_1, κ	562916	BD Biosciences
13	CD45	BV510	HI30 (RUO)	Mouse IgG_1, κ	563204	BD Biosciences
14	CD45	BV650	HI30 (RUO)	Mouse IgG_1, κ	563717	BD Biosciences
15	CD45RA	BV510	HI100 (RUO)	Mouse IgG_{2b}, κ	563031	BD Biosciences
16	CD45RO	BV605	UCHL1 (RUO)	Mouse IgG_{2a}, κ	562791	BD Biosciences
17	CD56	PERCP-CY5.5	B159 (RUO)	Mouse IgG_1, κ	560842	BD Biosciences
18	CD57	APC	NK-1 (RUO)	Mouse IgM, κ	560845	BD Biosciences
19	CD62L	APC	DREG-56 (RUO)	Mouse IgG_1, κ	559772	BD Biosciences
20	CD62L	BV605	DREG-56 (RUO)	Mouse IgG_1, κ	562719	BD Biosciences
21	CD69	PE-CY7	FN50 (FN50 (RUO))	Mouse IgG_1, κ	557745	BD Biosciences
22	CD127	BV510	HIL-7R- M21 (RUO)	Mouse IgG_1, κ	563086	BD Biosciences
23	CD197 (CCR7)	BV421	150503 (RUO)	Mouse IgG_{2a}	562555	BD Biosciences
24	CD279 (PD-1)	PE	EH12.1 (EH12) (RUO)	Mouse IgG_1, κ	560795	BD Biosciences
25	LAG-3	FITC	17B4	Mouse IgG_1	ALX-804-806-C100	Enzo Life Sciences
26	TIM-3	APC	344823	Rat IgG2A	FAB2365A	R & D Systems
27	Istotype control	BV650	X40 (RUO)	Mouse IgG_1, κ	563231	BD Biosciences
28	Istotype control	BV510	X40 (RUO)	Mouse IgG_1, κ	562946	BD Biosciences

All mAbs were purchased from BD Bioscience, Oxford UK with exception of LAG-3-FITC (Enzo Life Sciences, UK) and TIM-3-APC (R & D Systems, UK). On arrival, all mAbs were kept in the fridge and any reagent that expired in the course of time were removed and replaced with new vials of preferably similar batches and working volumes determined.

3.4 Establishment of FACS method for the study

The 3-tube multicolour FACS design illustrated in Figure 3-1 is aimed at defining the several T and NK cell subpopulations in the peripheral blood. Tube 1 contains 10 conjugated mAbs listed in Figure 3-1 designed to analyse for senescent T cells (CD57+CD28-), NKT and NK cells (CD56 & CD16) of the study cohort, as well as B cells (CD19+) and monocytes (CD14+) gated as controls.

TUBE #1	TUBE #2	TUBE #3
CD3-FITC (20)	CD3-FITC (20)	LAG-3-FITC (10)
CD56-PERCP-CY5.5 (5)	CD27-PERCP-CY5.5 (5)	CD3-PERCP-CY5.5 (2.5)
CD57-APC (10)	CD62L-APC (20)	TIM-3-APC (5)
CD8-AF700 (2.5)	CD8-AF700 (2.5)	CD8-AF700 (2.5)
CD4-APC-H7 (10)	CD4-APC-H7 (10)	CD4-APC-H7 (10)
-	CD197 (CCR7)-BV421 (10)	CD43-BV421 (10)
CD45-BV510 (5)	CD45RA-BV510 (10)	CD127-BV510 (5)
-	CD45RO-BV605 (2.5)	CD62L-BV605 (10)
CD19-BV650 (5)	CD45-BV650 (10)	CD45-BV650 (10)
CD28-PE (20)	CD28-PE (20)	CD279 (PD-1)-PE (10)
CD16-PE-CF594 (5)	CD25-PE-CF594 (10)	-
CD14-PE-CY7 (5)	CD11a-PE-CY7 (2.5)	CD69-PE-CY7 (5)
Senescent T cells	Naïve T cells	Exhausted T Cells
NKT cells	Central Memory T (T_{CM}) cells	
NK cells	Effector Memory T T_{EM}) cells	
Monocytes	Effector T cells	
B cells	Regulatory T Cells (Tregs)	

Figure 3-1: Illustrating the 3-tube design and the respective panels of conjugated mAbs.

The values in brackets represent the respective working volumes of the mAbs in microlitres.

Tube 2 contains 12 mAbs and is dedicated to examining the frequencies of the different differentiation stages to T cells including the naïve (CD45RA+CD45RO-CCR7+CD62L+), central memory (CD45RA-CD45RO+CCR7+CD62L+), effector memory (CD45RA-CD45RO+CCR7-CD62L-) and effector (CD45RA+CD45RO-CCR7-CD62L-) T cells. Terminally differentiated T cells (TEMRA) subtypes are also defined using CD27 and CD28 as well as those of regulatory T cells (Tregs) (CD4+CD25+). The tube 3, an 11 mAbs panel is committed to the analysis of T cell exhaustion markers including PD-1, LAG-3, and TIM-3.

Due to variations in the availability of material and the number of gated T cells (CD3+), the number of events acquired per tube was between 100,000 and 2,000,000, with majority of samples yielding 500,000 events and above. Isotype controls and single stained fluorescence histograms were used to gate for cut-offs for distinct lymphocyte populations on Diva software platform using respective surface markers.

The number of events, percent parent and percent total events of the various cell types and subtypes are automatically defined and displayed using FACSDiva software. Conventionally, this provides the needed values for measuring the percent of lymphocyte subpopulations within the lymphocyte gate. A comparative analysis of the median values of percent parent (relative) frequencies of the different lymphocyte subtypes according to the CMV status was carried out using a nonparametric Mann-Whitney U-test analysis to determine the significant difference. A p-value of < 0.05 is considered significant.

3.5 Results of T & NK cell subtypes

The analysis of cell surface markers has been used to define the various lymphocyte populations in stored PBMC samples using multiparametric flow cytometry. Essential FACS optimisation steps aimed at minimising errors and generating reliable results and subsequent interpretations were strictly adopted. Of note, the right approach to FACS data analyses and presentation of the cell population of interest are as important as cells acquisition design. Before commencement of the FACS experiments, optimal working volumes of fluorochrome-conjugated antibodies were determined, contour and dot plots were evaluated against polygon and rectangle gating strategies respectively, gating strategies for the three FACS tubes were evaluated, and fresh versus frozen FACS assessed.

3.5.1 An assessment of gating Strategies

Routinely, the dot or quantile contour plots are applied to display FACS data for gating and analysis. The contour plots have been shown to produce a more reliable data representation than dot plots by maintaining the contours in the quantile contour plots despite the number of observations displayed (Herzenberg et al., 2006, Tung et al., 2004). In dot plots, higher numbers of events tend to get the dot plots saturated resulting in difficulties in gating for the populations of interest for analysis (Tung et al., 2004).

The assessment of gating Strategies is set to evaluate the possible impact of using different gating strategies and plots on FACSDiva Software (BD Biosciences, Oxford, UK) in the analysis of the various cell populations and the overall analysis of data. Common antibody markers included in the three FACS tubes including anti-CD45, -CD3, -CD4 and -CD8 were considered for the assessment. A fresh PBMC from healthy volunteer sample coded 3457 was stained with the different monoclonal antibodies, and cells acquired using FACSDiva Software.

The first two upper rows (A & B panels) in Figure 3-2 depicts rectangle and polygon gating strategies for CD45-BV510 (Amcyan channel on Fortessa), CD3-Alexa Fluor 488, CD8-Alexa Fluor 700 and CD4-APC-Cy7 populations displayed on Contour plots. Interestingly, no pronounced difference in the percent population of the cells due to gating method observed. Similarly, the two lower rows (C & D panels) in Figure 3-2 illustrates rectangle and polygon gating strategies for CD45-BV510 (Amcyan channel on Fotessa), CD3-Alexa Fluor 488, CD8-Alexa Fluor 700 and CD4-APC-Cy7 populations displayed on dot plots. Again, no noticeable difference in the percentage cell populations was observed between the two gating techniques. Also, no huge difference in the percentage expression of gated cell types across the

two categories of plots is observed. Consequently, this study has adopted the use of both contour and dot plots and has applied both rectangular and polygon gating strategies individually or in combination depending on the evaluation type and the nature of discreteness of the cell populations of interest.

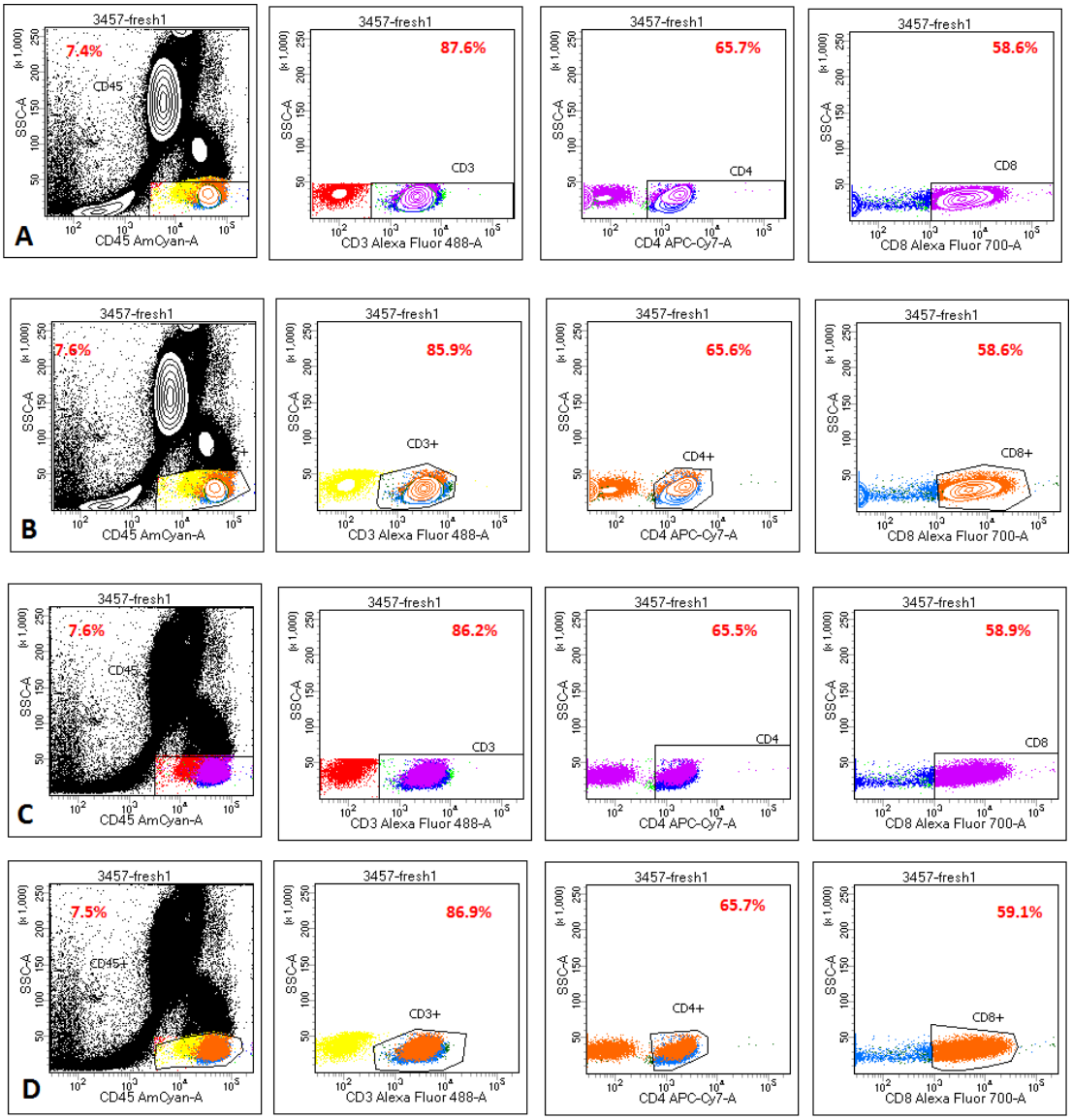


Figure 3-2: Evaluating gating Strategies.

Contour plots (A and B panels) and dot plots (C and D panels) against polygon gating and rectangle gating strategies for adoption in the analysis of data on Diva Software (BD Biosciences, Oxford, UK).

3.5.2 Illustrations of gating strategies for the FACS tubes

In the FACS design, consideration was made for the analysis of the various T cell populations by purposefully incorporating CD45, CD3, CD4 and CD8 markers in all the three FACS tubes to define hierarchy and facilitate data analysis. In the gating strategy for T-cell subpopulations and subpopulations, the first thing was to display cells based on the forward scatter (FSC) for size and side scatter (SSC) for cell characteristics to identify the various cell populations present in the suspension. In all the tubes, analysis commenced with the gating of CD45, being the leukocyte-common antigen (L-CA) and all the cells of interest possess CD45 surface marker.

The percentage circulating T cells was determined using the membrane determinant CD3. Due to high chance of dual positivity for CD4 and CD8 subtypes of CD3 surface markers, CD4-APC-Cy7 and CD8-Alexa Fluor 700 were identified and gated from the CD3 population independently on the FSC axis. Figure 3-3 demonstrates the gating strategy adopted for tube one, which is a 9-parameter, 10-colour FACS designs as defined by the hierarchical tree in Figure 3-4. Senescent CD4 and CD8 populations were defined by plotting CD57-APC versus CD28- PE and gating for CD57+CD28-. For NKT cells, CD56-PerCp-Cy5.5 versus CD16-PE-CF594 were gated from CD3-FITC and NKT cells population (CD3+CD56+CD16+) was defined. NK cell subcategories were determined from the CD45-BV510 population by gating CD56-PerCp-cY5.5 versus CD16-PE-CF594. This population is CD3-. Also, monocytes and B cells were also identified by gating for CD14-PE-Cy7 and CD19-BV650 from the CD45-BV510 respectively.

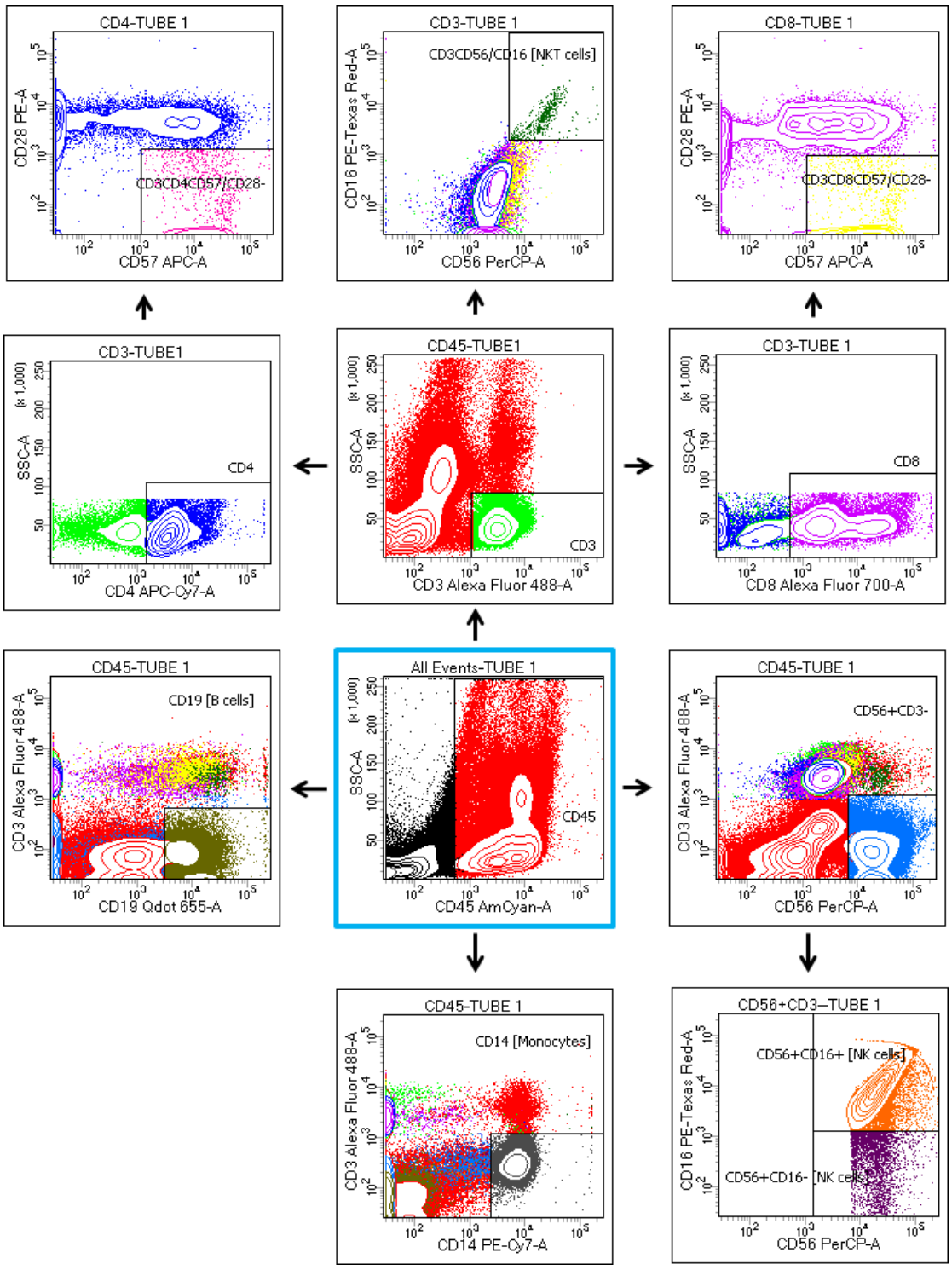
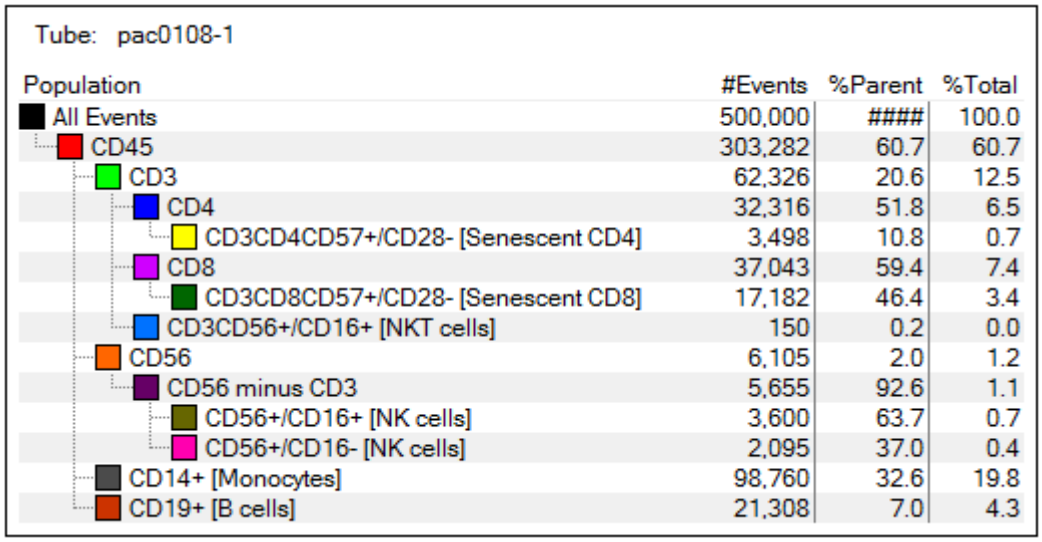


Figure 3-3: Gating Strategy adopted for tube 1 of FACS design.

Contour plots and rectangular gates identify populations of interest. All cell markers were gated from CD45 AmCyan-A (highlighted blue) and the arrows indicate the origin of parent cells while the markers on the top of each plot represent the parent name. The markers of interest for each plot are labelled on the x-and y-axis.

The arrows show the direction of the gating. From the gating strategy in Figure 3-3 and the hierarchy used in differentiating the various cells types as display in Figure 3-4, gating began with the identification of CD45+ population in all the three tubes on the FACS design. In tube 1, four main populations were gated from the leukocyte common antigen (CD45) including CD3-Alexa Fluor-488 (T cells), CD14-PE-Cy7 (Monocytes), CD19-Qdot-655 (B cells) and CD56-PerCP (NK cells). Other downstream cell populations that were subsequently gated from CD3+ populations include senescent T cells and NKT cell populations. Figure 3-4 illustrated the hierarchy through which different cell markers were used to identify the various cell types in the tube 1 of the FACS design.



Tube: pac0108-1

Population	#Events	%Parent	%Total
All Events	500,000	####	100.0
CD45	303,282	60.7	60.7
CD3	62,326	20.6	12.5
CD4	32,316	51.8	6.5
CD3CD4CD57+/CD28- [Senescent CD4]	3,498	10.8	0.7
CD8	37,043	59.4	7.4
CD3CD8CD57+/CD28- [Senescent CD8]	17,182	46.4	3.4
CD3CD56+/CD16+ [NKT cells]	150	0.2	0.0
CD56	6,105	2.0	1.2
CD56 minus CD3	5,655	92.6	1.1
CD56+/CD16+ [NK cells]	3,600	63.7	0.7
CD56+/CD16- [NK cells]	2,095	37.0	0.4
CD14+ [Monocytes]	98,760	32.6	19.8
CD19+ [B cells]	21,308	7.0	4.3

Figure 3-4: A hierarchical procedure used for analysis of cell types tested in tube 1.

A PACIFICO trial stored PBMC sample (pac0108) was used for this illustration showing number of events (#Events), percent parent cell (%Parent) and percent total (%Total) as displayed on FACSDiva software.

The contour plot gating approach adopted for tube 2 is shown in Figure 3-5, a 14-parameter, 12-colour FACS design. It is planned to define Naïve (N), central memory

(CM), effector memory (EM), Effector (E) T cells and terminally differentiated (TEMRA) T cells plus regulatory T cells (Tregs) as can be seen in the cells hierarchal distinctions in Figure 3-6. In this design, CD45RA-BV510 and CD45RO-BV605 were gated separately from CD4 and CD8 populations respectively due to the likely existence of double-positive CD45RA+CD45RO+ T cells (Sandberg et al., 2001). Broadly, this categorises CD4 and CD8 populations into Naïve and memory T cells, an important platform for further definitions of the subsets of T cells using appropriate markers. To further discriminate between CM and EM populations and between naïve and effector populations, CD197 (CCR7)-BV421, a chemokine receptor was gated from both CD45RA (naïve) and CD45RO (memory) of the CD4 and CD8 arms respectively. At this point, naïve (CD45RA+CCR7+), effector (CD45RO+CCR7-), CM (CD45RO+CCR7+) and EM (CD45RO+CCR7-) populations for CD4 and CD8 are defined. To increase the specificity of naïve and CM cells and characterization of the subtypes of effector and EM T cells, CD11a a mediator of leukocyte adhesion, and the cell surface expression of CD27 and CD28, costimulatory molecules were used. Therefore, CD11a-PE-Cy7 was gated from the FSC axis of the CD45RA+CCR7+ plots for both CD4 and CD8 arms. To define and quantify naïve T cells, CD27-PerCp-Cy5.5 versus CD28-PE was gated from the CD11a population, and CD27+CD28+ events on the top right quadrants were used to define the N-CD4 and CD8 T cell populations respectively. The CM T cells (CD27+CD28+) were gated from the CD45RO+CCR7+ plots. Also, to identify and quantify the subtypes of effector and EM populations, CD27-PerCp-Cy5.5 versus CD28-PE was gated from both CD45RA+ and CD45RO+ plots. The quadrants of the contour plots defined the T cell subtypes as pE1 (CD27+CD28+), pE2 (CD27+CD28-) and effector (CD27-CD28-) populations from the CD45RA+CCR7- of the CD4 and

CD8. In a similar approach, the subtypes of EM were defined by the number of events in the quadrants of the contour plots as EM1 (CD27+CD28+), EM2 (CD27+CD28-), EM3 (CD27-CD28-) and EM4 (CD27-CD28+).

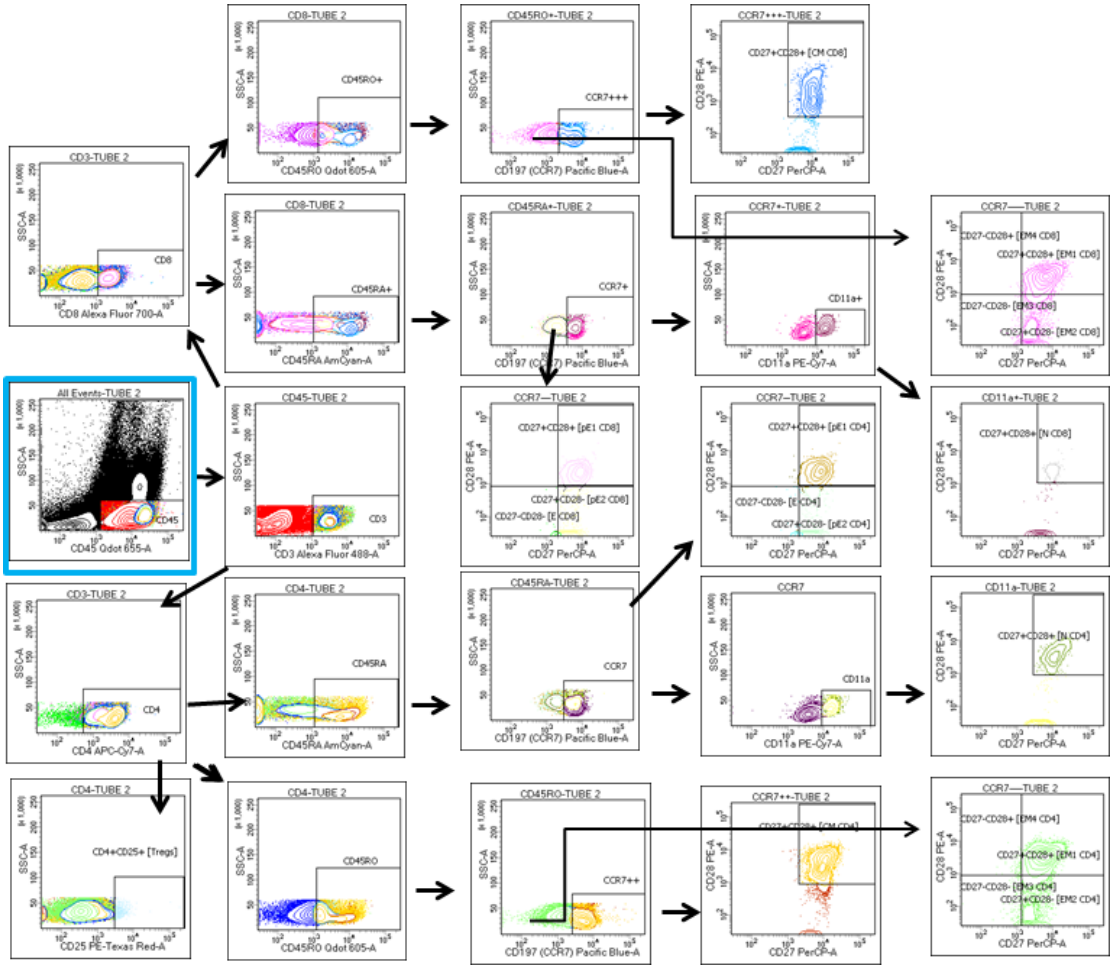


Figure 3-5: Gating Strategies adopted for tube 2 of FACS design.

Contour plots and rectangular gates identify populations of interest. All cell markers were gated from CD45 AmCyan-A (highlighted blue) and the arrows indicate the origin of parent cells while the markers on the top of each plot represent the parent name. The markers of interest for each plot are labelled on the x-and y-axis.

As a multicolour design comprising of cell surface markers, we could not include FOXP3, a specific marker for regulatory T (Treg) cells (Roncarolo and Gregori, 2008) to avoid subjecting the majority of the markers to the additional fixing and

permeabilizing requirements for the intracellular markers. Therefore, CD25-PE-CF594 was used to define the population of regulatory T cells (Tregs) gating from the FSC axis of the CD4+ plot as CD4+CD25+.

Tube: pac0108-2

Population	#Events	%Parent	%Total
All Events	500,000	####	100.0
CD45	291,941	58.4	58.4
CD3	63,649	21.8	12.7
CD4	28,439	44.7	5.7
CD45RA	16,909	59.5	3.4
CCR7	14,484	85.7	2.9
CD11a	13,936	96.2	2.8
CD27+CD28+ [N CD4]	13,235	95.0	2.6
CCR7-	2,425	14.3	0.5
CD27+CD28+ [pE1 CD4]	427	17.6	0.1
CD27+CD28- [pE2 CD4]	410	16.9	0.1
CD27-CD28- [E CD4]	1,067	44.0	0.2
CD45RO	20,246	71.2	4.0
CCR7+	13,183	65.1	2.6
CD27+CD28+ [CM CD4]	11,287	85.6	2.3
CCR7--	7,063	34.9	1.4
CD27-CD28+ [EM4 CD4]	2,722	38.5	0.5
CD27+CD28+ [EM1 CD4]	2,209	31.3	0.4
CD27-CD28- [EM3 CD4]	1,617	22.9	0.3
CD27+CD28- [EM2 CD4]	515	7.3	0.1
CD4+CD25+ [Tregs]	2,870	10.1	0.6
CD8	32,195	50.6	6.4
CD45RA+	24,274	75.4	4.9
CCR7++	1,400	5.8	0.3
CD11a+	1,358	97.0	0.3
CD27+CD28+ [N CD8]	788	58.0	0.2
CCR7---	22,874	94.2	4.6
CD27+CD28+ [pE1 CD8]	1,386	6.1	0.3
CD27+CD28- [pE2 CD8]	1,732	7.6	0.3
CD27-CD28- [E CD8]	16,936	74.0	3.4
CD45RO+	21,212	65.9	4.2
CCR7+++	1,204	5.7	0.2
CD27+CD28+ [CM CD8]	664	55.1	0.1
CCR7----	20,008	94.3	4.0
CD27-CD28+ [EM4 CD8]	4,518	22.6	0.9
CD27+CD28+ [EM1 CD8]	2,135	10.7	0.4
CD27-CD28- [EM3 CD8]	11,897	59.5	2.4
CD27+CD28- [EM2 CD8]	1,458	7.3	0.3

Figure 3-6: An example of hierarchical analysis of different cell types in tube 2.

A PACIFICO trial stored PBMC sample (pac0108) was used for this illustration showing number of events (#Events), percent parent cell (%Parent) and percent total (%Total) as displayed on FACSDiva software.

The tube 3 of the FACS design is a 6-parameter, 11– colour analysis of T cell markers of exhaustion and gating strategy is as outlined in Figure 3-7, while Figure 3-8 shows the main exhaustion markers analysed to include PD-1, LAG-3 and TIM-3 for both CD4+ and $CD8_+$ arms.

As part of T-cell subpopulations, gating for exhausted T cells commenced with the CD45+ populations through to CD4+ and CD8+ T cells as in Figure 3-7. CD43-BV421 versus CD62L-BV605 was gated from the CD4+ and CD8+ contour plots; CD127-BV510 versus CD69-PE-Cy7 was gated from the CD43+CD62L+ population. Exhaustion markers CD279 (PD-1)-PE, LAG-3-FITC and TIM-3-APC were gated individually from the CD127+CD69+ plot for CD4 and CD8 arms.

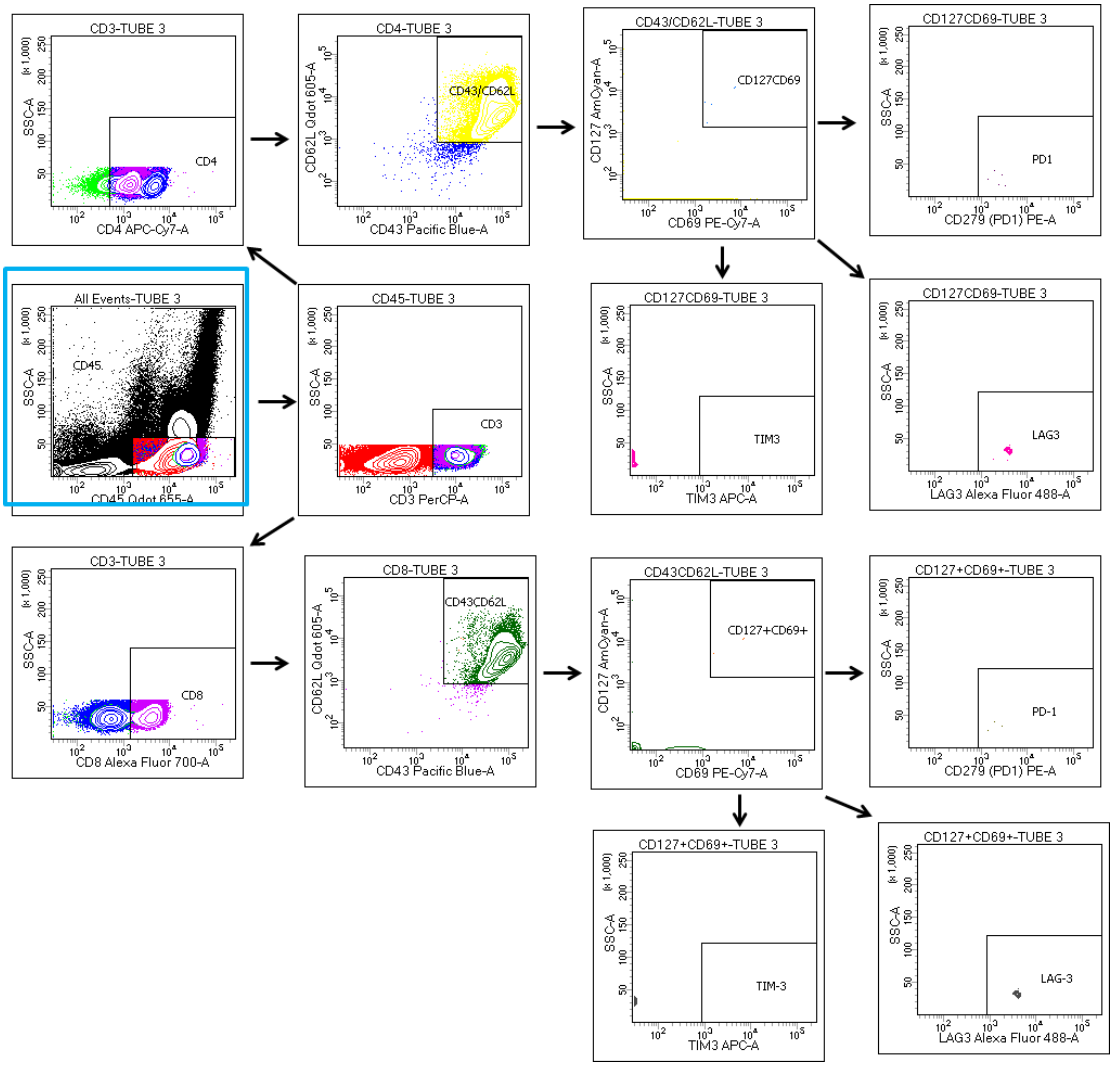


Figure 3-7: Gating Strategies for tube 3 of FACS design.

Contour plots and rectangular gates identify populations of interest. All cell markers were gated from CD45 AmCyan-A (highlighted blue) and the arrows indicate the origin of parent cells while the markers on the top of each plot represent the parent name. The markers of interest for each plot are labelled on the x-and y-axis.

Tube: pac0108-3

Population	#Events	%Parent	%Total
All Events	500,000	####	100.0
CD45	193,128	38.6	38.6
CD3	61,893	32.0	12.4
CD4	28,244	45.6	5.6
CD43+/CD62L+	2,552	9.0	0.5
CD127+/CD69+	4	0.2	0.0
PD-1	661	25.9	0.1
LAG3	4	0.2	0.0
TIM3	8	0.3	0.0
CD8	31,539	51.0	6.3
CD43/CD62L	925	2.9	0.2
CD127/CD69	4	0.4	0.0
PD1	125	13.5	0.0
LAG-3	4	0.4	0.0
TIM-3	5	0.5	0.0

Figure 3-8: An example of hierarchical analysis of different cell types in tube 3.

A PACIFICO trial stored PBMC sample (pac0108) was used for this illustration showing number of events (#Events), percent parent cell (%Parent) and percent total (%Total) as displayed on FACSDiva software

3.5.3 Fresh versus frozen FACS analysis

Because the PBMC samples used for this study were stored frozen at -80°C and for the purpose of possible clinical translational and interpretational relevance of the FACS data, it is crucial to evaluate the impact of freezing on PBMCs FACS analysis. Consequently, before the commencement of FACS analysis of the stored trial PBMC samples, a fresh sample was treated to mimic freeze-thaw experience and FACS analysis of the fresh, frozen and purified PBMCs were carried out to evaluate the effect of freeze-thaw on for the major subtypes of cells.

As demonstrated in Figure 3-9, panel A represents tube 1 of the FACS design. There was no significant variation in the percent parent cells of the various cell subpopulations except NK cells that have shown a clear reduction in the frozen and purified cells as compared to the fresh cells. By implication, FACS analysis of NK

cells may yield a more representative data by using fresh PBMC samples compared to stored PBMC samples. However, in this study, analysis of NK cell subtypes was carried out using the recoverable amount of NK cells in the thawed stored trial PBMC samples. For the FACS data from panel B (Figure 3-9; tube 2) and panel C in Figure 3-9 (Tube 3), minimal variations in the quantities of the cell types in the three phases of treated cell categories observed and importantly, all parameters are detectable in the frozen PBMCs as well as in the fresh samples. Therefore, all other parameters within the study design have been determined with an acceptable degree of confidence.

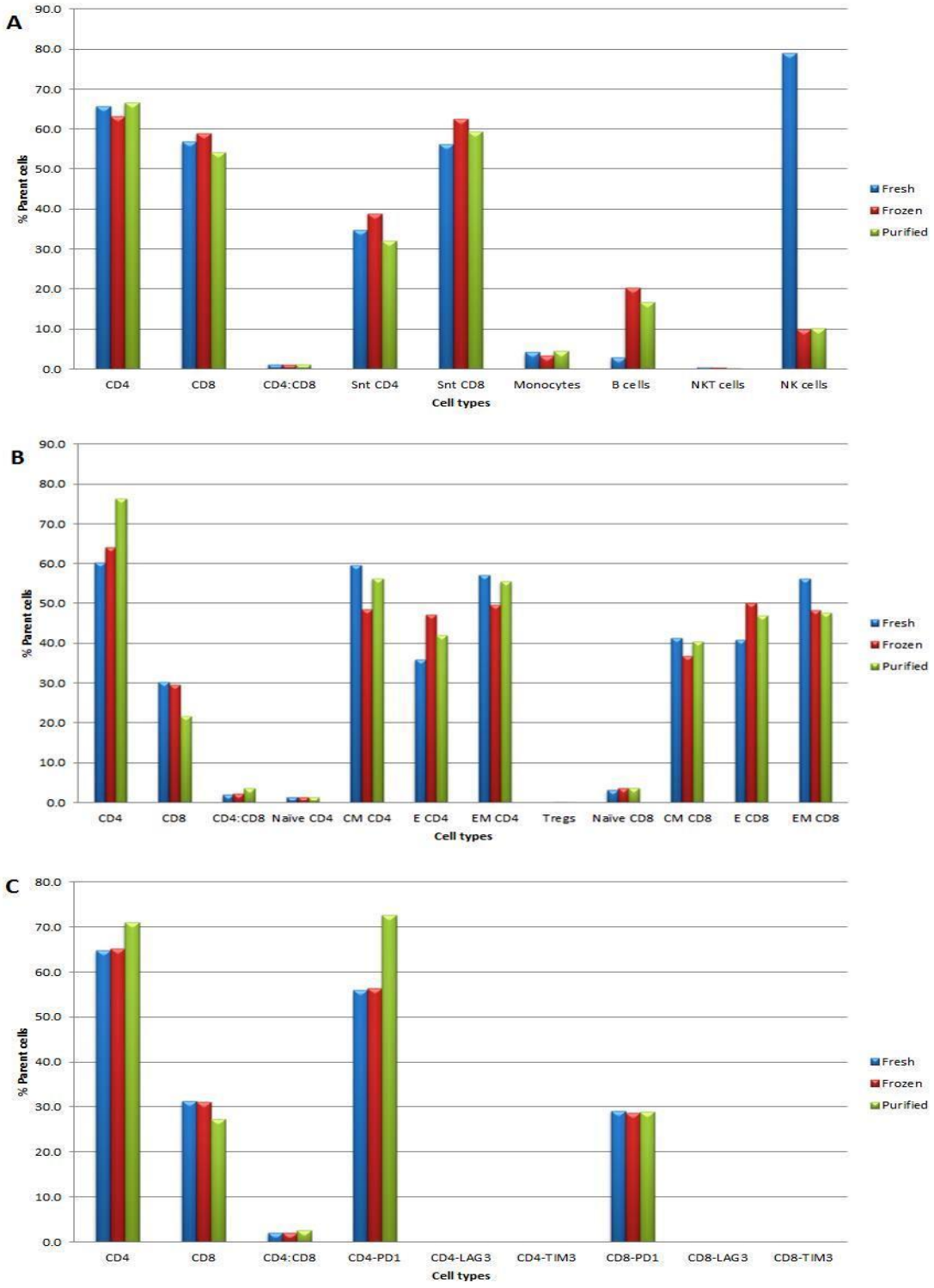


Figure 3-9: FACS comparison of fresh, frozen and purified PBMCs.

Local Biobank samples were used to compare the FACs staining and cell populations using fresh whole blood (blue bars), fresh PBMCs (green) and frozen PBMCs (red). Panels A, B and C have followed the hierarchy of cell types in Tubes 1, 2 and 3 of the FACS design.

3.5.4 The lymphocyte subtypes

Using the established flow cytometry method and analysis strategies, I successfully quantified relative percentages of T and NK cell populations from parent cells (as in Figures 3-4, 3-6 and 3-8) in samples taken from a cohort of patients with FL (see Table 3-3). Mann-Whitney U-test was used to compare the relative proportions (median percentage) of the various cell types between CMV-positive and CMV-negative groups. Of interest, statistically significant elevation in the frequencies of pre-effector-2 CD4 (pE2-CD4; CD45RA+CD27+CCR7-CD28-), effector-CD4 (E-CD4; CD45RA+CD27-CCR7-CD28-), effector memory-3 CD4 (EM3-CD4; CD45RO+CD27-CCR7-CD28-), effector-CD8 (E-CD8; CD45RA+CD27-CCR7-CD28-) and effector memory-3-CD8 (EM3-CD8; CD45RO+CD27-CCR7-CD28-) phenotypes were observed in CMV- positive compared to CMV-negative patients as shown in Table 3-3. The most striking common features of these T cell subcategories is the loss of CD28 and CCR7 (CD28-CCR7-) surface markers. The subtypes of effector cells express CD45RA+ isoform; those of the effector memory cells express CD45RO+.

Also, looking at the results in Table 3-3, a statistically significant increase in the frequency of NKT cells (p=0.031) was detected among CMV-positive patients compared to CMV- negative cohort. However, NKT cells are known to perform both protective and destructive consequences due to their abilities to produce cytokines that support either inflammatory responses or immune tolerance.

Marginal differences were observed in the percentages of CD4+ T cells and CD4-LAG3 subpopulations (p=0.051 and p=0.074; Table 3-3) due to CMV status. Also, a marginal difference is observed in the ratio of CD4+ and CD8+ T cell populations between CMV- Positive and CMV- negative groups (1.2 vs. 1.5, p=0.054; Table 3-3).

Table 3-3: The comparison of cell subtypes between patients with and without CMV infection

S#	Cell types	CMV Status			
		Total	Positive	Negative	P
		Median (IQR)	Median (IQR)	Median (IQR)	
1	Viability	78.4 (70.8-83.2)	77.9 (69.6-83.2)	80.6 (73.0-82.8)	0.458
2	CD4	72.4(56.1-88.4)	65.8 (46.3-75.0)	85.6 (68.6-93.0)	**0.051**
3	CD8	48.6 (28.8-66.1)	49.9 (38.3-64.5)	42.3 (22.5-70.0)	0.466
4	CD4:CD8	1.4 (0.9-2.6)	1.2 (0.7-1.7)	1.5 (1.0-3.3)	**0.054**
5	Snt-CD4	3.4 (0.7-10.8)	5.0 (1.0-13.2)	2.7 (0.6-7.1)	0.134
6	Snt-CD8	24.7 (15.5-46.3)	35.1 (15.5-55.1)	24.3 (16.5-37.2)	0.218
7	NKT Cells	1.15 (0.3-2.8)	1.6 (0.6-3.8)	0.5 (0.1-1.6)	**0.032**
8	Monocytes	9.9 (4.7-24.4)	15.9 (4.7-24.4)	8.6 (6.0-17.8)	0.571
9	B cells	9.2 (6.2-168)	9.3 (7.0-25.7)	9.1 (6.2-12.0)	0.589
10	N-CD4	91.5 (82.0-95.0)	90.7 (84.6-94.5)	91.5 (82.0-97.3)	0.850
11	pE1-CD4	33.9 (17.5-51.0)	33.3 (17.6-50.2)	34.5 (14.3-51.0)	0.920
12	pE2-CD4	10.6 (5.5-17.1)	10.8 (8.2-25)	7.3 (0.3-12.2)	**0.048**
13	E-CD4	18.8 (10.5-42.1)	20.2 (13.8-47.8)	11.9 (5.4-26.9)	**0.030**
14	CM-CD4	84.2 (69.6-91.7)	84.6 (71.2-91.3)	83.9 (69.6-91.7)	0.860
15	EM4-CD4	22.6 (4.0-39.0)	22.1 (9.2-35.7)	23.1 (0.4-57.6)	0.792
16	EM1-CD4	46.4 (15.8-65.7)	46.7 (37.7-57.9)	39.9 (13.2-70.0)	0.473
17	EM3-CD4	11.9 (2.3-22.1)	13.9 (9.4-33.6)	7.3 (0.7-13.7	**0.019**
18	EM2-CD4	6.2 (1.6-11.2)	6.8 (2.3-11.2)	5.2 (0.6-10.8)	0.521
19	Tregs	6.2 (2.4-15.7)	7.9 (3.8-10.9)	5.6 (1.1-17.5)	0.940
20	N-CD8	53.5 (12.0-76.5)	34.0 (12.0-63.4)	63.7 (26.8-81.6)	0.163
21	pE1-CD8	14.4 (6.1-34.6)	11.5 (6.3-31.1)	16.0 (2.1-36.1)	0.782
22	pE2-CD8	13.9 (5.4-28.9)	15.3 (6.2-25.2)	10.1 (0.8-31.1)	0.208
23	E-CD8	41.4 (14.6-64.1)	54.9 (38.1-72.1)	20.8 (7.4-49.1)	**0.035**
24	CM-CD8	62.2 (25.8-75.7)	59.4 (17.8-74.4)	63.6 (42.8-84.5)	0.521
25	EM4-CD8	10.9 (1.3-22.6)	7.1 (1.9-16.8)	17.6 (0.7-35.0)	0.279
26	EM1-CD8	25.6 (10.9-47.9)	20.9 (12.1-41.0)	26.6 (6.9-47.9)	0.850
27	EM3-CD8	31.6 (12.1-53.7)	46.5 (27.5-63.1)	15.5 (7.1-34.2)	**0.005**
28	EM2-CD8	8.7 (5.3-20.0)	9.4 (6.8-16.9)	7.6 (1.5-20.4)	0.563
29	PD1-CD4	49.7 (16.5-91.3)	62.5 (25.5-91.8)	43.3 (5.3-84.6)	0.151
30	LAG3-CD4	31.2 (2.6-83.3)	42.0 (11.1-95.7)	15.5 (2.0-66.7)	**0.074**
31	TIM3-CD4	0.3 (0.0-16.7)	2.0 (0.0 -26.0)	0.1 (0.0- 2.6)	0.172
32	PD1-CD8	25.0 (4.5-70.5)	29.5 (9.1-65.2)	12.5 (4.4- 70.5)	0.358
33	LAG3-CD8	6.4 (0.8-51.6)	12.8 (1.9- 82.5)	4.1 (0.0- 31.9)	0.278
34	TIM3-CD8	0.5 (0.0-25.0)	1.6 (0.0- 25.7)	0.4 (0.0 -5.6)	0.435
35	CD56+/CD16+	87.2 (68.8-95.4)	88.6 (69.5-95.3)	83.7 (68.8-95.9)	0.935
36	CD56+/CD16-	12.9 (4.6-31.9)	11.5 (4.7-30.5)	16.4 (4.1-31.9)	0.935

The table shows median relative percentages of cells types as determined from the parent populations in the hierarchical procedure in Figures 3-4, 3-6 and 3-8. The cell markers analysed include CD4, CD8, the ratio of CD4 to CD8 (CD4: CD8), senescent CD4 (Snt-CD4), CD8 (Snt-CD8), natural killer T cells (NKT cells), monocytes, B cells, Naïve CD4 (N-CD4), pre-effector - 1 CD4 (pE1-CD4), pre-effector - 2 CD4 (pE2-CD4), effector CD4 (E-CD4), central memory CD4 (CM-CD4), effector

memory -1 - CD4 (EM1-CD4), effector memory -2 - CD4 (EM2-CD4), effector memory -3 - CD4 (EM3-CD4), effector memory -4 - CD4 (EM4-CD4), regulatory T cells (Tregs), naïve CD8 (N-CD8), pre-effector - 1 CD8 (pE1-CD8), pre-effector - 2 CD8 (pE2-CD8), effector CD8 (E-CD8), central memory CD8 (CM-CD8), effector memory -1 - CD8 (EM1-CD8), effector memory -2 - CD8 (EM2-CD8), effector memory -3 - CD8 (EM3-CD8), effector memory -4 - CD8 (EM4-CD8), Programmed cell death protein- 1 CD4 (PD1-CD4), PD1-CD8, Lymphocyte-activation gene -3 CD4 (LAG3-CD4), Lymphocyte-activation gene -3 CD8 (LAG3-CD8), T-cell immunoglobulin and mucin-domain containing-3 CD4 (TIM3-CD4), T-cell immunoglobulin and mucin-domain containing-3 CD8 (TIM3-CD8), natural killer cells (CD56+/CD16+ and CD56+/CD16-).

The CD4+ T cells have shown a reduced frequency in the CMV- positive FL patients compared to CMV- negative group. Notably, CD4+ T cells alongside CD8+T cells forms the bulk of T-lymphocytes. The $CD4^{+}$T cells are critical in attaining a coordinated, efficient immune response to pathogenic agents. Following activation, CD4+T cells differentiate into distinct effector subtypes that participate actively in mediating immune response by secreting distinct cytokines and partake in many functions. The functional activities of CD4+ T cells cover from the activation of the cells of the natural immune system, B-lymphocytes, cytotoxic T cells, as well as nonimmune cells, and also play a crucial part in the elimination of immune response. Although no striking observation is made on the values of exhaustion surface markers analysed, higher expression values of CD4+ and CD8+ PD-1, LAG-3 and TIM-3 (Table 3-3) phenotypes are seen among CMV- positive cohort compared to CMV-negative arm. The CMV-negative group presents a marginal increase in the median value of CD4: CD8 ratio of 1.5 against 1.1 of CMV- positive FL patients.

Also, no significant difference in quantities is observed of senescent CD4+ and CD8+ T cells in the study population. However, the CMV-positive patients show an

increased expression trend for both senescent CD4+ and senescent CD8+ T cell populations compared to the negative FL patients.

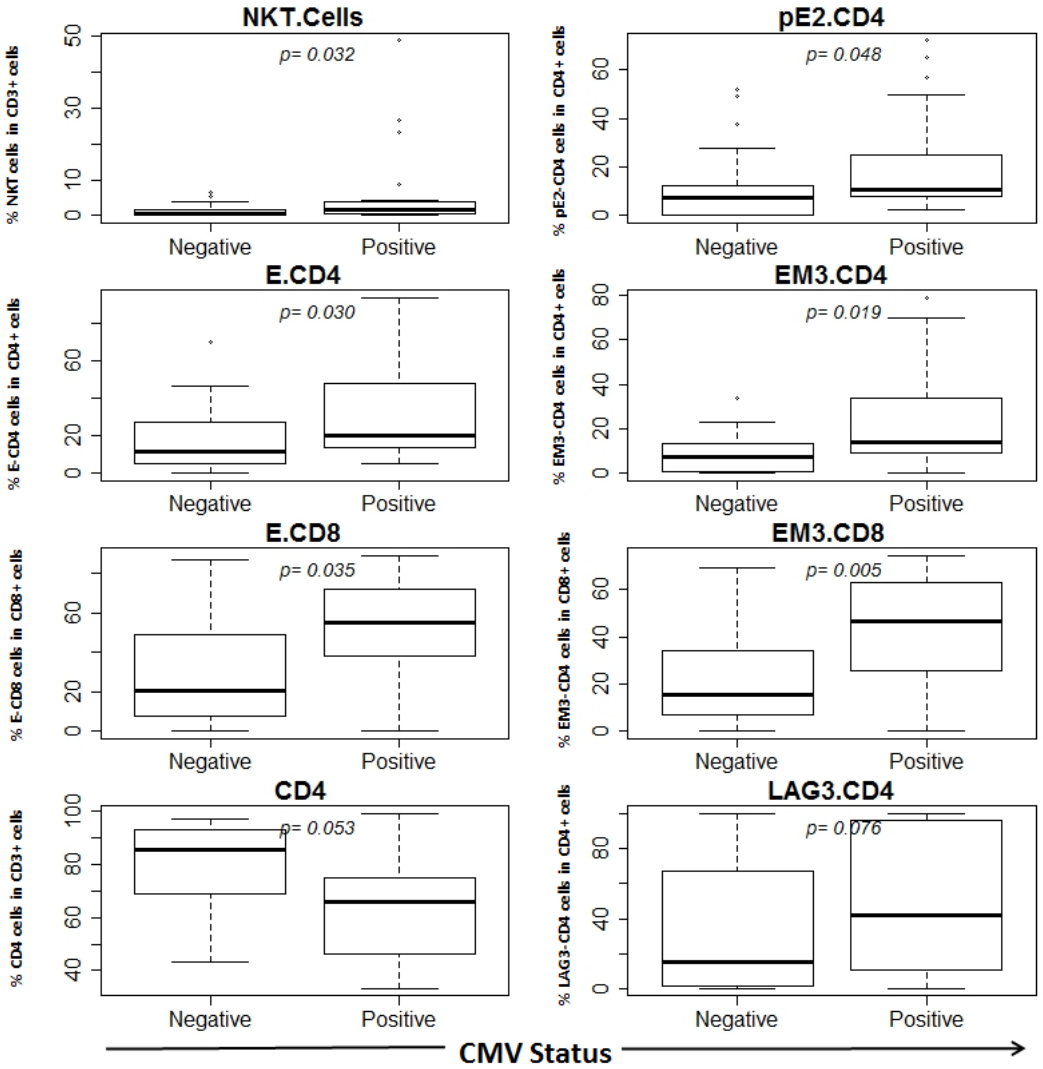


Figure 3-10: Boxplots of end-stage T cell subtypes and NKT cells that show significant difference between CMV-positive and -negative patients.

3.6 Discussions

The FACS analysis of the stored PBMC samples of patients have revealed elevated quantities of subtypes of end-stage terminally differentiated (TEMRA) T cells from both the CD4+ and CD8+ T cell compartments as well as NKT cells in the CMV-positive patients compared to the CMV- negative group. In details, these data show

that pre-effector-2 (pE2) (CD45RA+CD27+CCR7-CD28-), effector (CD45RA+CD27-CCR7-CD28-) and effector memory-3 (EM3) (CD45RO+CD27-CCR7-CD28-) subtypes of the CD4+ T cell compartment, as well as effector (CD45RA+CD27-CCR7-CD28-) and effector memory-3 (EM3) (CD45RO+CD27-CCR7-CD28-) phenotypes of the CD8+ T cell compartment accumulated more in the CMV-positive patients. Phenotypically, the terminally nonproliferating effector T cell subsets usually lack the costimulatory receptors CD27 and CD28 as well as lymph node homing receptors CCR7 and CD62L on the cells surface, and are variously referred to as TEMRA, CD45RA+ memory, terminally differentiated (T_{TD}) or end-stage or persisting effector T cells (Pawelec et al., 2005). As a result, these cells are characterised by diminished long-term memory potential, limited effector function and poor cytokine production resulting in impaired immune functions capable of causing increased vulnerability to infections (Wherry, 2011, Rothe et al., 2016).

CD4+ and CD8+T cells constitute the majority of the T-lymphocytes in the peripheral blood. Both of which possesses specific T cell receptors for efficient adaptive immunological responses. The high accumulation of nonproliferating versions of these cells portends immune deficits for the patients. Moreover, effector T cells that display TEMRA phenotypes have been reported to demonstrate senescence and end-stage differentiation and are found eminently increased in both CD4+ and CD8+ T cell arms, especially in association with ageing and CMV infection (Derhovanessian et al., 2011, Koch et al., 2008a). A close look at the differentiation phases of T cells in Figure 3-11 identifies terminal effector T cells (TEMRA) as the most differentiated subtype of memory T cells, which are known to be extremely susceptible to apoptosis and display high levels of perforin and Fas ligand (cytotoxic molecules) (Woodbury, 1996).

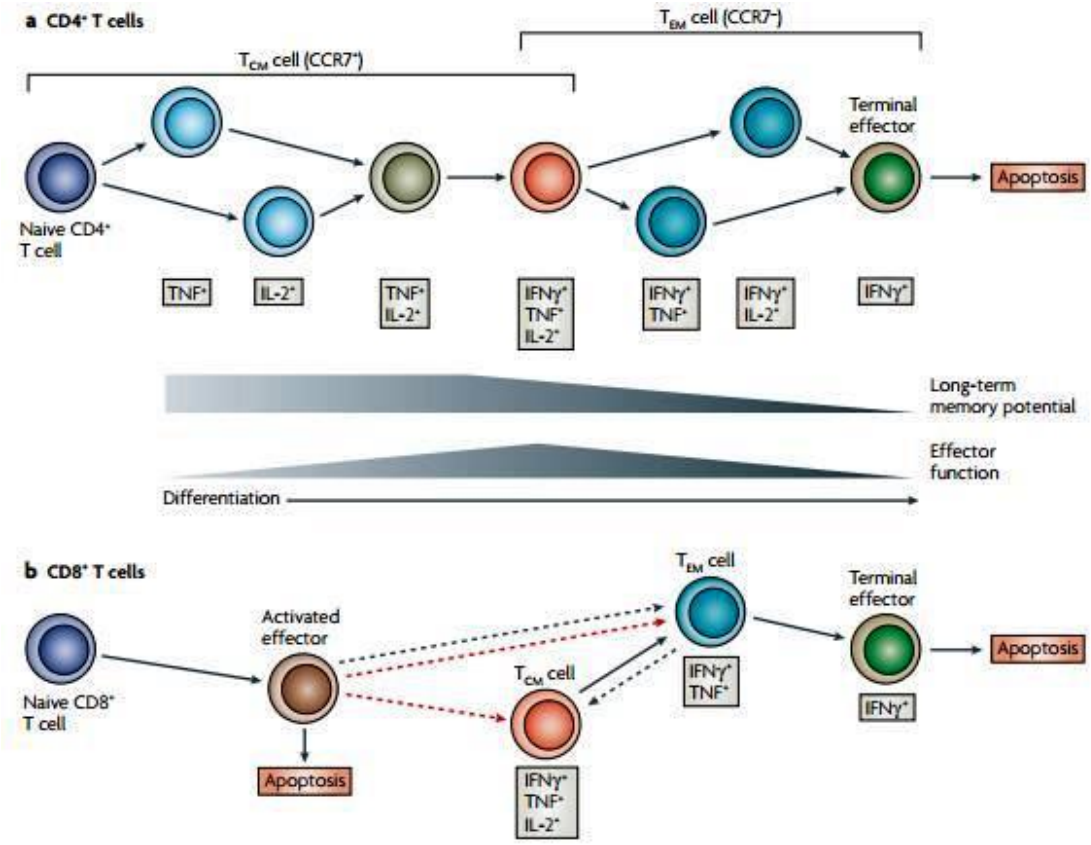


Figure 3-11: Illustrations of distinct and developmental phases of effector and memory T‑cells.

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Although higher proportion of NKT cells is observed in the CMV-positive FL patients, a functional analysis of these cells would be required to help in assessing the functional potential of the high frequency cells in FL. However, NKT cells are known to share properties of both NK cells and T cells. They act rapidly to stimuli, a characteristic of the innate arm of the immunity, and can produce cytokines such as IL-2, IFN-γ, TNF-α, and IL-4 typical of adaptive immunity to promote or suppress different immune responses (Liao et al., 2013). Of interest, the NKT cells have been shown to play a role in tumour immunity. Particularly, it has been demonstrated that

poor IFN-γ production due to defective NKT cells was observed in patients with progressive malignant multiple myeloma but not in non-progressive myeloma or premalignant gammopathy (Terabe and Berzofsky, 2008, Dhodapkar et al., 2003). Furthermore, an analysis of peripheral blood has reported a statistically significant reduction in the quantities of NKT cells in patients with some solid tumours in relation to healthy persons (Terabe and Berzofsky, 2008, Giaccone et al., 2002). Also, decreased production of IFN-γ or proliferation by type I NKT cells associated with cancer patients, and low circulating levels of type I NKT cells was an independent predictor of poor O/S and DFS in patients with head and neck squamous cell carcinoma (Molling et al., 2007). Conversely, the type II NKT cells mediates a regulatory opposite role in tumour immunity, for instance, activation of type II NKT cells by sulfatide enhanced tumour burden. But because we did not stratify the NKT cells into its subtypes, it is not possible to conclude which subtype of NKT cells is elevated in the CMV-positive FL patients and what impact of such increase on clinical outcome of these patients. However, drawing from the functional profile of NKT cells, it is also sensible to infer that the CMV-positive FL will either suffer more recurrent infections and AEs as well as poor response to therapies. It is also suggested that the increase in NKT cells in CMV- positive cases might be a compensatory response to the reduction in adaptive immunity resulting from reduced CD4+ T cells and accumulation of exhausted T cells reported in Table 3-3.

Conversely, although marginally significant, CMV-positive patients are associated with decreased CD4+T cells ($p=0.051$, Table 3-3) were associated with CMV-negative FL patients compared to the CMV-positive group. Of note, CD4+ T helper (T_H) and cytotoxic CD8+ T lymphocytes (CTL) constitute the bulk of T-lymphocytes. Importantly, CD4+ T cells which comprise of different lineages elicit immune

responses via the production of distinct cytokines and are capable of performing multiple functions including activation of a host of immune and non-immune cells such as B-lymphocytes and cytotoxic CD8+ T cells. In this regard, the CMV-negative FL patients possibly have some immunologic advantage over the CMV-positive patients. Furthermore, although there is no significant difference in the quantities of the Naïve-CD4+ and -CD8+ T cell phenotypes due to CMV status, the CMV- negative patient have shown a trend of higher median values for both naïve CD4+ and CD8+ T cells compared to the CMV- positive group (Table 3-3). It is important to mention that having sufficient quantities of naïve T cells provide a more virile response and protection against infections by novel pathogens (Pennock et al., 2013).

The multicolour flow cytometric analysis employed by the current study seems to demonstrate for the first time an analysis of a large number of subpopulations of T cells in FL, especially in relation to CMV infection. However, to secure a clear-cut interpretation of flow cytometric analysis of circulating lymphocyte subtypes, a functional analysis of the various T cell subsets is suggested. In the meantime, an exploratory analysis relating the quantities of the cell types to clinical data, involving treatment response status and toxicities records of the trial patients will help in providing a further understanding of the clinical implications of findings (see Chapter 7). Also, it is well known fact that in a deliberate effort to keep the CMV under checks, the immune profile of a latent CMV-infected individual is adversely affected, particularly leading to an expanded population of CMV-specific memory CD8+ T cells, in a memory inflation phenomenon (Klenerman and Oxenius, 2016). Unfortunately, it was not within the scope of this study to investigate this important

hallmark of CMV infection by evaluating for CMV-specific T cells and functional study to provide further support to findings.

Although not much has been reported on CD4 TEMRA cells, the phenomenon of expanded end stage T cells in the peripheral blood has been reported by researchers in relation to other clinical conditions. For instance, a study by Reinke et al has reported a significant correlation between elevated levels of end stage CD8 (+) effector memory T (TEMRA) cells in peripheral blood with delayed fracture healing (Reinke et al., 2013). Their study has provided a clear illustration of the role of T cells in modulating endogenous bone fracture repair even in the absence of infection. Another study that employed Cox regression model reported a 2-fold higher risk of late graft dysfunction among kidney transplant recipients who had increased levels of differentiated TEMRA CD8+ T cells before transplantation (Yap et al., 2014). Also, an increased accumulation of terminally differentiated CD8 T cells (TEMRA) has been reported in chronic graft-versus-host disease (cGVHD) as a consequence of continuous differentiation from naïve/central memory T cells to TEMRA cells following prolonged alloantigen exposure (D'Asaro et al., 2006). However, a study that also determined the frequency of subtypes of peripheral T-cells including CD8 TEMRA cells before kidney transplantation showed that advanced end-stage renal disease (ESRD) related with T-cell dysregulation is associated with a relative expansion of CD8 TEMRA cells guards against the acute rejection of kidney allografts (Betjes et al., 2012). Elevated percentages and absolute numbers of TEMRA cells in the subsets of CD4 and CD8 T that express CD26 were observed in type 1 diabetes and the number of TEMRA cells associated positively with indices of intermediate and long-term glycaemic control (Matteucci et al., 2011). The significant accumulation of TEMRA T cells in type 1 diabetes patients is thought to be caused by

a long-term and sustained antigenic stimulation consequent to a prolonged presentation of viral or other antigenic particles or could be a homeostatic deficit in the regulation/contraction of immunological responses.

Judging from the significant accumulation of end-stage T cells and high amount of senescence and exhausted T cells in the CMV-positive patients, one may speculate that the presence of CMV infection in FL patients is capable of altering the immune dynamics of patients. These findings support the hypothesis that CMV infection results in perturbation of the immune system in FL in a way that might increase the risk of infection, and this question is examined directly in Chapter 6.

Chapter 4 : Effect of CMV infection on Serum cytokines

4.1 Introduction

Immune cells in both the tumour microenvironment and in the peripheral circulation are known to secrete cytokines that perform critical roles in the control of fundamental pathways of the immune system (Labidi et al., 2010). The cytokine types and quantities may reveal some fundamental biological developments involving tumour–host interplays (Pedersen et al., 2005). Cytokines are incriminated in the pathogenesis of various lymphomas. For instance, elevated frequencies of several cytokines and angiogenic factors have been documented as biomarkers for utility in the diagnosis and prognosis of Hodgkin and NHLs (Casasnovas et al., 2007, Pedersen et al., 2005, Salven et al., 2000).

In this perspective, high serum quantities of VEGF and FGF-basic at diagnosis have been associated with poor outcome in NHL (Salven et al., 1997). Also, low baseline serum IL-6 and VEGF levels have been related to a good response rate and overall survival in NHL patients (Pedersen et al., 2005). This has been further echoed by a significant reduction of the serum IL-6 and VEGF in NHL patients that achieved CR following CHOP treatment(Pedersen et al., 2005).

Since CMV infection has been shown to elevate end-stage T cell subtypes in patients with FL as found in Chapter 3, cytokines produced by those cells or other cell types may reflect functions and regulations of those T cells in FL. CMV infection, therefore, may alter the cytokine profiles in the serum of patients with this disease. However, there is still a lack of sufficient experimental evidence for such a hypothesis. Thus, this chapter aimed to address this hypothesis with the analysis of 27 serum cytokines secreted mainly by T cells and other cells in patients with and

without CMV infection before the commencement of trial treatment. To achieve this, I applied the Luminex technique to measure those cytokines quantitatively. This technique was used because a profoundly sensitive readout of the bulk of cytokines is achievable with a tiny amount of the sample. Also, Luminex technology allows for simultaneous measurement of multiple proteins (cytokines), thereby reduce sample handling, for instance, the freezing/thawing cycles as well as decreased processing time (Ellington et al., 2009). Although Multiplex assay is comparable with other immunoassays (Dias et al., 2005, Wang et al., 2005), some of the problems that may be encountered with introducing multiplex assays in routine clinical use in comparison with the single-plex platforms such as sELISA may include the determination of the optimum working sample dilution that can cut across the spectrum of all analytes being measured and the freezing-thawing stability across analytes (Jani et al., 2016). As these challenges further increase the complexity of validating the performance of the Multiplex assay for its intended use, this study was planned to examine the agreement between Luminex assay and sELISA technique in order to examine the quality of the cytokine data generated from Luminex as well as to evaluate the impact of freeze-thaw cycles on the sELISA results of serum cytokines. The outcomes of these experiments will inform whether results of cytokine data generated using either of the techniques can be reliably used for cytokine analysis in the routine clinical patient care.

4.2 Laboratory Approaches & Reagents

As stated above, the Bio-Plex Pro Human Cytokine 27-plex Assay (Luminex, Bio-Rad Laboratories Ltd, Hertfordshire, UK)) platform was used to measure the expression levels of serum cytokines of the FL patients used in the FACS analysis in Chapter 3. The 27 cytokines determined before the treatment in serum samples of 42

FL patients comprising of 21 CMV- positive and 21 CMV- negative are listed in Table 4-1. The choice of the 27-plex was based on the availability of commercial kits for the evaluation of the right blend of cytokines and mediators that spans across T_H1 (IL-12, IL-15, INF-γ, TGF-β) and T_H2 (IL-4, IL-5, IL-10, IL-13) cytokine types based on their sources (Ellyard et al., 2007, Cui and Florholmen, 2008, Kantola et al., 2012). By function, the test kit has proinflammatory cytokines such as IL-6, IL-8, IL-17, and GM-CSF, anti-inflammatory for instance IL-4, IL-10, IL-13, IL-1ra; as well as haematopoietic growth factors (GM-CSF, G-CSF) and chemoattractants (IL-8, MIP-1α, and MIP-1β). Included in the assay kits are 96-well microplates, coupled magnetic beads, detection antibodies, standards, Bio-Plex Pro™ Streptavidin-PE reagents, and diluents for the detection of 27 human cytokines (refer to section 2.1). The two patient groups were carefully selected to ensure that any difference observed in the results between CMV- negative and CMV- positive groups would be not attributable to differences in their baseline clinical and pathological characteristics. Experiments were performed in the GCLP Facility owing to the legal requirement for trial samples to be processed and analysed to stringent criteria. To adjust the cytokine concentrations in samples within the range required for the test, 50µl of each serum sample were diluted with 150µl of sample diluent according to pre-optimised working condition in the GCLP laboratory. For the validation exercise, PeproTech sandwich ELISA Development Kits (EDK) containing essential components for the quantitative measurement of human cytokines in a sandwich ELISA format was used (refer to section 2.6.2). To evaluate the impact of freezing and thawing on serum sample sELISA results, we undertook sELISA analyses of IL-4, IL-6, IL-8 and IL-9 using respective serum samples from healthy volunteers that were previously subjected to a planned thawing and freezing cycles 1 to 3. Furthermore, to evaluate the agreement

between Luminex and sELISA techniques for the quantification cytokine levels in serum samples, serum IL-9 concentrations of the 42 trial tested with Luminex assay were analysed with sELISA and evaluated using Pearson's correlation coefficient (r).

4.3 The results

In this section, the serum cytokine data generated from the Multiplex assay, the data on the impact of thawing and freezing of serum samples using sELISA and the results of correlational analysis between serum IL-9 values determined by Luminex and sELISA are presented. The median and the interquartile range (IQR) values of serum cytokines (in pg/ml) are presented according to CMV status as shown in Table 4-1. Boxplots in Figures 4-1 and the heat map representations in Figures 4-2 to Figure 4-5 are displayed with p-values and hierarchical clustering respectively to enhance the visibility of the data. Also, a statistical Table 4-2 that demonstrates the relationship between groups expressing high and low levels of cytokines taken from the heat map and the baseline clinical features is presented.

The data on the thawing and freezing of serum samples on the results of sELISA is presented in section 4.3.2 (Bar charts in Figure 4-6) while the data on the correlation between serum IL-9 reports determined by Luminex method and sELISA technique is presented in Section 4.3.3 (Figure 4-7).

4.3.1The cytokine expression profiles in CMV- negative and CMV- positive patients with FL

Levels of the 27 cytokines were measured in baseline serum samples from 21 CMV-negative and 21 CMV- positive patients. The clinical and pathological characteristics of patients selected for the study are outlined in Table 3-1 of Chapter 3, and showed no statistical differences between the two cohorts.

To understand whether the differences between the two cohorts presented in Table 4-1 were statistically significant, Mann-Whitney U-test using SPSS version 20.0 (SPSS, Inc., Chicago, IL, USA) was used for comparison. Overall, the serum levels of 10 out of the 27 cytokines appeared to be different between CMV- negative and CMV-positive FL patients. Among them, IL-17A, IL-9, IL-8, IL-6, MIP-1α, MIP-1β and FGF-basic were significantly different with a two-sided P value ranging from 0.01 to 0.04. The remaining IL-4, GM-CSF and IL-1β also showed a trend of difference although the P values (0.06 ~ 0.07) were slightly bigger than the pre-set α level. Interestingly, levels of all of the 10 cytokines decreased in the CMV- positive cohort compared to those from the CMV- negative population as shown in Figure 4-1. To show a general picture of relative levels of the 27 cytokines studied, we compared the mean rank of each cytokine level in CMV-positive group to that of the CMV-negative. Surprisingly, as shown in Figure 4.2, the levels of all cytokines except IP-10 reduced (by 4 – 40%) in the former cohort, with the most profound reduction (by 31% to 40%) observed in the 7 with statistical significance following by that in the 3 borderline different cytokines (by around 28%).

This negative association is easily visualised even in an unsupervised cluster analysis shown in Figure 4-4. We undertook a hierarchical clustering analysis of the cytokine data. Firstly, the unsupervised clustering analysis of the data revealed a variable cytokine expression among these 42 patients (data not shown). In a subsequent supervised clustering analysis, the differences in the cytokine expression profile between the two cohorts of patients became clear as presented in the heat map shown in Figure 4-3. In a further analysis of this unsupervised data, the proportion of CMV-positive patients was found much higher than those with CMV- negative in patient group showing low levels of the 10 cytokines. This was statistically confirmed in χ^2

test, no matter the comparison was performed between cases with the lowest and highest cytokine levels ($p = 0.008$) when all the 42 cases were equally divided into three groups, or with low and high levels ($p = 0.031$) when they were equally divided into two groups according to the expression levels (Figure 4-5). Importantly, such a difference was not associated with clinical and laboratory characteristics (including age, Hb level, level of blood LDH and number of enlarged lymph nodes) of patients as summarised in Table 4-2.

Regarding expression level of individual cytokines, the median serum IL-9 in the CMV- positive cohort was decreased from 42.6pg/ml to 22.2pg/ml ($p = 0.006$), IL-17A from 43.9pg/ml to 23.0pg/ml ($p = 0.020$), FGF-basic from 72.3pg/ml to 55.9pg/ml ($p = 0.028$), MIP-1α from 10.1pg/ml to 7.4pg/ml ($p = 0.039$), MIP-1β from 294.4pg/ml to 225.0pg/ml ($p = 0.029$), IL-6 from 19.6pg/ml to 11.5pg/ml ($p = 0.029$) and IL-8 from 534.2pg/ml to 168.9pg/ml ($p = 0.037$) as compared to the other cohort. Also, decreases in the levels of IL-1β from 4.0pg/ml to 3.3pg/ml ($p = 0.068$), IL-4 from 5.1pg/ml to 4.2pg/ml ($p = 0.066$) and GM-CSF from 72.9pg/ml to 37.6pg/ml ($p = 0.070$) were also observed among those exposed to CMV infection compared the CMV- negative population, despite of the marginal significance. There were no statistical differences in levels of other 17 cytokines, although all of them except IP-10, IL-12 and MCP-1(MCAF) showed the same changes as the 10 with or nearly with statistical significance in CMV- positive patients (Table 4-1).

Table 4-1: Cytokine levels (pg/ml) in serum samples of FL patients in CMV-positive and negative patients with FL

S#	Cytokines (pg/ml)	CMV Status			
		Total	Positive	Negative	P
		Median (IQR)	Median (IQR)	Median (IQR)	
1	PDGF-bb	5977.06 (4122.61-7437.84)	5630.0 (3676.3-6457.3)	6640.7 (4461.7-8224.6)	0.170
2	IL-1b	3.8 (3.1-5.6)	3.3 (2.8-4.8)	4.0 (3.5-5.8)	**0.068**
3	IL-1ra	194.0 (139.4-264.5)	182.6 (146.8-212.9)	229.3 (139.4-310.6)	0.119
4	IL-2	0.0 (0.0-9.8)	0.0 (0.0-6.9)	3.0 (0.0-13.6)	0.248
5	IL-4	4.3 (3.9-5.5)	4.2 (3.9-5.1)	5.1 (4.1-5.6)	**0.066**
6	IL-5	2.34 (1.67-2.82)	2.3 (1.7-2.7)	2.5 (1.7-3.1)	0.831
7	IL-6	16.2 (10.7-26.9)	11.5 (9.7-17.5)	19.6 (12.1-35.1)	**0.029**
8	IL-7	10.8 (8.2-15.4)	10.2 (8.4-13.7)	10.9 (7.5-16.2)	0.359
9	IL-8	268.2 (66.3-715.5)	168.9 (48.8-338.8)	534.2 (135.3-1077.1)	**0.037**
10	IL-9	28.8 (20.1-44.0)	22.2 (17.5-29.9)	42.6 (28.4-56.1)	**0.006**
11	IL-10	12.4 (6.1-19.4)	8.3 (5.7-19.8)	14.0 (9.0-18.5)	0.222
12	IL-12 p70	62.0 (49.8-97.1)	62.5 (35.7-75.9)	61.4 (52.5-123.6)	0.571
13	IL-13	8.4 (5.7-11.4)	8.2 (5.4-10.2)	9.1 (6.2-11.4)	0.443
14	IL-15	0.0	0.0	0.0	0.269
15	IL-17A	31.2 (9.5-66.8)	23.0 (6.3-35.7)	43.9 (19.7-69.1)	**0.020**
16	Eotaxin	160.5 (114.7-225.9)	158.9 (114.6-205.3)	191.0 (122.7-242.0)	0.333
17	FGF basic	61.7 (43.2-75.8)	55.9 (36.9-66.8)	72.3 (56.8-86.8)	**0.028**
18	G-CSF	60.3 (48.9-76.0)	55.6 (45.9-67.2)	64.2 (52.8-77.6)	0.213
19	GM-CSF	62.0 (18.4-86.5)	37.6 (7.9-73.0)	72.9 (50.1-92.9)	**0.070**
20	IFN-g	175.9 (149.0-225.1)	174.0 (146.3-202.3)	198.5 (155.2-231.4)	0.187
21	IP-10	1804.1 (1074.2-2894.2)	1827.5 (1319.1-3030.7)	1788.3 (1054.2-2253.2)	0.308
22	MCP-1(MCAF)	67.9 (44.3-124.3)	68.6 (36.8-93.9)	67.3 (57.2-178.4)	0.320
23	MIP-1α	8.7 (6.4-12.5)	7.4 (5.8-9.9)	10.1 (7.5-21.4)	**0.039**
24	MIP-1β	258.2 (209.2-330.3)	225.0 (198.6-296.8)	294.4 (228.2-419.6)	**0.029**
25	RANTES	61692.1 (32722.9-90443.7)	61249.3 (32722.2-75198.7)	62134.9 (37391.4-92008.7)	0.521
26	TNF-α	48.8 (35.5-59.8)	45.3 (35.8-57.0)	52.2 (35.5-62.9)	0.346
27	VEGF	197.1 (135.4-283.0)	170.0 (102.6-254.9)	243.5 (176.7-369.3)	0.110

Comparison of median cytokine levels of CMV- positive and CMV-negative groups.

P< 0.05 is considered statistically significant.

IQ R = Interquartile Range

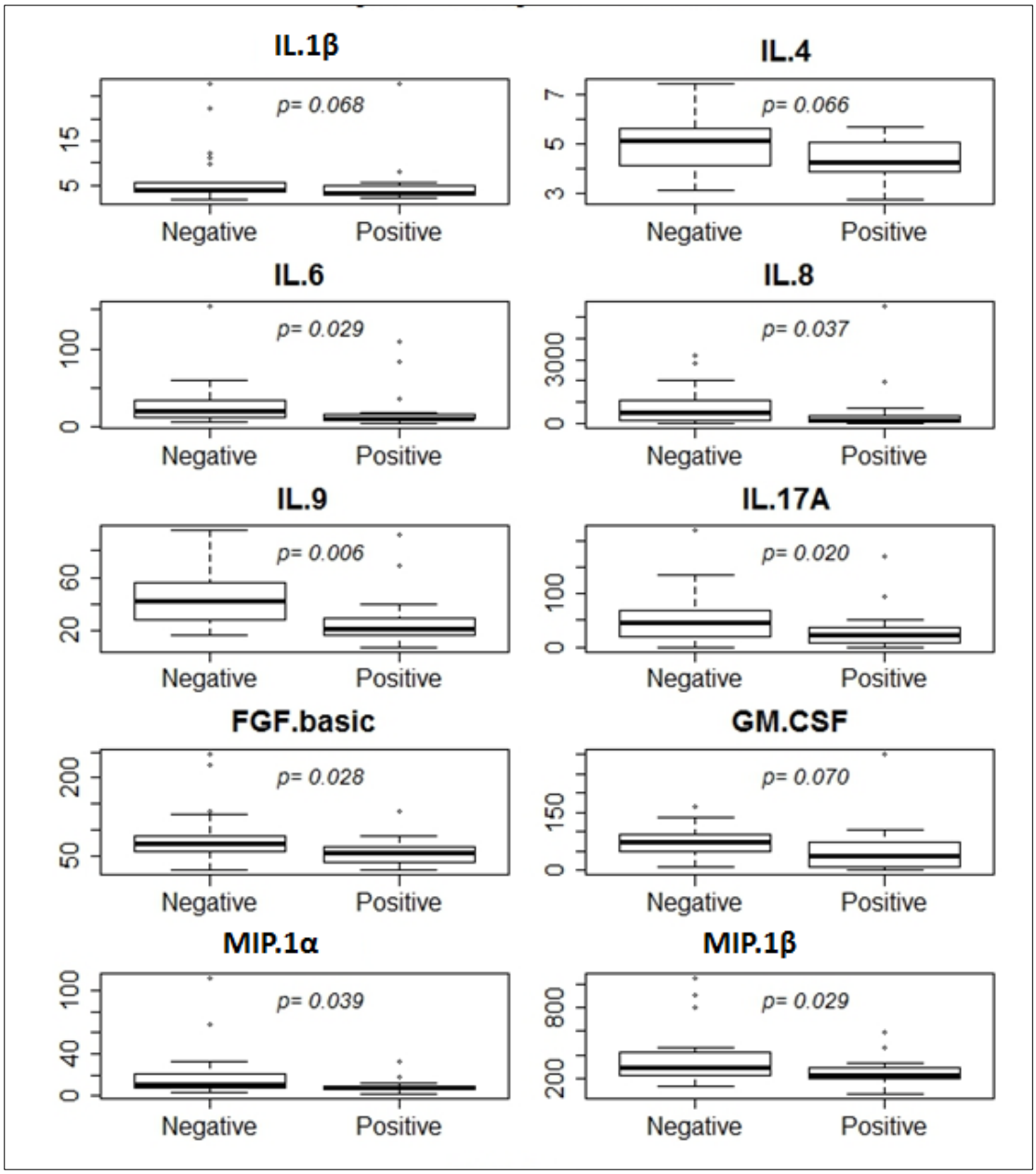

Figure 4-1: The box plot/whiskers graphs of concentrations of 10 cytokines measured using the Luminex method that shows significant difference between CMV- positive (n=21) and CMV- negative (n=21) FL patients.

The P-values were from the two-sided Mann-Whitley U test.

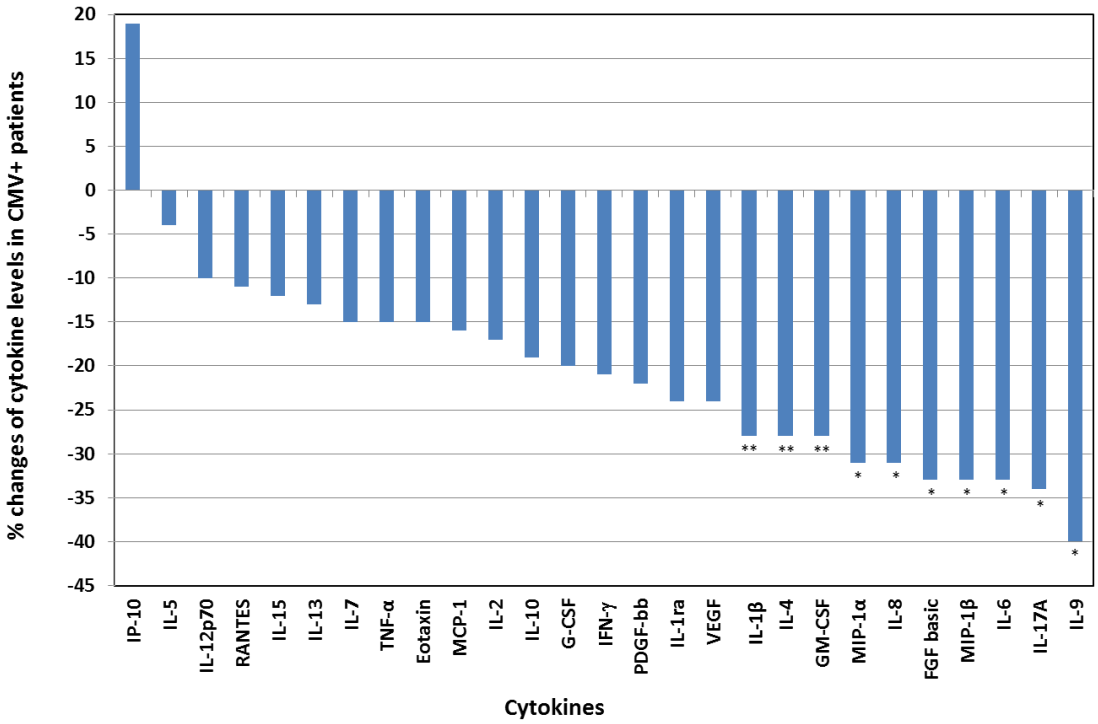

Figure 4-2: The relative changes in cytokine levels in patients with CMV infection as compared to those without infection by this virus.

The mean rank of concentration of each of the 27 serum cytokines in the 21 CMV-positive patients are compared to that in the 21 CMV-negative cases (set as 0). The expression of cytokines marked by * was significantly different ($P<0.05$), and those by ** board-line different ($P=0.066\sim0.070$) between the two groups of patients.

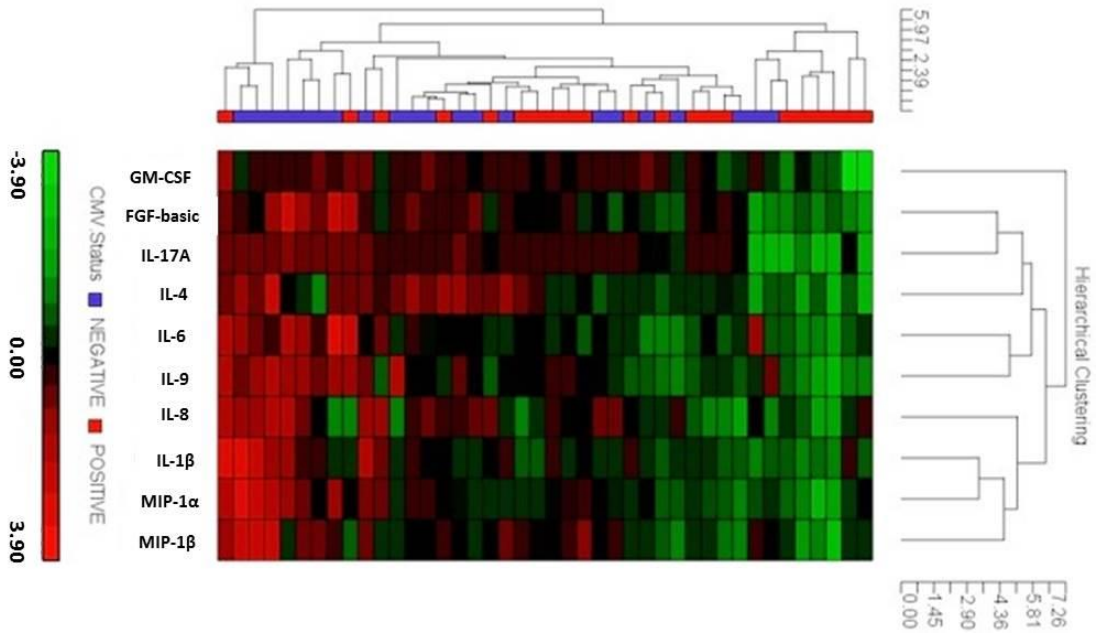

Figure 4-3: The unsupervised heat maps showing expression levels of the 10 cytokines with statistically differential expression between the two groups in 42 patients.

In the heat map in Figure 4-3, cytokine data was log transformed to 0.01 and cytokine expression was scaled from the minimum level (-3.90) in green and maximum level (3.90) in red. Patients were coded red for CMV- positive and blue for CMV- negative. The hierarchical clustering of the ten cytokines of interest versus the 42 FL patients has grouped cytokines according to their functional combinations of chemokines (IL-8, MIP-1α, & MIP-1β) and IL-1β, T_H2 cytokines (IL-4, IL-6 and IL-9), T_H17 (IL-17A) and Vascular remodelling (FGF-basic & GM-CSF).

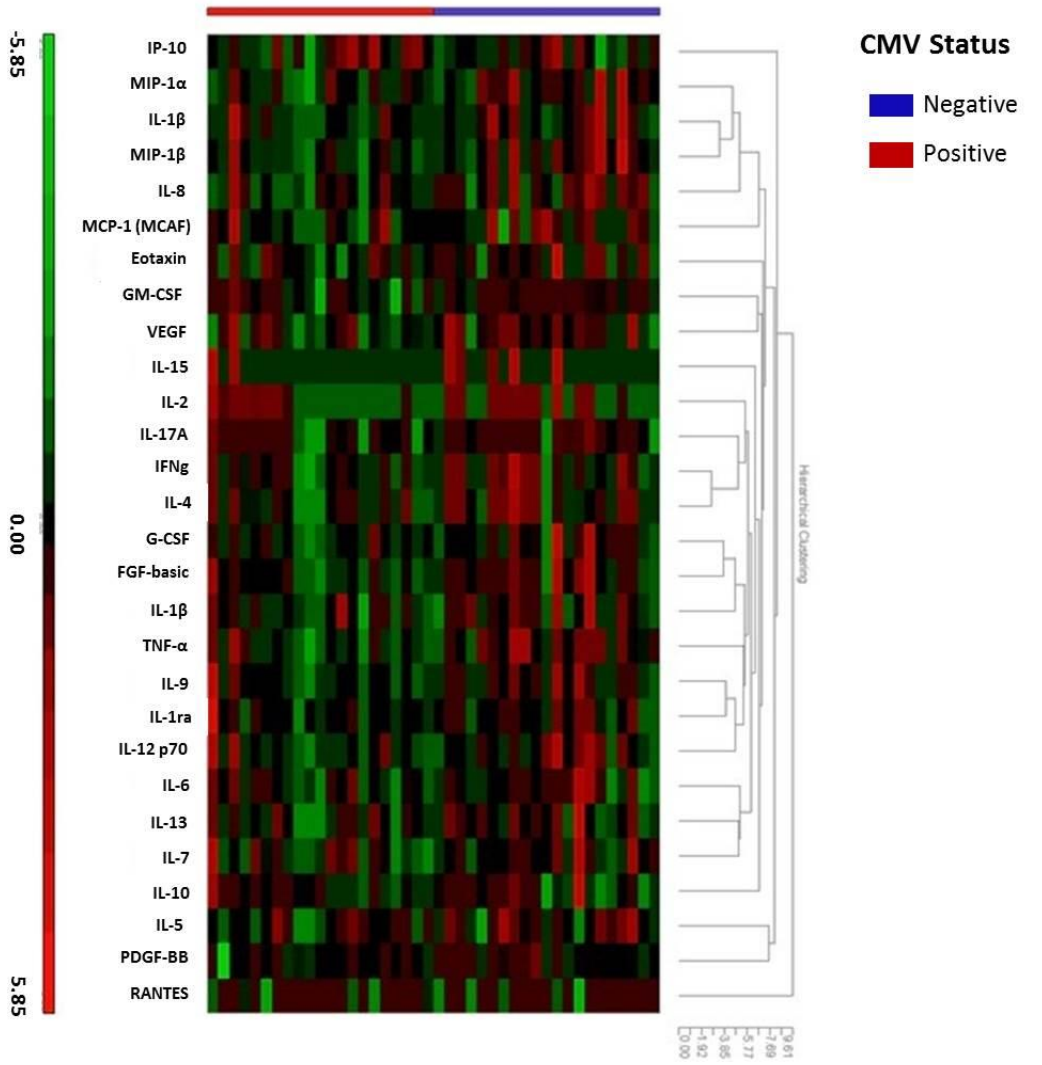

Figure 4-4: The supervised heat map showing levels of the 27 cytokines measured using the Luminex method between CMV- positive and CMV- negative groups in the 42 patients.

Cytokine data was log transformed to 0.01 and cytokine expression was scaled from the minimum level in green and maximum level in red. The CMV- positive and CMV- negative patients are under the red and blue bars, respectively.

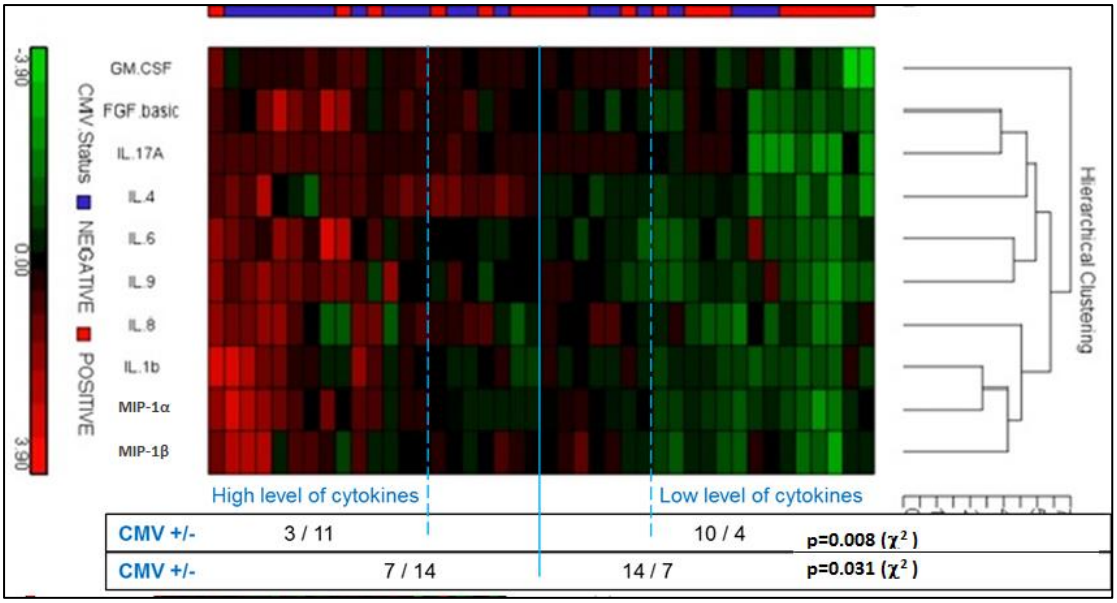

Figure 4-5: The heat maps of 42 patients versus 10 cytokines grouped patients according to expression levels of cytokines.

The middle line on the unsupervised heat map in Figure 4-5 separates patients into two halves of 21 each. The left half can be seen to be dominated by red map, signifying high levels of cytokine expression and comprises of 7 CMV- positive patients against 14 CMV-negative. Green map for low levels of cytokines dominates the right half and consists of 14 CMV- positive and 7 CMV- negative patients. The Chi-square test revealed a difference ($p = 0.031$) in the cytokine expression between the left (high expression) and right (low expression) halves. Also, Chi-square test comparing the first 14 and the last 14 patients on the heat map revealed a statistical difference ($p = 0.008$). This finding shows that there is a statistical difference between the levels of cytokine expressed by 14 patients on the extreme left and the 14 on the right-wing extreme of the heat map.

Table 4-2: Cytokines in heat map and clinical features

Parameter		n=14 Low	n=14 Intermediate	n=14* High	Parameter		n=14 Low	n=14 Intermediate	n=14* High
Age	Mean	65.93	69.14	67.5	Ann Arbor Score	Mean	3.43	3.36	3.36
	SD	6.55	7.5	6.94		SD	0.76	0.74	0.74
	Median	67.5	69.5	66.5		Median	4	3.5	3.5
	Range	51-74	53-81	56-78		Range	2-4	2-4	2-4
	t-test			LvsH, p=0.543		t-test			LvsH, p=0.803
	M-W test			LvsH, p=0.910		M-W test			LvsH, p=0.804
Hb	Mean	12.61	13.34	12.87	FLIPI Score	Mean	1.64	1.64	1.57
	SD	2.24	1.76	1.63		SD	0.63	0.63	0.65
	Median	12.95	13.95	13.6		Median	2	2	2
	Range	9.2-16.4	9.4-15.5	9.8-14.6		Range	0-2	0-2	0-2
	t-test			LvsH, p=0.733		t-test			LvsH, p=0.770
	M-W test			LvsH, p=0.685		M-W test			LvsH, p=0.769
LDH	mean	359.79	432.71	390.93	CIRS Score	Mean	2.71	5.07	2.71
	SD	155.89	193.54	160.94		SD	2.16	3.32	1.38
	Median	357.5	406	433.5		Median	3	4.5	3
	Range	128-741	166-834	180-699		Range	0-7	0-11	1-5
	t-test			LvsH, p=0.607		t-test			LvsH, p=1.000
	M-W test			LvsH, p=0.427		M-W test			LvsH, p=0.769
No. of LNs	mean	5.5	5.14	5.36	* n=13 for Hb in the H group				
	SD	1.09	1.35	1.45					
	Median	6	6	6					
	Range	3-6	2-6	1-6					
	t-test			LvsH, p=0.770					
	M-W test			LvsH, p=0.982					

4.3.2 The effect of Sample Thawing/Freezing Cycles on sELISA Results

In order to validate results from the Luminex test with an alternative method sELISA, some of these 42 serum samples had to be repeatedly frozen and thawed as no aliquots were stored initially. To learn whether the number of cycles of such thawing/freezing affect results of the cytokine measurement, we collected serum samples from four healthy donors and stored them in 3 aliquots at -80^{o}C for 3 days per cycle of thawing/freezing. After experiencing 1, 2 and 3 cycles, the serum aliquots from donors 1, 2, 3 and 4 were used for testing concentrations of IL-4, IL-6, IL-8 and IL-9, respectively. Surprisingly, the results changed with the cycle number of thawing/freezing increased. The concentration measured for IL-4 and IL-9 became higher after each cycle. Thus, after the 3rd cycle, it was 4.6 and 1.5 folds in samples measured for IL-4 and IL-9, respectively, as compared to that in the sample

experienced only one cycle (Figure 4-6: A and D). The value of IL-8 was also bigger after 2 cycles (1.3 folds), but with no further increase after the 3rd cycle (Figure 4-6: C). However, the concentration of IL-6 was reduced by 16% from 3438pg/ml to 2904pg/ml (Figure 4-6: B). These results suggested that the repeated thawing and freezing for serum samples changed measurement values of these cytokines, with an increase in most cases. The reasons for increased in the values of these cytokines following repeated thawing and freezing have not been investigated.

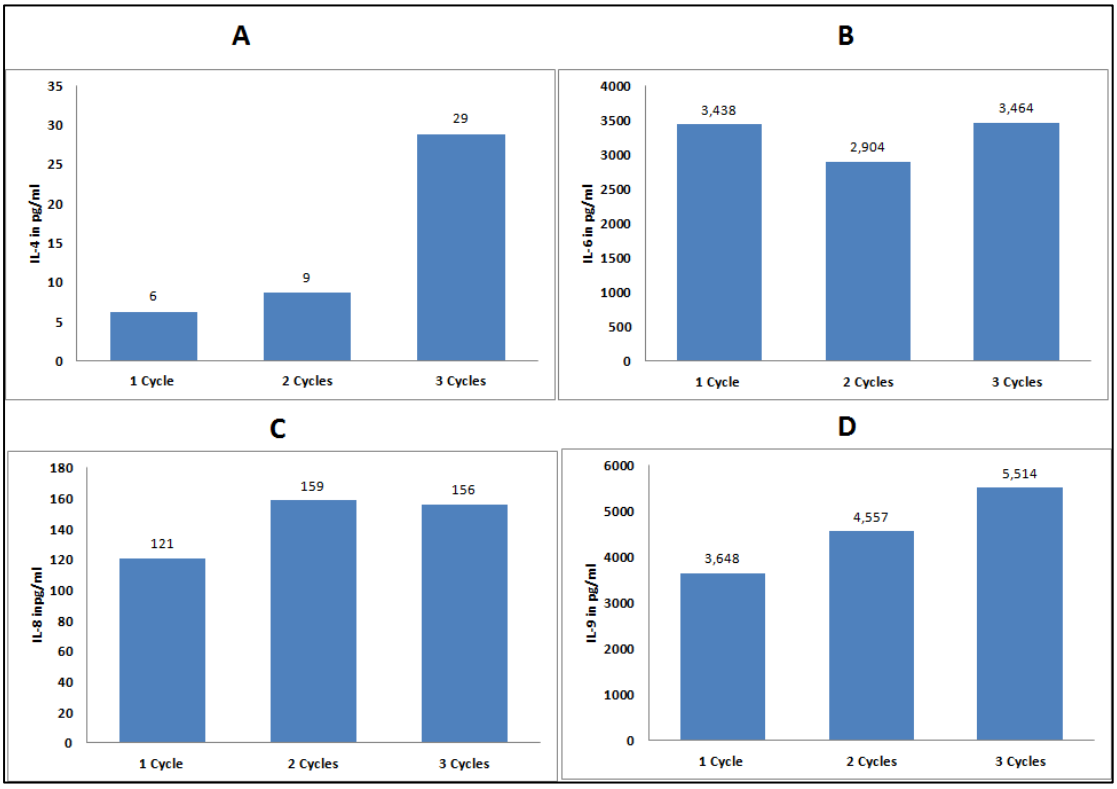

Figure 4-6: Cytokine levels measured with sELISA in serum samples after different cycles of thawing/ freezing.

Aliquots of serum samples from 4 healthy donors were subjected to experience of 1, 2, and 3 cycles cryopreserving storage. Each aliquot of donors 1, 2, 3, and 4 were then used for detecting IL-4 (A), IL-6 (B), IL-8 (C) and IL-9 (D), respectively, in sELISA experiments.

4.3.3 A positive correlation between IL-9 concentrations measured with Luminex and sELISA

The alternations of cytokine measurement by sELISA in serum samples experienced different cycles of thawing and freezing hampered the use of such type of samples for validation of cytokine results from the Luminex experiment. However, the results shown in Figure 4-6D suggested that such changes for IL-9 were relatively small and consistent with increase in the cycle number. This implied a possible correlation existing between concentrations of this cytokine measured by the two methods, if the Luminex results were reliable. We therefore used the serum samples from all of the 42 patients after the 3rd cycle of storage to measure IL-9 with sELISA and then compared the results with those previously measured with the Luminex.

As shown in Figure 4-7, the Pearson's correlation analysis revealed a positive linear correlation existing between the two methods, although not perfect ($r = 0.374$, $p = 0.015$). Expectedly, the average concentration of the cytokine measured by sELISA was over 7 folds of that as measured by the Luminex (280pg/ml versus 36pg/ml). This might partially be explained by the effects of repeated sample freezing and thawing as noticed in our previous experiment (Section 4.3.2). Those results suggested that the relative cytokine levels in patients of this cohort as presented in Table 4-4 were consistent with the results of the sELISA experiment, and therefore reliable.

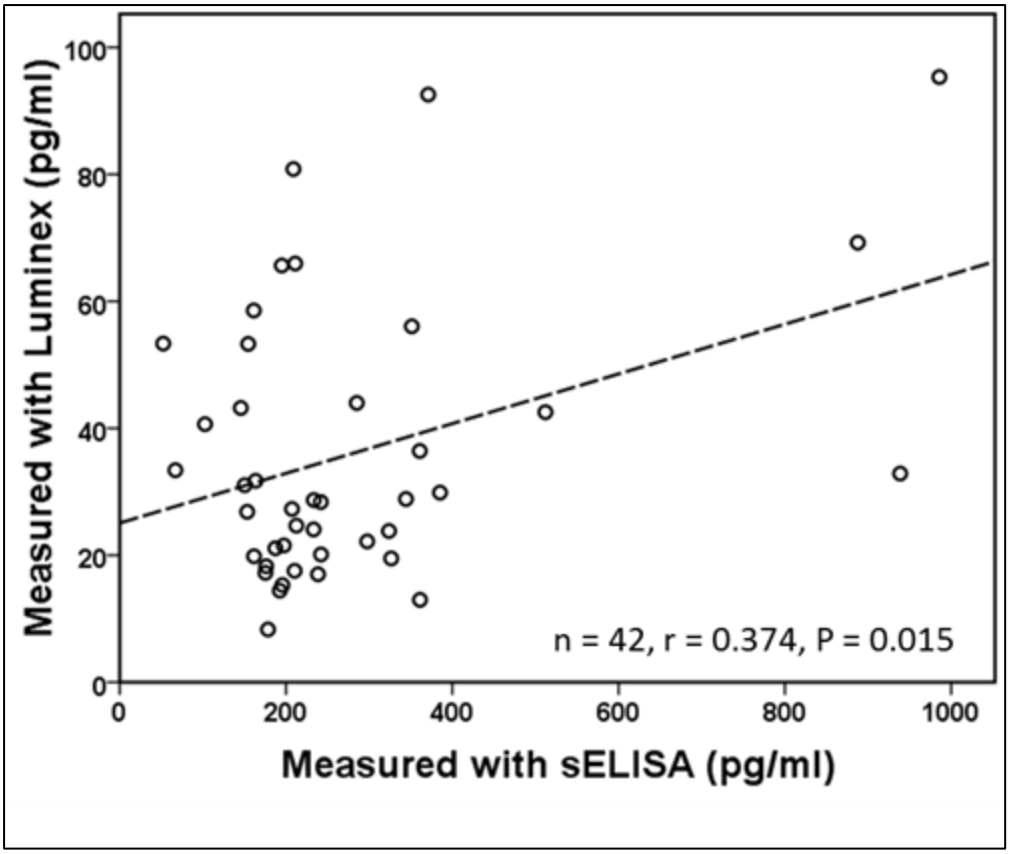

Figure 4-7: A positive correlation between the Luminex and sELISA in measuring IL-9 concentrations in patient serum samples.

The concentrations of IL-9 measured with the Luminex (Y axis) and sELISA (X axis) in all of the 42 patients studied in this chapter are compared in the two-sided Pearson's correlation analysis. Note that the serum samples used for the sELISA test experienced two more cycles of thawing/freezing than those for the Luminex test.

4.4 Correlation Analysis of cytokines and cell subtypes

This section presents a correlational analysis of results of the lymphocytes obtained in Chapter 3 and cytokines detected with the Luminex in this Chapter. The analysis was expected to provide an indirect approach to assessing the functional potential of the quantitative T and NKT cells data in Chapter 3 with regards to cytokine production. It is also hoped that the correlation analysis will provide an insight into the relationship between the lymphocyte subtypes and specific cytokines. In addition, the measurement of both lymphocytes and cytokines provides an opportunity for cross-

validation between the two Chapters to increase confidence that the results of both Chapters are trustworthy.

The graphical representation of Spearman's correlation coefficient matrix for cytokines and FACS data is presented in Figure 4-8. The legend on the right side summarises the colour meaning: the blue shades are used for positive correlation and the red shades for the negative correlation. The more the variables are correlated; the more is the intensity of the colour. The results do not show a high correlation between cytokines and the T and NKT cells. However, all the 10 cytokines seem to be positively correlated among themselves, especially between FGF-basic with IL-17A ($r = 0.969$, $p < 0.001$) and between IL-9 with IL-17A ($r = 0.916$, $p < 0.001$). Furthermore, positive correlations exists among some cell types, especially between E-CD4 with EM3-CD4 ($r = 0.872$, $p < 0.001$) and between E-CD8 with EM3-CD8 ($r = 0.899$, $p < 0.001$) cell subtypes. Differently from the cytokines, that show only positive correlation among themselves, some cell types seem to correlate negatively with others, as between CD4 with EM3-CD8 ($r = -0.672$, $p=0.003$) and between CD4 with E-CD8 ($r = -0.631$, $p=0.009$).

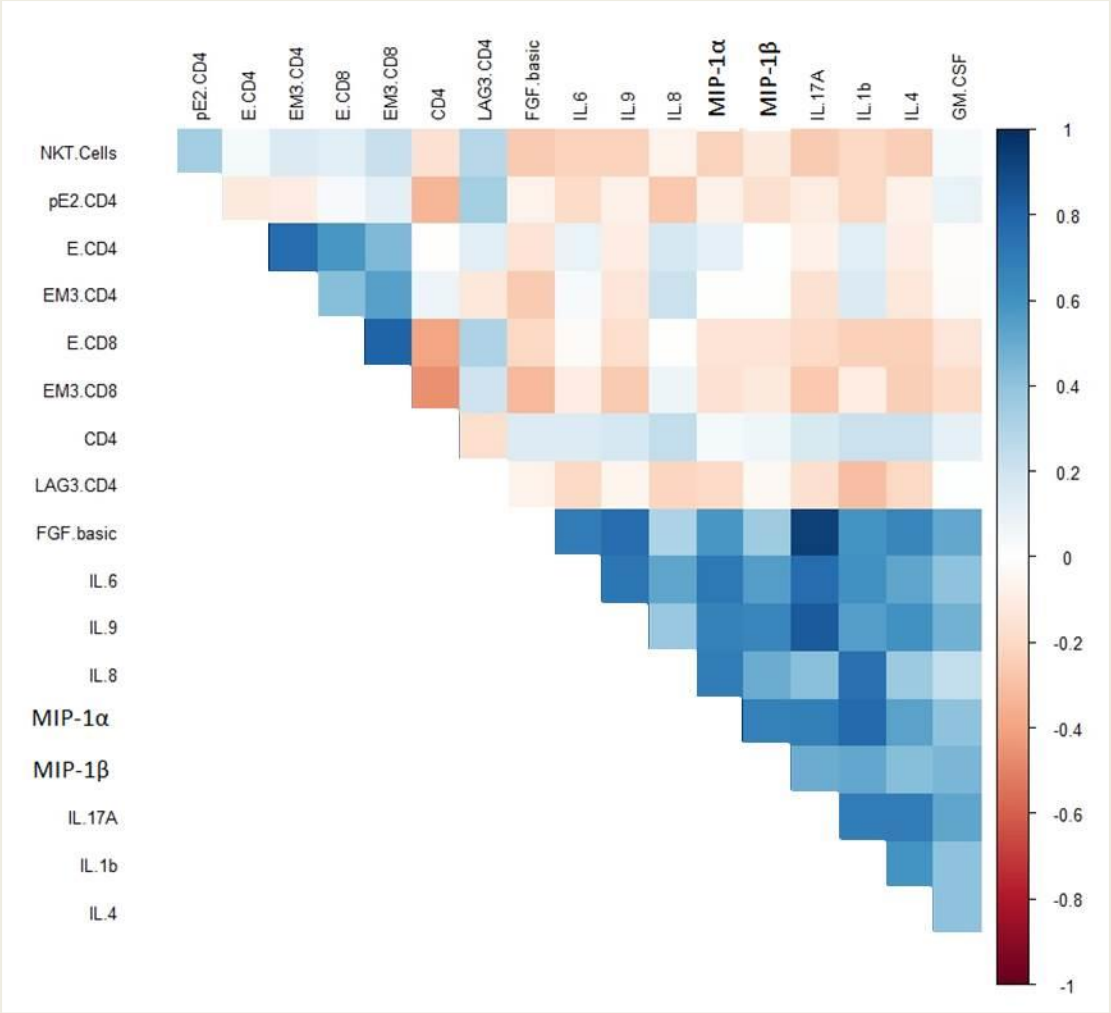

Figure 4-8: A matrix of correlation coefficients between cell types and cytokines.

Correlation coefficients were calculated in the Spearman's correlation analysis and presented in heating colours.

4.5 Discussions

Being that T cells are known to be the major source of cytokines, a quantitative analysis of pre-treatment serum cytokine levels of the PACIFICO trial patient samples was carried out as a strategy to evaluate the functional potential of the elevated terminally differentiated T cell subtypes observed in the CMV- positive FL patients in Chapter 3.

Strikingly, Luminex data reveal decreased levels of all the 27 serum cytokines studied among the CMV- positive FL patients compared to the CMV- negative patients, except for IP-10, IL-12 and MCP-1(MCAF). As depicted in Figure 4-1, significantly diminished levels of serum IL-9, IL-17A, FGF-basic, MIP-1α, MIP-1β, IL-6 and IL-8 were observed in the CMV- positive group compared to the CMV- negative counterparts ($P < 0.05$). Also, marginally significant inferior median quantities of IL-1β, IL-4 and GM-CSF are seen in the CMV-positive compared to the CMV- negative ($P = 0.066 \sim 0.070$) (Table 4-1). Moreover, there were no significant variations in clinicopathological features among patients with different status of cytokine production (Table 4-2). The reduced cytokine levels observed among the CMV-positive FL patients could be attributed to the marginally decreased numbers of CD4+ T cells in addition to the significant increase in the quantities of end stage T cells among patients reported in Chapter 3. T cells being the primary source of cytokines, one of the characteristics of the terminally differentiated T cell phenotypes is the poor cytokine production potential (Wherry, 2011, Rothe et al., 2016). Meanwhile, the reduced CD4+ T cells can further compound this deficiency since CD4+ T cells are needed to support the activity of other immune cells including the production of cytokines to suppress or regulate immune responses. These observations could suggest that the presence of CMV infection in FL may portend some functional immune deficit for the patients' subject patients to heightened vulnerability to other infections and increased AEs as captured in our hypothesis.

Furthermore, the categorisation of patients into low, medium and high (Figure 4-5) according to the median cytokine values has revealed a significant difference between patients with CMV- positive and negative status. However, there were no significant disparities in pre-treatment clinicopathological features among patients with different

status of cytokine production. These features include age, Ann Arbor staging, Hb values, LDH, FLIPI score, CIRS scale, gender, and the number of lymph nodes (LNs) involved (Table 4-2). This finding could suggest some degree of homogeneity in the participating patients and implies that baseline clinical and pathological characteristics of trial patients may not be responsible for the variations in the cytokine levels.

Although Multiplex assay (Luminex technology) has been shown to be comparable with other immunoassays (Dias et al., 2005, Wang et al., 2005), this study has evaluated the impact of thawing and freezing of serum samples on the results of sELISA. This was with the view to validation of the Luminex cytokine data for use in the subsequent relevant sections of the thesis. Interestingly, it was observed that different cytokines respond differently to the thawing and freezing effects. For instance, IL-4 and IL-9 can be seen to maintain an increasing trend in the levels of expression through 1 cycle of thawing and freezing to 3 cycles (Figure 4-6: A and D) while inconsistencies in the levels of IL-6 and IL-8 can be seen in the respective cycles of thawing and freezing (Figure 4-6: B and C). This result is in agreement with the findings studies by others. Using Milliplex® MAP (multi-analyte profiling) kit, Lee JE and his group assessed the variations in the concentration of some serum cytokines following repeated thawing and freezing and found significant increase or decrease in the concentrations of IL-15, IL-17A, TNF-a, and VEGF during repeated freezing-thawing cycles (Lee et al., 2015). Another study that used EVIDENCE 180 Biochip Analyser system and Cytokine Array I kits revealed a 3‑5-fold increase in IL‑1β, IL‑4 and IL‑10 levels following 1 freeze/thaw cycle but levels were seen to stabilise during the additional 9 freeze/thaw cycles (Guo et al., 2013). In one more study, aliquots of the same sample were subjected to multiple freezing - thawing

cycles and cytokines were measured with Bio-Rad Multiplex Immunoassay. In the report, IL-6 and IL-10 were found to be stable throughout the multiple freeze-thawing cycles, but IL-4, IL-13, IL-15, IL-17, TNF, IFN and CXCL8 levels were found to either increase (IL-4 and TNF) or decrease (IL-13, IL-15, IL-17, IFN and CXCL8) after one or more freeze - thawing cycles (de Jager et al., 2009). Therefore, our study supporting findings by others suggested that for reliable results of cytokines, serum samples should avoid repeated freeze - thawing cycles before the tests.

Undoubtedly, the application of multiplex assays in the routine clinical measurement of analytes is not devoid of problems. To resolve them, it requires to determine the optimum working sample dilution that may cover a broad spectrum and identify effects of the cycles of freezing-thawing serum samples on reliability of results (Jani et al., 2016). Despite of this, the Luminex methods was selected for the cytokine studies in this chapter. This was because the Luminex is a multiplex platform which allowed us to measure 27 cytokines simultaneously in one experiment, and also avoid result alterations caused by use of samples experienced repeated cycle of storage as found in this chapter. In fact, this method has been successfully applied in other cytokine studies. For instance, cytokine data from multiplex assays have been utilised in Wei-Lin-Weissfeld regression models to define the relationship between serum cytokine concentrations and oral HPV clearance (Lam et al., 2016). Multiplex bead assay was employed to analyse archived maternal serum samples that were collected at the time of delivery for cytokine levels, and results were used to suggest that elevated maternal levels of anti-inflammatory cytokines throughout the perinatal time may guard against the psychotic episodes (Allswede et al., 2016). Data of 77 inflammation markers generated by Luminex bead-platform were subjected to weighted Cox models and conditional logistic regression to estimate cumulative risks

and odds ratios (ORs) respectively, which revealed that certain levels of circulating inflammatory marker are associated with prospective lung cancer risk (Shiels et al., 2013). In another study, prediagnostic serum cytokine levels of B-cell NHL cases were measured by Luminex platform and relevant statistical analysis of data revealed an association between a moderately raised risk of all B-cell NHL in women and elevated levels of the cytokines (Edlefsen et al., 2014).

In addition, the Luminex assay had been well established in our GCLP laboratories for cytokine studies before this project. Finally, in this study, a positive linear correlation was found between IL-9 concentrations measured using this method and sELISA in all of the 42 patients studied ($r = 0.374$, $p = 0.015$). This result was in agreement with other studies. For instance, Wang et al. revealed perfect correlations between Luminex multiplex technology and ELISA platform in measuring levels of IL-1β, IL-4, IL-5, IL-6, IL-10, IFN γ, and TNF α (Wang et al., 2005). Others also found the Luminex assay highly reproducible and reliable compared to ELISA in quantifying cytokines (Richens et al., 2010, Codorean et al., 2010)

The results in this chapter therefore provided one more piece of evidence to support that both the Luminex and sELISA methods can be used to determine serum cytokine levels among patients. However, due to the limitation to use serum samples without experience of repeated storage cycles, cytokine data from the Luminex assay was used for subsequent analysis in this thesis.

Over fifty different chemokines are recognised and classified in humans, and they act mainly on neutrophils, monocytes, lymphocytes, and eosinophils and play a crucial responsibility in host immune defence mechanisms. It has been suggested that chemokines play fundamental roles in the growth, homoeostasis, and functionality of

the immune system. The classification of chemokines into four subgroups is based on the structural composition of cysteine residues in their amino-terminal portion: CXC, CC, CX3C and XC (Springael et al., 2005). Chemokines can also be categorised into functional subgroups as the homeostatic and inflammatory categories (Zlotnik and Yoshie, 2000, Ben-Baruch, 2002). For instance, the homeostatic chemokines such as CCL14, CCL19, CCL20, CCL21, and CCL25 control the directed migration of leukocytes in the processes of haematopoiesis and growth of lymphoid organ (Sallusto, 2002), on the other hand, inflammatory chemokines such as CXCL-8, CCL2, CCL3, CCL4, CCL5, CCL11, CXCL10 facilitate leukocyte infiltration to scenes of inflammation and they are induced principally by proinflammatory cytokines (Hadden, 1999).

The hierarchically distinct functional clusters of cytokines in Figure 4-4, revealed 10 cytokines down-regulated in CMV- positive patients. Among them, MIP-1α (CCL-3), MIP-1β (CCL_4) and IL-8 (CCL8) are members of a group of structurally related small molecules of about 8–14 kDa involved in the regulation of cell trafficking via interactions with a subgroup of transmembrane G-protein–coupled receptors (Zlotnik and Yoshie, 2000, Shrivastava et al., 2014). They belong to the inflammatory category of chemokines, which have been reported to be produced in high frequencies during infection or injury as they regulate the movement of inflammatory cells into the site of injury (Graham and Locati, 2013).

The second distinct cluster shown in Figure 4-4 comprised of IL-6 and IL-9. It is not surprising that IL-6 and IL-9 clustered together since both cytokines tend to perform similar functions and were originally grouped as T_H2 cytokines. The T_H2 cells secrete several cytokines such as IL-4, IL-6 and IL-9 to activate and sustain humoral immunity mainly targeted at extracellular bacterial and parasitic challenges, plus

allergens and toxins. Aside from being T_H2 cytokines, both IL-6 and IL-9 are described as pleiotropic and further identified as proinflammatory cytokines (Kishimoto, 2010). The IL-6 and IL-9 as pleiotropic cytokines have the capacity to influence the activities of multiple cell types leading to a broad spectrum of biological functions in oncogenesis, inflammation and autoimmunity, immune regulation and haematopoiesis (Kishimoto, 2010). The deficit observed in the frequencies of IL-6 and IL-9 among the CMV- positive FL patients could be thought to cause suppressed humoral immune responses among others; thereby causing heightened vulnerability to infections and illnesses.

The third cytokine cluster identified on the heat map in Figure 4-4 is the one including IL-4, IL-17A and FGF-basic. IL-4 is an autocrine cytokine that is marginally reduced among CMV- positive FL patients compared to the CMV- negative group. This is an essential cytokine for T_H2 priming and maturation; and high concentrations can prevent the production of T_H1 cells from the immature T cells. IL-4 is also vital in stimulating B cell proliferation and maturation of plasma cells and regulates the class switching of antibodies. The IL-17A is a proinflammatory cytokine and a member of the IL-17 cytokine family in addition to IL-17B, IL-17C, IL-17D, IL-17E (IL-25), and IL-17F (McAllister et al., 2005). It provides a strong defence against extracellular microbes and bacterial and fungal infections and adds to the pathogenesis of several autoimmune disorders (Yao et al., 1995, Kolls et al., 2003). It also regulates the formation and recruitment of neutrophils to the sites of inflammation due to its ability to cause the release of CXC chemokines (Moseley et al., 2003) and coordinating the expression of G-CSF (Jones and Chan, 2002). The FGF-basic and GM-CSF can be grouped under vascular remodelling cytokines. In a situation where there is no signal, FGF-basic is known to remain in basement membranes of the subendothelial

extracellular matrix of blood vessels. It is assumed that during both normal wound healing process and in tumour development, FGF-basic is activated by the action of heparan sulphate degrading enzymes and triggers angiogenesis (Kühn et al., 2012). Additionally, studies have demonstrated that FGF-basic protects the heart from injury connected to heart attack, reduction of tissue death and encouraging enhanced function following reperfusion (House et al., 2003). Also, low levels of FGF-basic has been reported to play a vital role in the occurrence of extreme anxiety (Perez et al., 2009). A monomeric glycoprotein, GM-CSF operates as a cytokine by activating stem cells to produce granulocytes and monocytes (Francisco-Cruz et al., 2014). The polymorphs are known to play pivotal roles, especially in the innate arm of the immunity, while the monocytes egress the circulation and relocate into tissues where they develop into macrophages and dendritic cells and participate in the characteristic immunological processes of defending the body and combating infections (Gasson, 1991). GM-CSF can, therefore, be said to promote the development of the immune system.

The weak negative correlation observed between levels of almost all cytokines and numbers of NKT and CD8+ E and EM3 cells is in keeping with the idea that these cells are (a) not the main source of the cytokines, (b) are not very good at making cytokines (supporting the idea that they are exhausted), and (c) in the case of NKT cells, their accumulation may be compensatory and secondary to depletion of competent T cells. Also, the weak positive correlation between E-CD4+ and EM3-CD4+ cells and IL-6, IL-8 and IL-1β suggest that these cells make pro-inflammatory cytokines, albeit not very effectively.

Therefore, it is logical to speculate that the ability of the end-stage effector T cells seen among the CMV-positive patients to produce cytokine is poor. By removing all

other confounders, the reduced cytokine levels in the serum of the CMV-infected FL patients can be attributed to the disruptive influence of the virus on T cell repertoires being the primary cellular source of cytokines. In another way, it is also rational to observe higher levels of end-stage (TEMRA) T cells in the circulating blood of CMV-positive FL patients due to the sustained antigenic stimulations of T cells over time and induction of premature immune senescence by the virus. Inferring from the above outlook, cytokine data can be said to portray danger for the CMV- positive FL patients as compared the CMV- negative. By implication, the lower expression levels of cytokines in this study can impact on both innate and adaptive arms of the immune system and possess a grave danger to patients exposed to CMV, and can be attributable to reduced secretions by relevant cells that produce them. The generally lower cytokine levels observed in the CMV- positive patients may potentially make them more vulnerable to adverse events and recurrent infection in the course of therapy compared to the CMV- negative. It is speculated that a functional study on T-cell subtypes, especially the end-stage T cell types may provide a further understanding of T cell plasticity and open the open the mystery of re-engineering and reverting end stage T cells to functional subtypes. To further unravel the impact of CMV infection in FL lymphoma biology, probing into the FL microenvironment to evaluate quantitative intensities of accessory cells according to the CMV infection status is thought to be complimentary. Hence, Chapter 5 is devoted to studying surface protein markers of key accessory infiltrating cells in the FL microenvironment. Besides, as part of this study, an evaluation of the relationship of the levels of cytokine expression and clinical data of trial patients, including toxicities and response status is explored in Chapter 7. Therefore, Chapter 7 of this thesis is

aimed at providing further illumination on the wider clinical relevance of the reduced cytokine levels in FL as seen in CMV- positive patients.

Chapter 5 : Effect of CMV on accessory cells in tissues

5.1 Introduction

This chapter is designed to examine key markers of cellular infiltrates in the FL microenvironment and to relate the findings to data from the FACS analysis in Chapter 3 and the serum cytokine data in Chapter 4 of this thesis. The result of the intensities of accessory cell markers in the tissues according to CMV status is related to patients' efficacy and toxicity data. It is anticipated that by associating laboratory findings to treatment response status and AEs experienced by the trial cohort, laboratory data can provide a useful pointer for better understanding of the clinical and biological relevance of the presence and frequencies of cellular markers within the follicular microenvironment.

The tumour microenvironment (TME) is comprised of normal cells, molecules, and blood vessels that surround and feed the tumour cell. A tumour can change its microenvironment, and the microenvironment can affect the growth and spread of tumours. The abundance of immune cells in the secondary lymphoid organs in TME of haematologic malignancies makes it distinctly different from solid tumours that exhibit decreased numbers of immune cells. Similarly, the number and function of immune cells vary greatly among patients with the identical type of tumour and has been proven to influence patient outcomes (Yang and Ansell, 2012a).

This study is aimed to unravel and evaluate the effect of CMV infection on the intensities and colocalization of non-malignant immune cells in the FL microenvironment. Most immunological assays including IF and IHC adopt the fundamental principle of antigen binding to its specific antibody for the detection and quantification of small quantities of antigenic molecules of biological interest such as

proteins, peptides, hormones, or antibody in fluid samples. Therefore, IHC was used as a platform to determine the optimal working concentrations (dilutions) of the primary antibodies before moving on to use IF to stain for more than one marker on each slide.

5.2 Experimental approaches

After determining the working dilutions of the primary antibodies based on the degree of positivity and distribution of targets of interest as outlined in sections 2.6.10.1- 2, Table 2-4 and illustrated in Figure 2-6, the working dilutions of the fluorochrome-conjugated secondary antibodies were determined. Three different dilutions around the manufacturers' recommended working dilutions for Alexa Fluor 488 (AF488) - Donkey anti-Mouse, and Alexa Fluor 647 (AF647)-Donkey anti-Rabbit antibodies were used to stain for sections of defined dilutions of Mouse anti-human CD56 (1:100) and Rabbit anti-human CD8 (1:500). Following a thorough assessment of the staining characteristics, optimal working dilutions of 1:2000 for AF488-Donkey anti-Mouse antibody and 1:100 for AF647-Donkey anti-Rabbit antibody were determined. These dilutions were used for the immunofluorescence staining of the PACIFICO FFPE tissue sections. Nine cell markers were evaluated per patient. Four sections were stained for markers expressing CD4/CD8, CD56/CD16, CD68/CD163 and PD1/FOXP3. However, a section was stained for cells expressing CD21. Although forkhead box protein P3 (FOXP3) is also expressed in recently activated human T cells (Magg et al., 2012), it is used to quantitate regulatory T (Tregs) cells.

5.3 The Results

5.3.1 Patients and materials

FFPE tissue biopsies from PACIFICO FL patients for this chapter comprised of 20 CMV positive and 22 CMV-negative patients. The selection of patient samples was based on the size and availability of lymph node biopsy material and not clinicopathological characteristics. A summary of demographic and baseline characteristics of this study cohort are described in Table 5-1. Patients' risk factors and the prognosis are stratified according to the Follicular Lymphoma International Prognostic Index (FLIPI) score into three risk categories: good (0 or 1), Intermediate (2) and poor (3-4). The FLIPI score is determined using five adverse prognostic factors including Ann Arbor stage (III-IV vs. I-II), age (> 60 years vs. ≤ 60 years), haemoglobin level (< 120 g/L vs. ≥ 120 g/L), number of nodal areas (> 4 vs. ≤ 4), and serum LDH level (above normal vs normal or below) (Solal-Céligny et al., 2004). A slightly higher proportion of CMV positive patients are on the high risk FLIPI grade compared to the CMV negative (65.0% vs. 59.1%). The Cumulative Illness Rating Scale (CIRS) is used to predict survival in lymphoma patients under 13 near independent areas. It is a 5-point rating of the degree of severity scale that ranges from none (1) to extremely severe (5) (Linn et al., 1968). There is no significant difference in the proportion of patients in Table 5-1 due to CMV status.

Table 5-1: Demographic and baseline characteristics of study cohort according to CMV status

Characteristics	Total (n=42) *	CMV Status (n=42)		
		Positive (n=20) *	Negative (n=22) *	P **
Age Median (IQR)	70 (64 – 75)	70.0 (64 – 75)	71 (64 – 75)	0.646
Gender N (%)				0.128
Male	23 (54.8)	8 (40.0)	15 (68.2)	
Female	19 (45.2)	12 (60.0)	7 (31.8)	
Haemoglobin (g/dL), Median (IQR)	13.3 (11.7 – 14.0)	13.5 (11.5 - 14.2)	13.2 (12.2 – 13.9)	0.929
Ann Arbor stage N (%)				0.448
2	4 (9.5)	1 (5.0)	3 (13.6)	
3	21 (50.0)	12 (60.0)	9 (40.9)	
4	17 (40.5)	7 (35.0)	10 (45.5)	
CIRS Score N (%)				1.000
≤5	31 (73.8)	16 (80.0)	15 (68.2)	
>5	11 (26.2)	4 (20.0)	7 (31.8)	
No. lymph nodes N (%)				0.666
1	1 (2.4)	1 (5.0)	0 (0.0)	
2	1 (2.4)	1 (5.0)	0 (0.0)	
3	4 (9.5)	0 (0.0)	4 (18.2)	
4	4 (9.5)	1 (5.0)	3 (13.6)	
5	5 (11.9)	2 (10.0)	3 (13.6)	
6	27 (64.3)	15 (75.0)	12 (54.5)	
LDH Median (IQR)	353 (229-447)	354 (263-446)	351 (228-453)	0.758
FLIPI Score N (%)				0.286
0-1 (low risk)	3 (7.5)	2 (10.0)	3 (13.6)	
2 (intermediate risk)	11 (27.5)	5 (25.0)	6 (27.3)	
3-5 (high risk)	26 (65.0)	13 (65.0)	13 (59.1)	
Histology N (%)				1.000
1	8 (19.0)	4 (20.0)	4 (18.2)	
2	24 (57.1)	11 (55.0)	13 (59.1)	
3	10 (23.8)	5 (25.0)	5 (22.7)	

*For continuous variables this refers to the Mean (SD) or Median (IQR). **P value for χ^2 test or Fisher's exact test for the difference between categorical variables or t - test for the difference between two means.

5.3.2 Evaluating of crossreactivity

The primary antibodies used in this double labelling study are Mouse anti-human in combination with Rabbit anti-human on a section and Donkey anti-Mouse secondary antibody conjugated to AF488 and mixed with Donkey anti-Rabbit secondary antibodies conjugated to AF647. Consequently, this study evaluates the cross-reactivity of a rabbit anti-human IgG and Mouse anti-human IgG1 as a representative of the antibodies used for the IF analysis of cellular markers on FFPE of FL patients. The aim of this experiment is to define if there is any cross-reactivity between the two secondary antibodies to exclude non-specific binding from interfering with the analysis.

To rule out the effect of crossreactivity, we undertook a crossreactivity assessment of the Mouse anti-human CD4 and Rabbit anti-human CD8. As shown in the cross-reactivity appraisal illustrated in Figure 5-1, 3-micron tonsil sections were stained thus: Sections A and D were stained with Mouse anti-CD4, while sections B and C were stained with Rabbit anti-CD8. Then sections A and B were counterstained with Donkey anti-Mouse secondary antibody conjugated to AF488 (for section B to be negative) and sections C and D were counterstained with Donkey anti-Rabbit conjugated to AF647 (making D a negative control).From the images in Figure 5-1, CD4+ (Figure 5-1A) and CD8+ (Figure 5-1C) cells are well defined with blue nuclear staining and surrounding red and green stains. Minimal cross reactivity was seen in Figure 5-1B and D for both secondary antibodies.

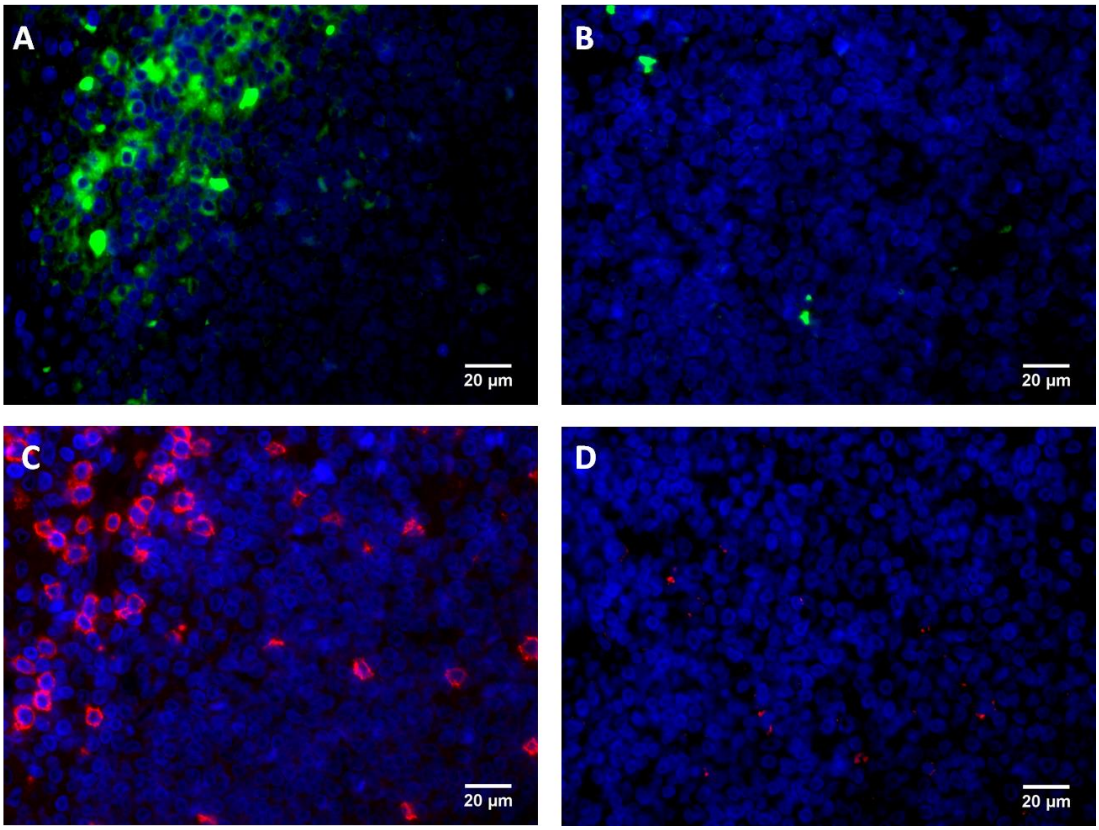

Figure 5-1: Images from the crossreactivity assessment.

Section A was stained with Mouse anti-CD4 and section B stained with Rabbit anti-CD8, and both sections were counterstained with Donkey anti-Mouse-AL488 (Green). Sections C and D were stained with Rabbit anti-CD8 and Mouse anti-CD4 respectively, and both were counterstained with Donkey anti-Rabbit-AF647 (Red). FFPE tonsil sections were used for the evaluation. (Original magnification: x40).

5.3.3 Evaluating simultaneous and sequential double IF

With the understanding that there was minimal crossreactivity in our design and as part of optimising the immunofluorescence protocol, an experiment to assess whether to use simultaneous or sequential double immunofluorescence staining was undertaken using an Autostainer. FFPE tonsil sections were stained for CD4 and CD8 markers and detected using fluorochrome-conjugated secondary antibodies, Donkey anti-Mouse-AF488 and Donkey anti-Rabbit-AF647 using the Dako AutostainerPLUS. The images from both methods are displayed in Figure 5-2 as composites, A and D

(CD4/CD8/DAPI), and single antibody fluorochromes with DAPI (CD4/DAPI and CD8/DAPI). No pronounced difference between the qualities of images from the two staining methods was observed. However, being that the simultaneous staining method reduces the chances of tissue detachment (as fewer steps), as well as the exposure to ambient light, this method was chosen for analysing the patients' tissue samples.

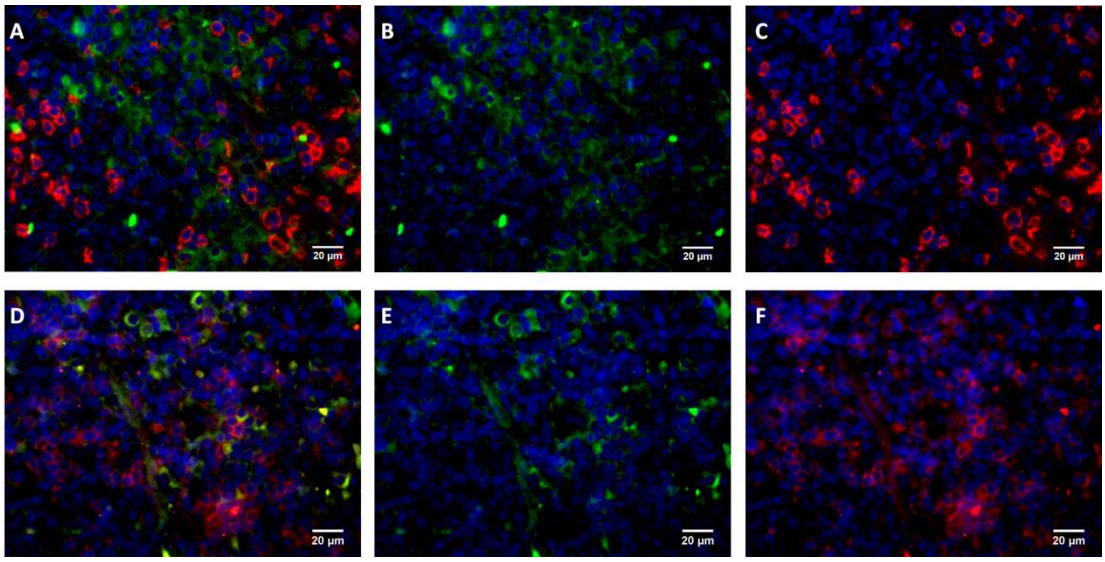

Figure 5-2: Evaluating Immunofluorescence staining methods.

Images of double stained of FFPE tonsil sections with antibodies directed against CD4 (green) and CD8 (red) by sequential (upper panel) and simultaneously (lower panel) methods. (Original magnification: x40).

This project adopts a simultaneous double labelling IF technique of targets on FFPE tissue sections to analyse for the expression levels of CD4+ and CD8+ (T cells), CD68+ and CD163+ (macrophages), FOXP3+ (regulatory T cells), CD21+ (primarily FDCs) PD1+ (exhausted T cells), CD16+ and CD56+ (NK cells) populations in FFPE tissue biopsies from FL patients.

To answer our hypothesis that CMV infection modulates FL microenvironment with the possibility of influencing rituximab-chemotherapy, we quantified and compared the expression of cellular markers between CMV- positive and negative patients.

After staining the 3-micron sections from the PACICIFO FFPE tissue biopsies the coverslips were sealed to the slide with nail varnish and stored in the fridge until ready for image capturing and analysis. A total of 8 images were captured per section using an Olympus BX63 motorised upright microscope with a 40× objective, equipped with a Photometrics CoolSnap camera and MicroManager acquisition software (Edelstein et al., 2014). Images were analysed with ImageJ (Schneider et al., 2012) using in-house written macros and Java plugins. The representative images of the antibody staining of five different tissue sections are displayed in Figures 5-3 to 5-7.

In Fig 5-3, higher intensities of CD8 (cytotoxic) expressing T cells compared to CD4 (helper) expressing T cells seen in A & C. Figures 5-4 shows PD1+ cells (a surface protein marker) stained with FOXP3+ cells (a nuclear protein). In this study, PD1 expression is used as a marker to assess for exhausted T cells in the FL microenvironment. Higher expression of FOXP3 can be seen in C & E. The expression of CD21, a FDC marker in FFPE sections, revealed a meshwork pattern of CD21+ staining Figure 5-5as commonly demonstrated by FDC in lymphoid tissue (Alio et al., 2015). Sections were stained for CD56/CD16 and CD68/CD163 expressing cells in the FFPE to quantitate for intratumoral NK cells and macrophages. Weak colocalization was demonstrated in CD68 and CD163 in Figures 5-7 and as evaluated in section 5.3.4*:*

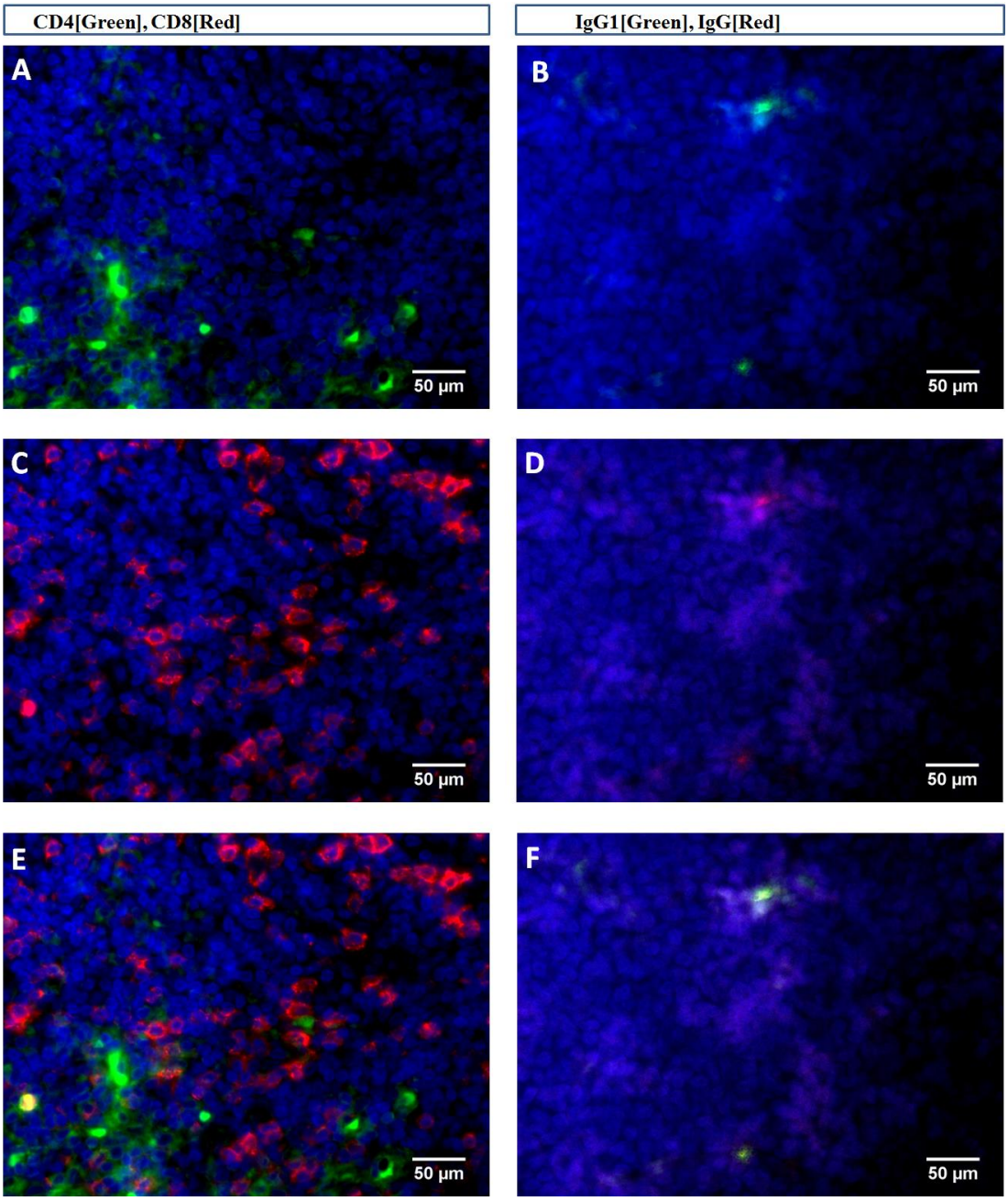

Figure 5-3: Double labelled immunofluorescent image for CD4/CD8 expression.

Immunofluorescent images of CD4/DAPI (A), CD8/DAPI (C) and a merged image of CD4/CD8/DAPI (E) alongside corresponding Isotype controls (B, D & F). The secondary antibodies comprised a mixture of Donkey anti-Mouse-AF488 dye (green) and Donkey anti-Rabbit-AF647 (a bright and photostable far-red dye). (Original magnification: x40).

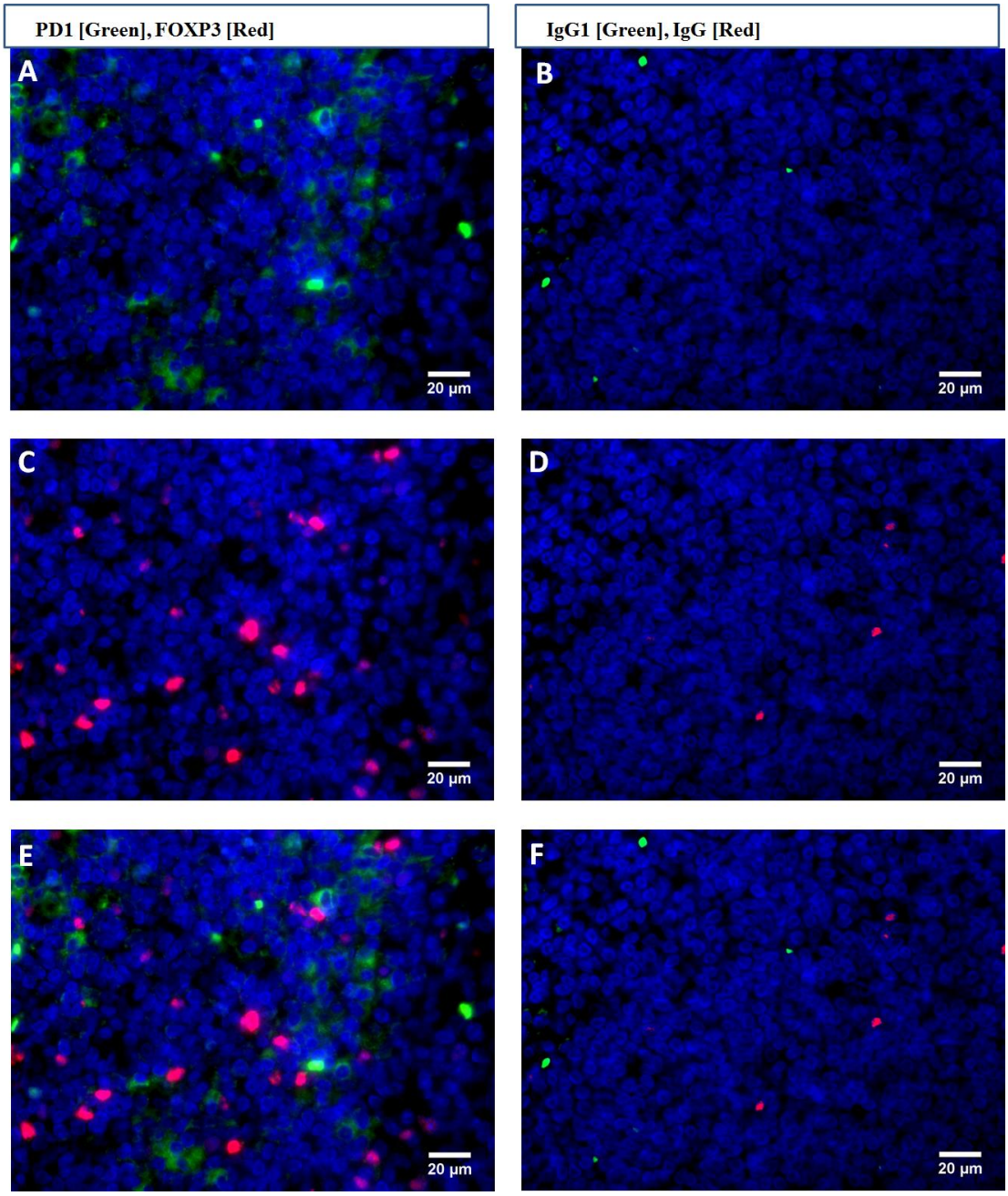

Figure 5-4: Double labelled immunofluorescent image for PD1/FOXP3 expression.

Immunofluorescent images of PD1/DAPI (A), FOXP3/DAPI (C) and a merged image of PD1/FOXP3/DAPI (E) alongside corresponding Isotype controls (B, D & F). The secondary antibodies comprised a mixture of Donkey anti-Mouse-AF488 dye (green) and Donkey anti-Rabbit-AF647 (a bright and photostable far-red dye. (Original magnification: x40).

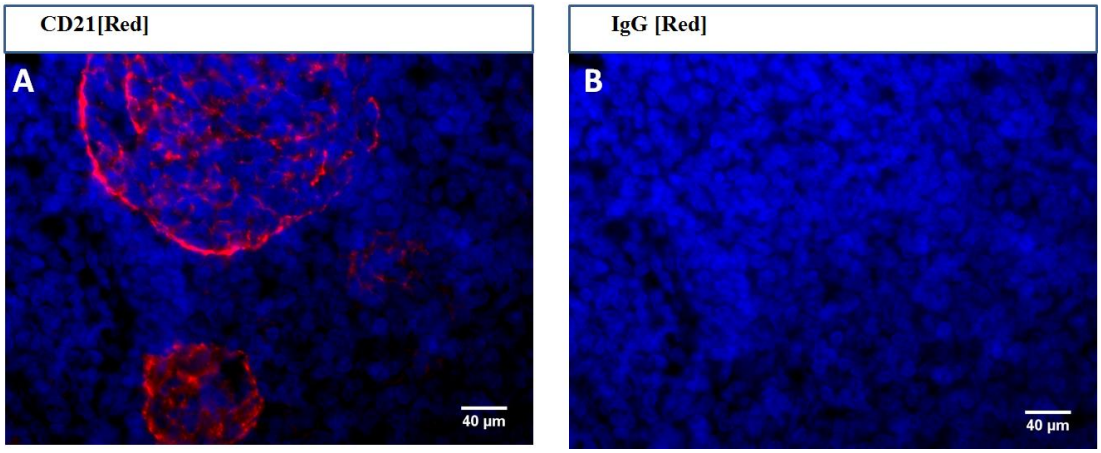

Figure 5-5: A single labelled immunofluorescent image for CD21 expression.

Immunofluorescent image for CD21/DAPI (A) and Isotype control (B). The secondary antibody used is Donkey anti-Rabbit-AF647 (a bright and photostable far-red dye). (Original magnification: x40).

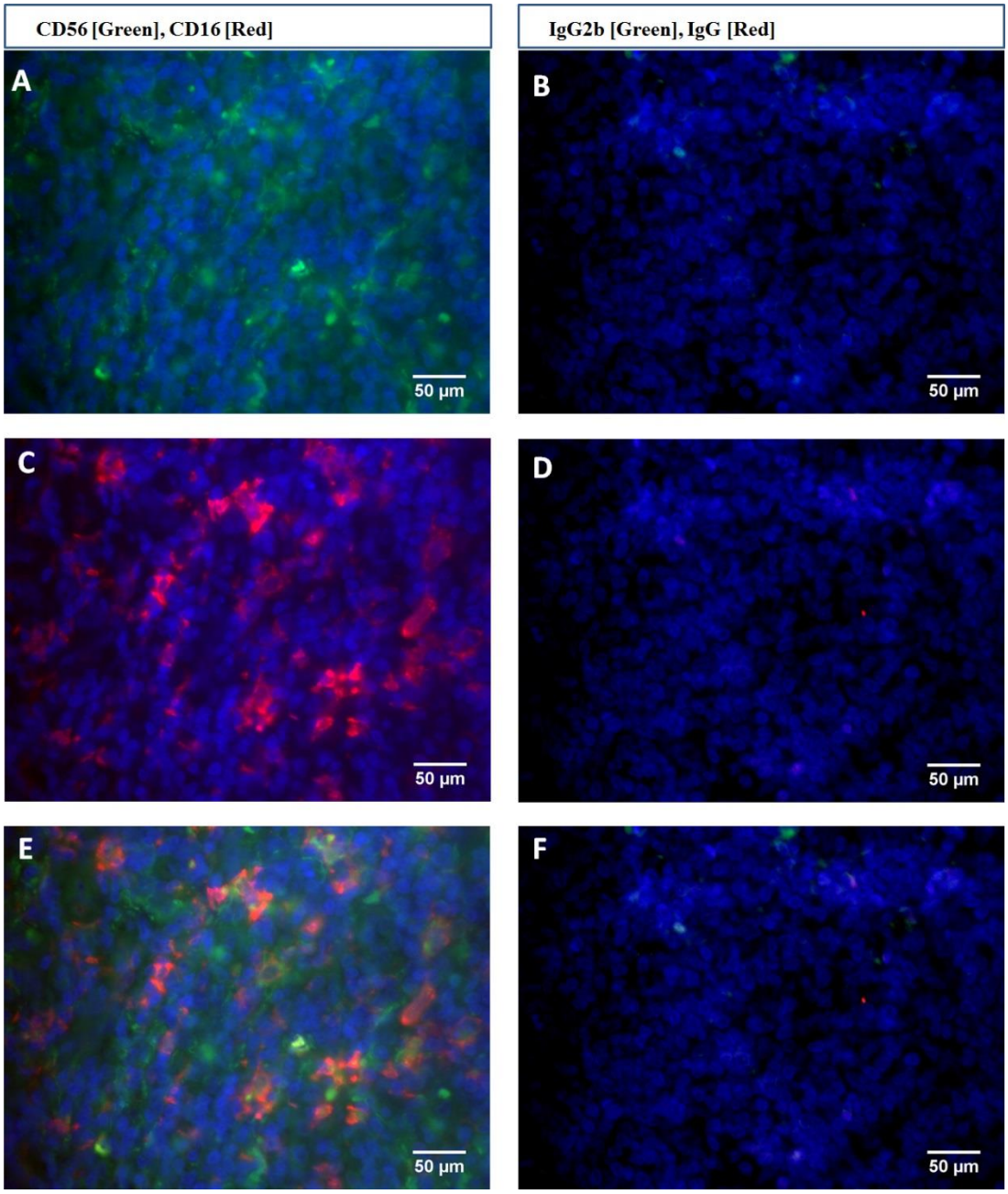

Figure 5-6: Double labelled immunofluorescent images for CD56/CD16 expression.

Immunofluorescent images of CD56/DAPI (A), CD16/DAPI (C) and a merged image of CD56/CD16/DAPI (E) alongside corresponding Isotype controls (B, D & F). The secondary antibodies comprised a mixture of Donkey anti-Mouse-AF488 dye (green) and Donkey anti-Rabbit-AF647 (a bright and photostable far-red dye). (Original magnification: x40).

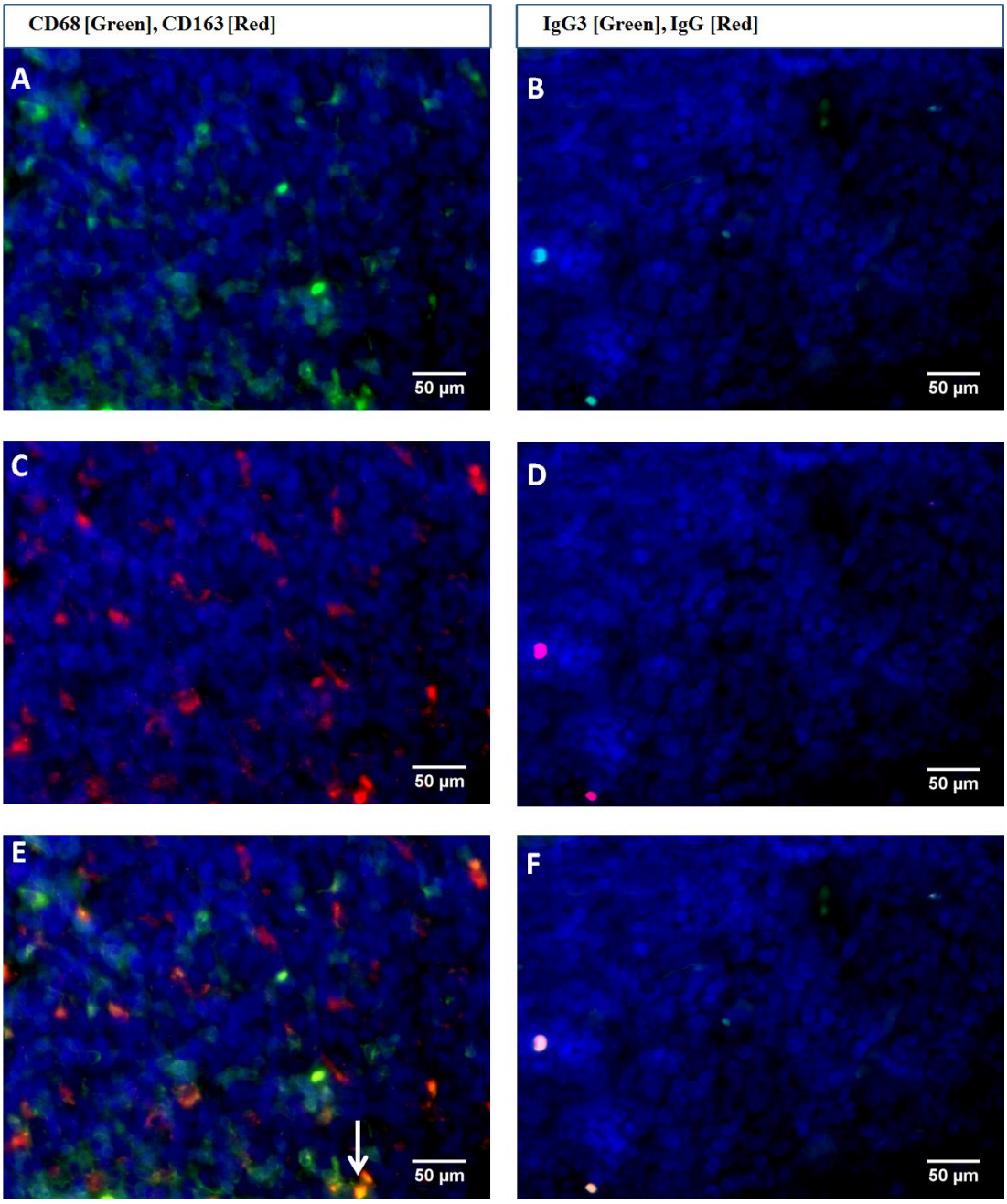

Figure 5-7: Double labelled immunofluorescent images for CD68/CD163 expression.

Immunofluorescent images of CD68/DAPI (A), CD163/DAPI (C) and a merged image of CD68/CD163/DAPI (E) alongside corresponding Isotype controls (B, D & F). The secondary antibodies comprised a mixture of Donkey anti-Mouse-AF488 dye (green) and Donkey anti-Rabbit-AF647 (a bright and photostable far-red dye). Arrow points at Colocalization (E; yellow). (Original magnification: x40).

5.3.4 Evaluating colocalization

The distribution of fluorescent signals between the paired markers per section (CD4/CD8, CD68/CD163 and CD56/CD16) was analysed quantitatively and statistically using the chi-squared (χ^2) test to determine the correlation coefficient (r) between pairs. This analysis revealed whether two markers associate with the same structures (not the same cell). This information is anticipated to provide an insight into possible co-expression of two markers on a cell.

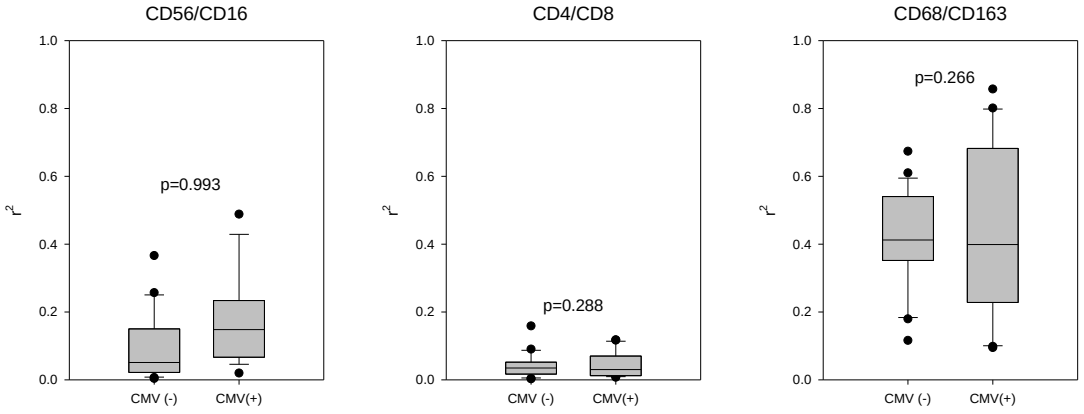

Figure 5-8: Spearman Rank Order Correlation for paired markers.

Figure summarises colocalisation of CD56/CD16, CD4/CD8 & CD68/CD163 according to CMV status. CMV- depicts CMV- negative while CMV+ represents CMV- positive.

5.3.5 Median intensities of markers of accessory in FL

The median expression levels of the accessory cell markers according to CMV infection status are as displayed in Table 5-2. The Mann-Whitney U test was used to analyse the intensities of markers of accessory cells in the FL microenvironment. Figure 5-9 revealed a statistically significant elevated median intensity of CD21 expression among CMV- positive (432.59) compared to the CMV- negative cohort (340.97) with a p-value of 0.043. Also, the sections from CMV- positive patients show marginally increased intensities of FOXP3 (p-values=0.069) and PD1 (p-

value=0.097) expression compared to CMV- negative. No other markers demonstrate a significant difference due to CMV status. However, from the table the CMV-negative group has shown a trend of increased median values of CD4+, CD8+, CD16+, CD56+, CD68+ and CD163+ expression.

Table 5-2: Descriptive table of intensities of cellular infiltrates in the FL microenvironment determined by immunofluorescence

S#	Cell Markers	CMV Status (n=42)			
		Total (n=42)	Positive (n=20)	Negative (n=22)	P
		Median (IQR)	Median (IQR)	Median (IQR)	
1	CD4	1672.8 (1452.9 - 1892.8)	1653.2 (1298.4 - 2008.0)	1691.5 (1394.9 - 1988.1)	0.784
2	CD8	473.5 (427.8 - 519.1)	461.3 (403.4 - 519.2)	485.1 (410.3 - 559.9)	0.969
3	CD16	332.1 (294.9 - 348.6)	329.9 (298.3 - 347.4)	333.6 (297.1 - 353.7)	0.796
4	CD21	390.8 (355.7 - 425.8)	426.0 (374.1 - 477.8)	358.8 (311.6 – 406.0)	*0.043*
5	CD56	1501.7 (1339.9 - 1663.5)	1453.6 (1195.3 - 1711.9)	1541.1 (1318.0 - 1764.3)	0.471
6	CD68	1059.0 (904.5 - 1430.0)	948.6 (867.8 - 1282.0)	1137.0 (981.3 - 1430.0)	0.162
7	CD163	599.7 (558.8 - 678.6)	583.8 (549.1 - 655.6)	609.2 (578.1 - 696.2)	0.345
8	FOXP3	114.7 (110.0 - 301.6)	232.2 (110.9 - 337.3)	111.1 (109.3 - 119.0)	*0.069*
9	PD1	193.4 (175.1 - 1053.0)	291.3 (187.2 - 1223.0)	187.9 (172.0 - 221.2)	0.097

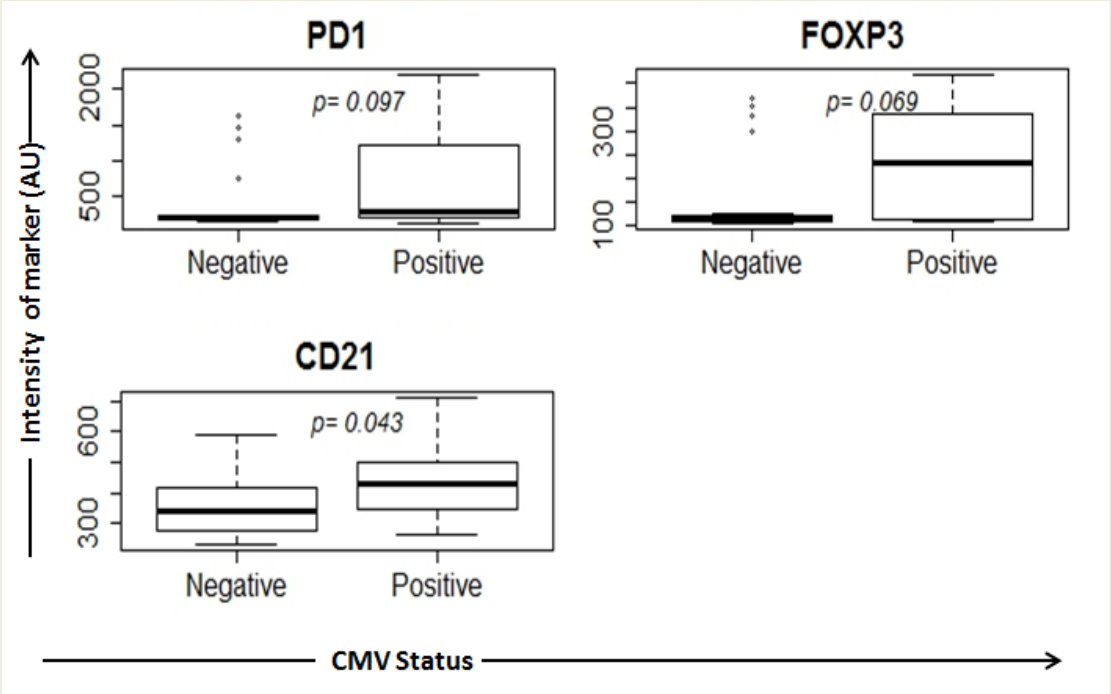

Figure 5-9: Median intensities of accessory cell markers in the FL microenvironment.

The p-values are determined by Mann-Whitney U-test.

5.4 Discussions

Cellular composition and quantities of infiltrating accessory cells in the FL microenvironment are imperative in determining the overall outcome of the disease. This Chapter has evaluated the intensities of essential cell markers of the FL microenvironment with a view to identify any conceivable differences in the tumour microenvironment due to CMV infection. Analysis of the median intensities of cell markers in the FL microenvironment revealed a statistically significant elevation in the level of CD21 (markers of FDCs) among CMV- positive FL patients compared to the CMV- negative group (p=0.043). Also, marginal increase in the expression of FOXP3 and PD1 were observed in CMV- positive than negative (p=0.069 & p=0.097 respectively).

By morphology, the FL is described as a proliferation of malignant GCBs surrounded by a variety of nonmalignant cells such as follicular helper T (T_{FH}) cells, follicular dendritic cells (FDCs), follicular reticular cells (FRCs) and macrophages, as illustrated in Figure 5-10 that can potentially affect the fate of tumour (Kridel et al., 2012). On the other hand, a 2-fold role of the FL microenvironment has been described to include supporting tumour growth/survival and suppressing the antitumoral immune response.

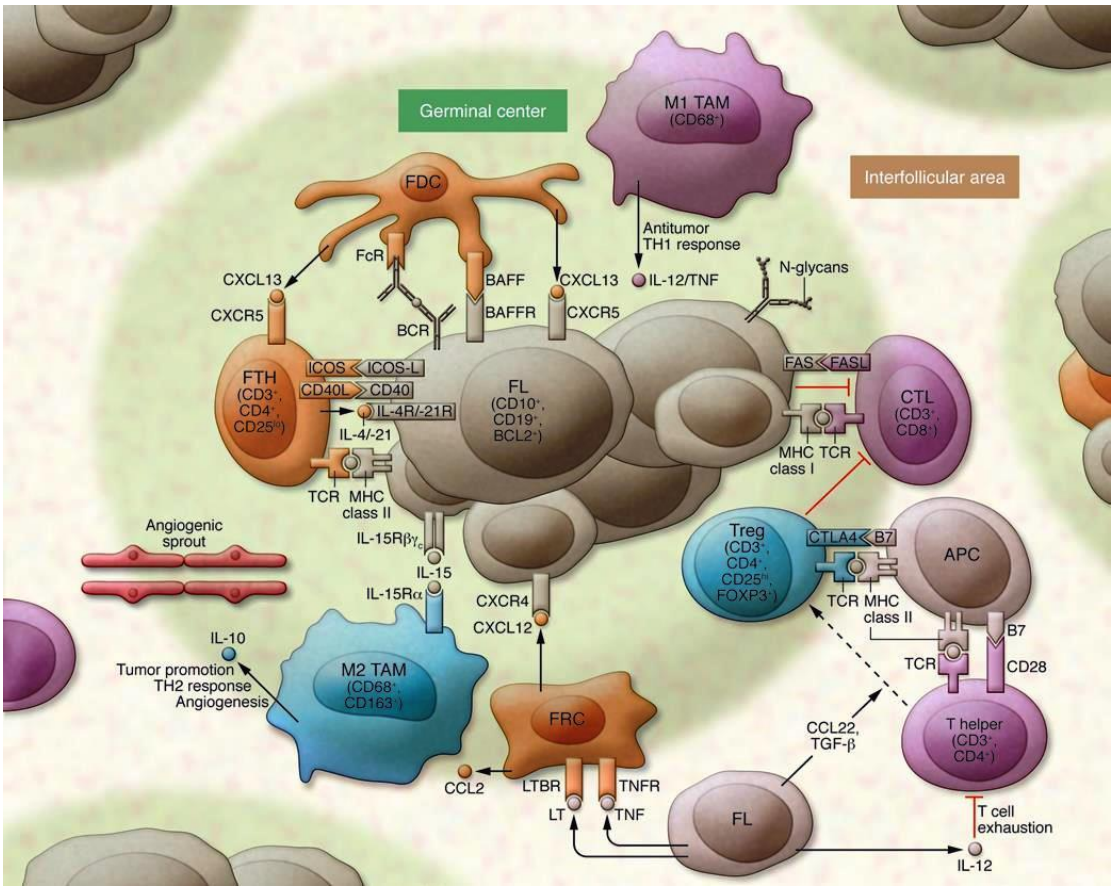

Figure 5-10: A model of the FL microenvironment.

This figure is reproduced with permission from (Kridel et al., 2012) and copyright clearance.

CD21 is a marker for FDCs that forms a complex with CD19, CD225 and CD81 in the membrane of mature B cells; which results in a considerably enhanced B cell

response to the antigen. FDCs are not a subtype of normal dendritic cells (DCs) as they are stromal in origin and develop from vascular mural cells, while the traditional DCs are haematopoietic (Aguzzi et al., 2014). While DCs activate naïve T cells by presentation of processed antigen via major MHC molecules, FDCs appear to present unprocessed antigen in the form of trapped immune complexes (Krautler et al., 2012). This attribute, along with the availability of chemokines, adhesion molecules, and trophic factors, enables FDCs to shape B cell responses and fashion the local microenvironment (Kridel et al., 2012).

Furthermore, FDC links innate and adaptive (B cell) responses. The ability of the FDCs to perceive innate stimuli through Toll-like receptors elaborates specific immune responses (Krautler et al., 2012). Also, FDCs control the elimination of apoptotic GC B cells. Though, FDCs can also contribute to autoimmune diseases, the FDC-mediated ingestion of dying B cells plays a crucial role in preventing autoimmunity. An immunohistological study has demonstrated that the dense mesh-like networks of follicular dendritic cells (CD21+) that contain growing neoplastic B-cells in low-grade B-cell lymphomas disappear in high-grade lymphomas (Shiozawa et al., 2003). The prognosis was found to be statistically better for patients with low-grade tumour than for those with high-grade tumour ($p = 0.026$), and there was a pattern of poorer survival among CD21-negative cases ($p = 0.186$). Along these lines, the presence of CD21-positive follicular dendritic cells in neoplastic follicles may predict the capability of FL to change to DLBCL (Shiozawa et al., 2003).

The model in Figure 5-10 also illustrates the variety of accessory infiltrating cells in a typical FL microenvironment, although quantities and types of cells in the microenvironment vary from one case to another and their clinical relevance. FL growth and survival signals may include IL-4 and IL-21. These supporting factors can

bind to the interleukin receptors on FL cells such as IL-4R/IL-21R or CXCL12 and CXCL13 secreted by the stromal cell subtypes. FDCs being professional antigen presenters can mediate BCR signalling, which can also take place via the action of an innate immune system (Janeway et al., 1997). As illustrated in Figure 5-10, it can be seen that tumour cells are capable of suppressing the immune responses against them from macrophages, CTLs and T helper cells (Kridel et al., 2012).

FL cells can survive the cytotoxic effects of CTLs by causing a T-cell immune dysfunction and induction of T cell exhaustion by secreting IL-12 (Kridel et al., 2012). Also, Tregs and M2 polarised macrophages have been reported to suppress an efficient immunological response against FL cells (Mantovani et al., 2017, Chung et al., 2011, Ha, 2009). The enhanced Tregs in the FL microenvironment weakens the T cell response.

FL contributes to the preferential conversion of T helper cells into Tregs by proteins such as TGF-β or CCL22 (Ha, 2009). Whereas classically activated TAMs, the M1 polarised CD68+ control malignant growth by inducing a T_H1 response; M2-polarized TAMs exerts a tumour-promoting function through angiogenesis and induction of an immunosuppressive T_H2 response (Quatromoni and Eruslanov, 2012).

There are well-documented pieces of evidence that point to the fact that the malignant B cells depress the function of the various T cell subtypes in the FL microenvironment so that they can escape immunosurveillance (Kridel et al., 2012). Studies have also revealed that elevated quantities of CD68+ -TAMS correlated with inferior prognosis and poor outcome in FL. (Clear et al., 2010, Taskinen et al., 2007). Although no statistical difference is observed due to CMV status, the CMV- negative FL patients had higher expression intensities for CD68 and may lead to a poor

outcome compared to the CMV- positive patients. It was also demonstrated that IL-12 secretion by malignant B cells induces T cell exhaustion (Yang et al., 2012).

Elevated numbers of tumour infiltrating FOXP3 (regulatory T cells) have been shown to associate with enhanced overall survival in FL (Carreras et al., 2006). This may suggest that CMV- positive patients are likely to fare better than the CMV- negative FL patients. It has also been reported that FL cells effect the transformation of effector T cells into FOXP3-expressing Tregs, which suppress the proliferation and action of both CD4+ and CD8+ T cells (Kridel et al., 2012, Ai et al., 2009). FDCs are professional APCs and increased frequencies in the microenvironment of FL patients with chronic CMV infection may be logical in enhancing BCR signalling, which is achievable either via the innate immune system, provoking BCR through N-glycans, or via antigen processing and presentation by FDCs (Kridel et al., 2012). Furthermore, it has also been reported that FL cells affect the transformation of effector T cells into FOXP3 (Tregs), which suppress the proliferation and action of both CD4+ and CD8+ T cells.

Our data may be vaguely interpreted as enhanced antigen presentation activity due to CMV, increased T cell regulation and raised T cell exhaustion following chronic and sustained antigenic stimulation.

The present study has not been able to characterise the various cell types in the FL microenvironment that express PD-1, however, two subcategories of T-cells are known to express PD-1 based on the expression levels of the marker. The PD-1^{high}CXCR5+ T_{FH} cells phenotype which are exclusively expressed by the CD4+ T cells have been reported to promote the survival of B cells. On the other hand, the PD-1^{low}TIM-3+ exhausted T cells category can be expressed on both CD4+ and CD8+ T

cells, and this subtype has lost the ability to secrete cytokines and to convey cell signalling (Yang et al., 2015). Therefore, there is no clear understanding of the functional or clinical relevance of the marginally elevated median intensities of PD-1 in the CMV- positive FL patients compared the CMV- negative counterparts.

Taking together, FL patients with CMV infection can be seen to express higher intensities of markers for FDCs (CD21+), regulatory T cells (FOXP3+) and exhausted T cells (PD1+) in the tumour microenvironment.

A Longitudinal study will further illuminate the relevance of findings. Also, a study that incorporates other differentiating markers for instance, TIM-3 can offer more information regarding the significance of the elevated intensities of CD21, PD-1 and FOXP3 markers in the FL microenvironment due to CMV infection. In the meantime, the explorative analysis of the relationship between clinical data (treatment efficacy and toxicity) and CMV status (Chapter 6) as well as laboratory data (Chapter 7) will provide an insightful perspective on the clinical relevance of the laboratory data.

Chapter 6 : Effect of CMV on treatment efficacy and toxicity

6.1 Introduction

The overall hypothesis for this thesis was that CMV infection in FL alters immune function in two ways: (1) altering the lymphoma microenvironment in a way that could modulate the behaviour of the disease as well as its response to therapy; and (2) accelerating immune senescence and thereby increasing the risk of infection following immunosuppressive chemo-immunotherapy. By showing that CMV infection was associated with the accumulation of exhausted T cells, reduction of some cytokines in the blood, and expansion of follicular dendritic cells in affected lymph nodes, the laboratory data presented in Chapters 3-5 support both components of this hypothesis. The aim of the present Chapter was to explore the potential impact of CMV infection in patients with FL at the clinical level by relating CMV infection status to therapy response and toxicity, especially infection.

6.2 Approach

Clinical data were related to CMV status using explorative statistics in 3 separate cohorts of patients: those whose samples were used for the FACS/cytokine analysis (n=42), those whose samples were used for the immunofluorescence study (n=42 including 6 in FACS/cytokine cohort), and all patients of known CMV serostatus who were recruited into the PACIFICO trial (n=269). As previously explained, samples used for lymphocyte/cytokine profiling and tissue staining were specifically selected (a) to provide an equal number of CMV-positive and CMV-negative cases and (b) to be well balanced in other respects. It was considered important to examine the effect of CMV status in the overall trial cohort in order to increase statistical power and therefore the chances of finding significant differences between the CMV-positive

and CMV-negative groups. On the other hand, there was a risk that CMV-positive and CMV-negative patients might not be balanced in other respects, potentially introducing bias. At the point of data lock, 360 patients had been randomised into the PACIFICO study. Of these, 269 (75%) were informative for CMV status and constituted the overall trial cohort. 191 patients were only in the overall cohort whereas the rest were also in FACS/cytokine cohort and/or IF cohort. Figure 6.1 shows the relationship between FACS/cytokine, IF and overall trial cohorts.

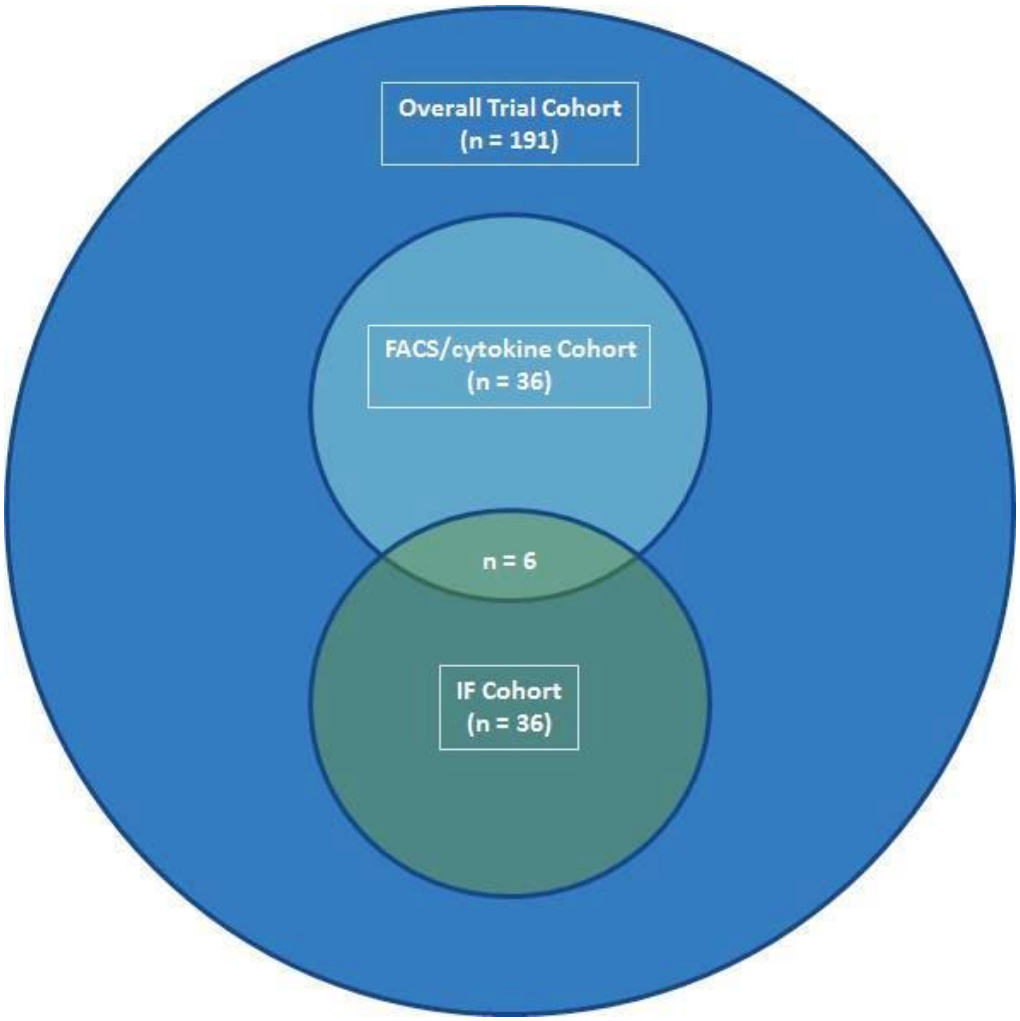

Figure 6-1: Venn diagram showing the number of patients and relationship between the three cohorts analysed.

The proportion of CMV-positive cases in FACS/cytokine, IF and overall trial cohorts was 50%, 48% and 40.5%, respectively, the remainder being CMV-negative.

The two main clinical events of interest were treatment response and toxicity. Long-term outcomes such as PFS and OS were not available as the trial is still in follow-up. Treatment response after 4 and 8 cycles of treatment was captured through CRFs provided by local sites and collated by the Clinical Trials Unit (CTU). The patients' response to treatment was evaluated based on Revised Standardised Response Criteria for Malignant Lymphoma (Cheson et al., 2007). In this criteria, an anatomical response is assessed by contrast-enhanced computed tomography (CT), which defines the degree of shrinkage of the affected lymph nodes and essentially classifies response into Complete Remission (CR), Partial Remission (PR), Stable Disease (SD) and Progressive Disease (PD) (Subramaniam, 2015, Cheson et al., 2007). As the name implies, CR is reported when there is no evidence of disease confirmed by the disappearance of palpable nodules in spleen and liver. PR, on the other hand, denotes regression of measurable disease and no new sites involvement. This is defined by a ≥ 50% reduction in the sum of the product of the diameters of up to 6 largest dominant nodal masses (Subramaniam, 2015, Cheson et al., 2007). Meanwhile, a failure to achieve CR, PR or a PD during therapy is described as SD. In this situation, no new locations identified on CT; with no change in the dimension of early lesions on CT. Finally, PD refers to a treatment response status characterised by the appearance of a new lesion(s) > 1.5 cm in any axis or increase by ≥ 50% of previously involved sites from nadir. Toxicity was captured via Adverse Event (AE) and Serious Adverse Event (SAE) reports submitted by local sites and collated by the CTU, and categorised in accordance with version 4 of the universally applied National Cancer Institute (NCI) Common Terminology Criteria for Adverse Events (CTCAE). Since CMV infection can accelerate immune senescence, the study focused on infection. However, to take into account the possibility that infections might arise in the context

of chemotherapy-induced neutropenia, the rate of febrile neutropenia (reported by some local sites as “neutropenic fever” or “neutropenic sepsis”) was also examined.

Summary statistics and independence hypothesis tests were conducted to explore any differences in treatment efficacy and toxicity due to CMV status. Toxicity was quantified in two different ways: either as the proportion of patients who experienced the event of interest, or as the total number of events of interest (thereby taking into account that one patient may experience multiple infections). To minimise bias due to differences between CMV-positive and CMV-negative patients that could affect the reporting of Adverse Events (AEs)/Severe Adverse Events (SAEs) (e.g. duration of treatment exposure), the rate of infection and febrile neutropenia was expressed as a proportion of all reported toxicity within each group being analysed.

The results of the analyses are presented under 3 main sections: baseline characteristics of the 3 cohorts used for the study; effect of CMV on treatment efficacy; and impact of CMV on infection and febrile neutropenia.

6.3 The baseline characteristics of study cohorts

The baseline characteristics of patients in the FACS/cytokine and immunofluorescence (IF) cohorts used for lymphocyte profiling/cytokine analysis and tissue studies respectively have already been presented in Table 3-1 (Chapter 3) and Table 5-1 (Chapter 5). In both cohorts, there was no difference between CMV-positive or CMV-negative groups in a range of variables with the potential to impact on treatment efficacy and/or toxicity. These variables included age, stage, Hb, LDH, number of affected lymph node groups and FLIPI score. Table 6-1 shows baseline characteristics of the overall trial cohort broken down by CMV status. Of the 269 patients, 160 (59%) were CMV- negative, and 109 (41%) were CMV- positive. FL

Patients' risk factors and the prognosis are stratified by the Follicular Lymphoma International Prognostic Index (FLIPI) score into three risk categories: good (0 or 1), Intermediate (2) and poor (3-4). The FLIPI score is determined using five adverse prognostic factors including Ann Arbor stage (III-IV vs. I-II), age (> 60 years vs. ≤ 60 years), haemoglobin level (< 120 g/L vs. ≥ 120 g/L), number of nodal areas (> 4 vs. ≤ 4), and serum LDH level (above normal vs normal or below) (Solal-Céligny et al., 2004). The Cumulative Illness Rating Scale (CIRS) is used for the physical assessment of impairment of patients under 13 near independent areas. It is a 5-point rating of the degree of severity scale that ranges from none (1) to extremely severe (5) (Linn et al., 1968). There is no significant difference in the proportion of patients in Table 6-1 due to CIRS. It can be seen that the two groups were mostly well balanced for a range of clinical features that have the potential to influence treatment efficacy and toxicity, although a higher proportion of patients in the CMV-positive group had a high-risk FLIPI score (70% versus 61%), potentially biasing this group towards a worse treatment outcome.

Table 6-1: Baseline clinicopathological characteristics of the overall trial cohort according to CMV status

Characteristics	Total (n=269) *	CMV Status (n=269)		
		Positive (n=109) *	Negative (n=160) *	P **
Age Median (IQR)	70 (66 – 75)	69 (67 – 76)	70 (65 – 75)	0.192
Gender N (%)				0.114
Male	123 (45.7)	43 (39.4)	80 (50)	
Female	146 (54.3)	66 (60.6)	80 (50)	
Haemoglobin (g/dL), Median (IQR)	13.0 (12.0 – 14.2)	13.0 (12.0-14.1)	13.1 (12.0 - 14.2)	0.423
Ann Arbor stage N (%)				0.419
2	33 (12.2)	10 (9.3)	23 (14.4)	
3	121 (45.0)	52 (48.6)	69 (43.1)	
4	113 (42.0)	45 (42.1)	68 (42.5)	
CIRS Score N (%)				0.963
≤5	223 (82.9)	91 (83.5)	132 (82.5)	
>5	46 (17.1)	18 (16.5)	28 (17.5)	
No. lymph nodes N (%)				0.666
1	11 (4.1)	6 (5.5)	5 (3.1)	
2	17 (6.3)	6 (5.5)	11 (6.9)	
3	21 (7.8)	4 (3.7)	17 (10.6)	
4	22 (8.2)	12 (11.0)	10 (6.3)	
5	31 (11.5)	12 (11.0)	19 (11.9)	
6	167 (62.1)	69 (63.3)	98 (61.3)	
LDH Median (IQR)	358 (225-467)	336 (215-437)	356 (221-470)	0.502
FLIPI Score N (%)				0.229
0-1 (low risk)	26 (9.7)	7 (6.4)	19 (11.9)	
2 (intermediate risk)	69 (25.6)	26 (23.9)	43 (26.9)	
3-5 (high risk)	174 (64.7)	76 (69.7)	98 (61.3)	
Histology N (%)				0.352
1	75 (29.1)	36 (34.0)	39 (26.6)	
2	128 (49.6)	49 (46.2)	79 (52.0)	
3	55 (21.3)	21 (19.2)	34 (22.4)	

*For continuous variables this refers to the Mean (SD) or Median (IQR)

**P value for χ^2 test or Fisher's exact test for the difference between categorical variables or t - test for the difference between two means.

6.4 Effect of CMV infection on treatment efficacy

6.4.1 FACS/cytokine Cohort

Response data was available for 37 of the 42 patients in this cohort. The stacked bar chart in Figure 6-2 shows treatment response post cycle 4. Out of the 18 CMV-negative FL patients, only 2 (11%) achieved complete remission (CR), and 16 (89%) responded partially (PR). On the other hand, out of 19 CMV-positive patients, 9 (47%) showed CR and 9 (47%) partial remission with 1 (5%) patient showing stable disease (SD). Thus the CR rate was significant higher in CMV-positive group than that in CMV- negative group after 4 cycles of treatment, as analysed with χ^2 test (P = 0.016) or Fisher's exact test (P = 0.029). As expected, response (CR or PR) was recorded in all cases whose treatment lasted for 8 cycles. The CR rate increased in both groups after that. In a total of 35 patients with information available, it was achieved by 12 of the 18 CMV-positive (67%), and 9 of the 17 CMV-negative (53%) patients (Figure 6.3). Although the CR rate was still higher in the former group, the difference became small and lacked a statistical significance (χ^2 test P = 0.407; Fisher's exact test P = 0.500).

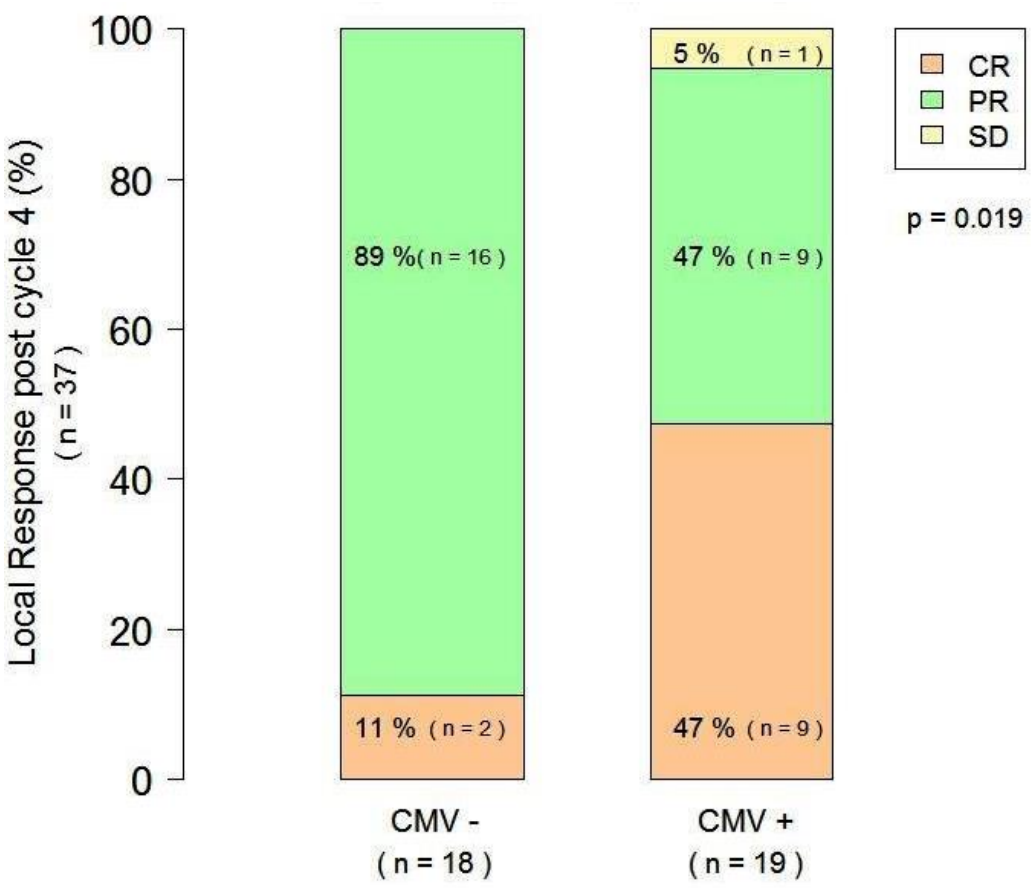

Figure 6-2: Stacked bar charts illustrating the proportion percentages of patients' local response after post cycle 4 by CMV status (FACS/cytokine Cohort).

CR= Complete Remission, PR= Partial Remission, SD= Stable Disease. There were no patients showing Progressive Disease (PD) response after post cycle 4.

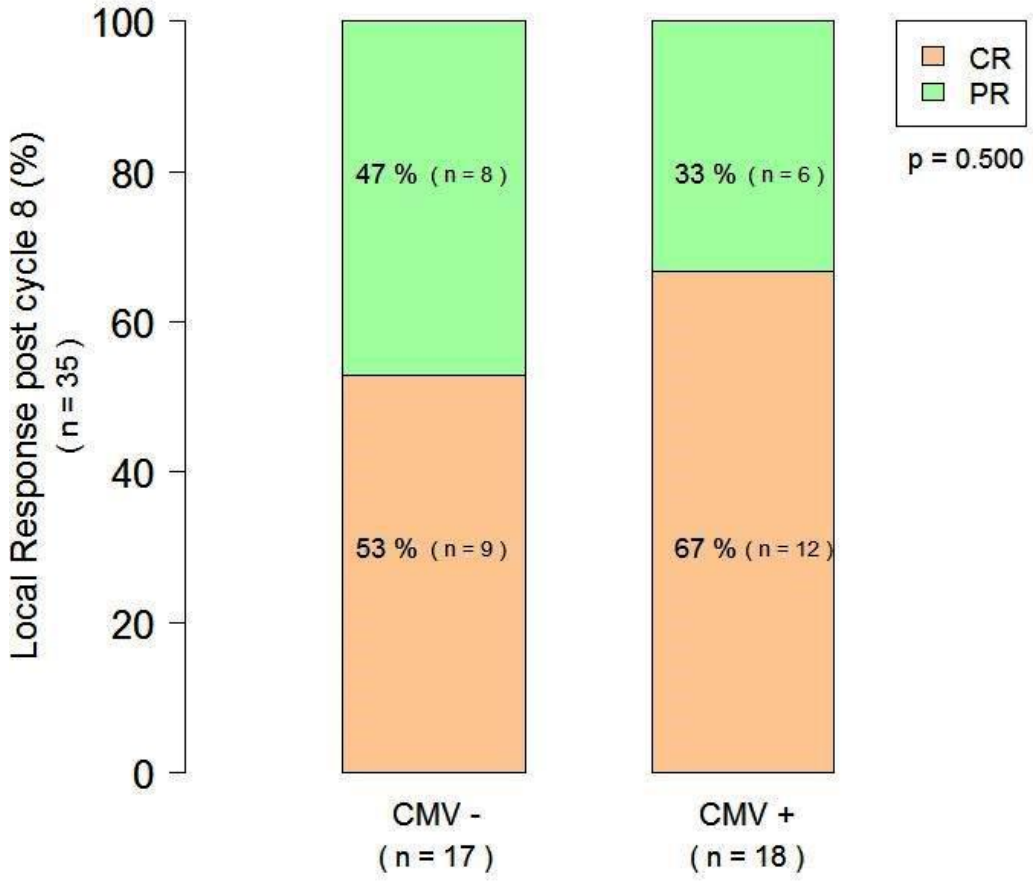

Figure 6-3: Stacked bar charts illustrating the proportion percentages of patients' local response after post cycle 8 by CMV status (cytokine/FACS.

CR= Complete Remission, PR= Partial Remission. There were no patients showing Stable Disease response or Progressive Disease response after post cycle 8.

The trend of CR achievement after 4 and 8 cycles of treatment in both groups are presented in Figure 6-4, which shows a favourable effect of CMV infection on the quick response.

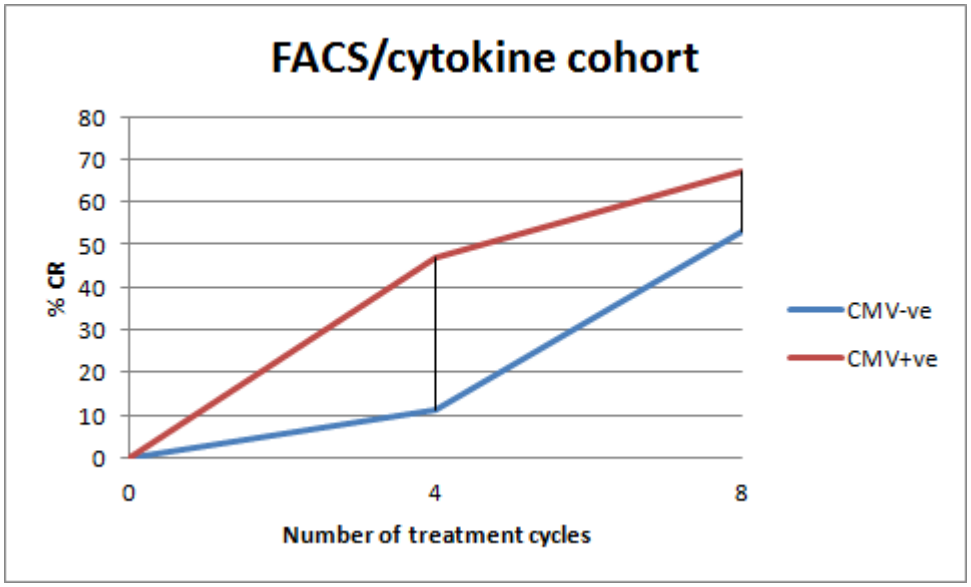

Figure 6-4: The line graph shows the proportion of CMV-positive and CMV-negative patients who achieved a CR at both time points (FACS/cytokine cohort).

6.4.2 Immunofluorescence cohort

Response data was available for 38 of the 42 patients in this cohort. The stacked bar chart in Figure 6-5 shows treatment response at post cycle 4. Out of the 20 CMV-negative FL patients, only 3 (15%) responded completely (CR) to therapy, 16 (80%) responded partially (PR) and 1 had a stable disease (SD). While among the CMV-positive patients, 4 (22%) achieved CR, 12 reached PR, and 1 (6%) each showed SD and PD, respectively. Although the CR rate in the CMV-positive group was bigger than that in the negative group, the difference did not reach a statistical significance ($p = 0.687$, Fisher's exact test). This was the same when the rate of CR+PR was compared between the two groups ($p = 0.595$).

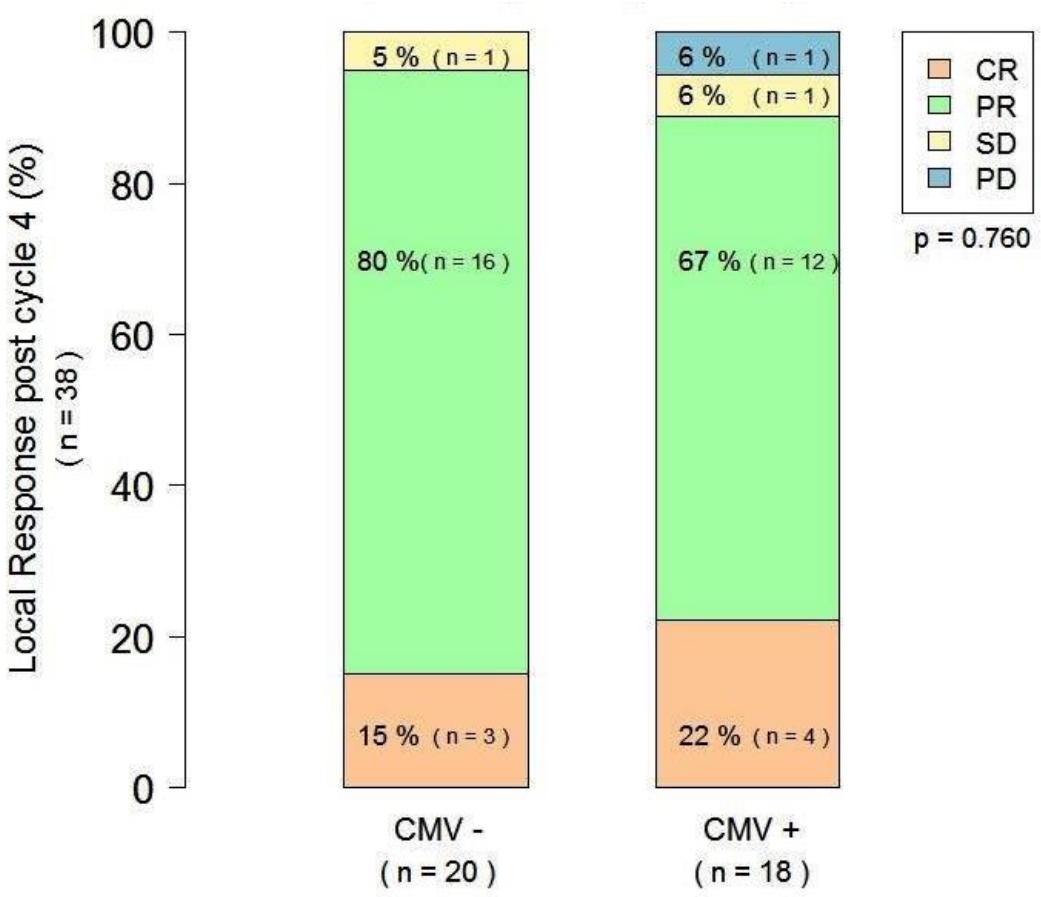

Figure 6-5: Stacked bar charts illustrating the proportion percentages of patients' local response to therapy after post cycle 4 by CMV status (Immunofluorescence).

CR= Complete Remission, PR= Partial Remission, SD= Stable Disease, Progressive Disease (PD).

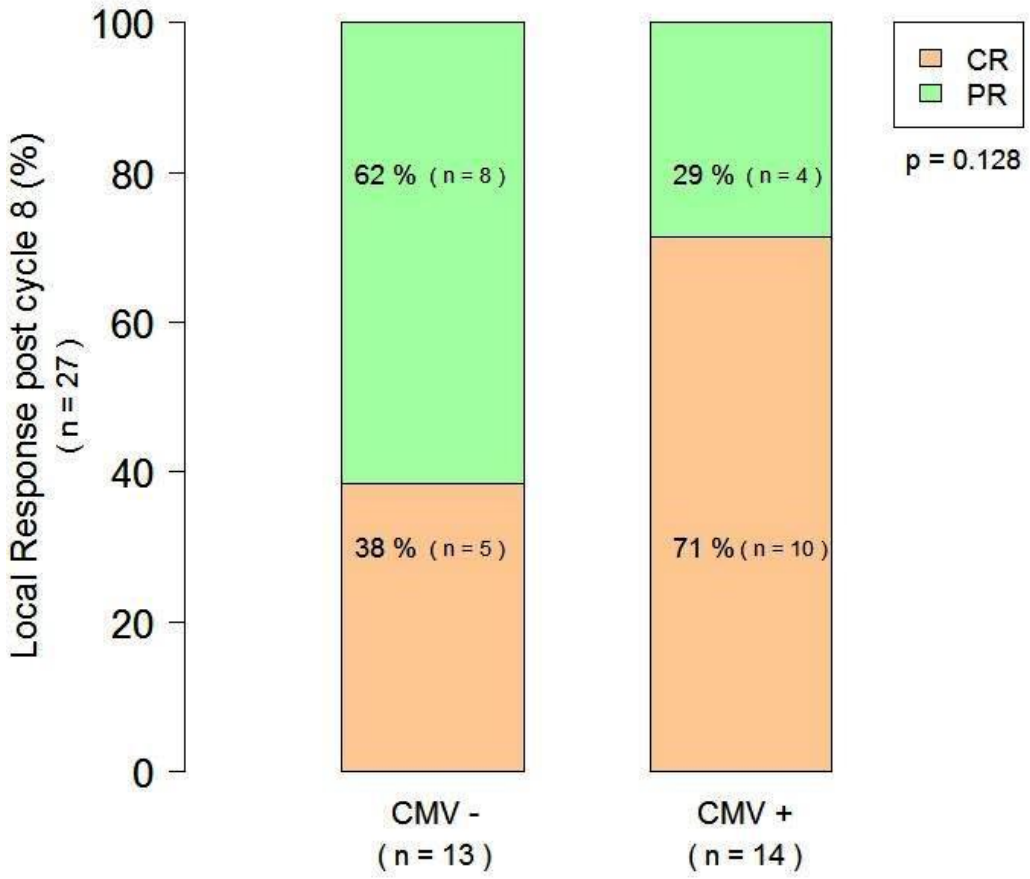

Figure 6-6: Stacked bar charts illustrating the proportion percentages of patients' local response to therapy after post cycle 8 by CMV status (Immunofluorescence).

CR= Complete Remission, PR= Partial Remission, SD= Stable Disease, Progressive Disease (PD).

Similar to what was found in the FACS/cytokine cohort, the CR rate increased when the treatment lasted for 8 cycles in both CMV-positive and negative groups. However, such increase was less profound in the negative group, leading to a bigger difference from the positive groups (38% versus 71%, $p = 0.085$, χ^2 test) (Figure 6-6). Thus, more patients in the positive group are likely to achieve CR. As summarised in Figure 6-7, the CR rates recorded at the two time points post treatment were generally in agreement with that found in the previous cohort, suggesting CMV infection is associated with good response to the treatment in the trial (detailed in 6.4.1).

However, compared the previous cohort, CR rate increased slower in patients with CMV negativity and did not reach to the same level at 8 cycle post treatment in this cohort. The reason for the difference was not clear, but might be due to the differences in patients between the two cohorts. (For example, more male patients were CMV-negative in this cohort (68.2% versus 38.1%, p = 0.069, Fisher's exact test).

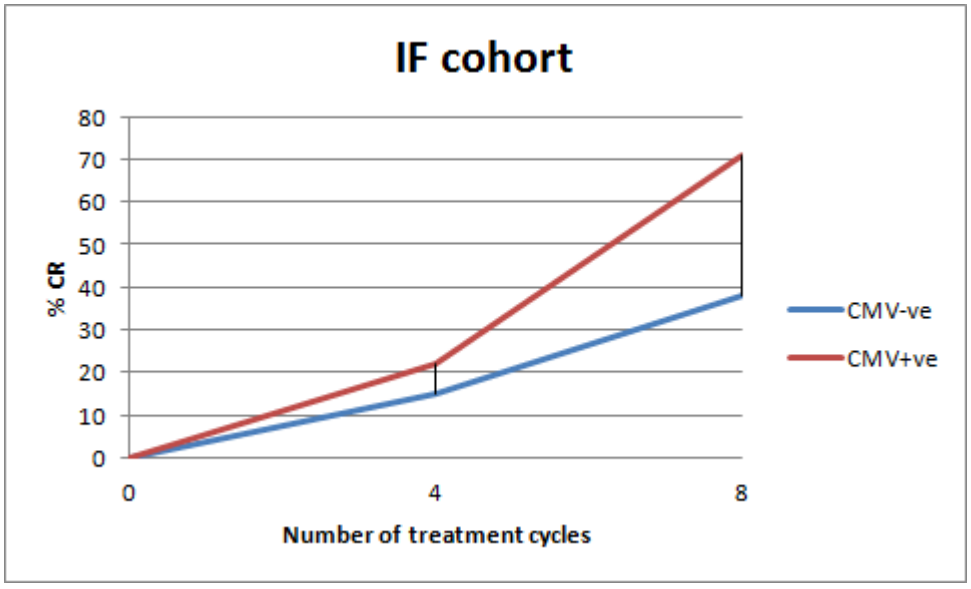

Figure 6-7: The line graph shows the proportion of CMV+ve and CMV-ve patients who achieved a CR at both time points (IF cohort).

CMV-ve denotes CMV- negative, while CMV+ve denotes CMV- positive.

Furthermore, the logistic regression analysis of contributors to the higher CR rate not only further confirmed the role of the prolonged treatment (p = 0.003), but also revealed a likely relationship with CMV infection (p = 0.103), as shown in Figure 6-7.

6.4.3 The PACIFICO overall trial cohort

Response data was available for 223 of the 269 patients in this cohort. Figures 6-8 and 6-9 give graphical summaries of the relationship between CMV status of patients and response to therapy at post cycles 4 and 8 respectively. The stacked bar chart in Figure 6-8 shows that after 4 cycles of therapy, among the 134 CMV- negative FL

patients, 21 (28%) had a complete response (CR), 102 (76%) had a partial response (PR) and 4 (3%) had stable disease (SD), whereas among the 89 CMV- positive, 20 (22%) had a CR, 66 (74%) a PR, 2 (2%) SD and 1 (1%) PD. In summary, there was no association between CMV status and response post cycle 4.

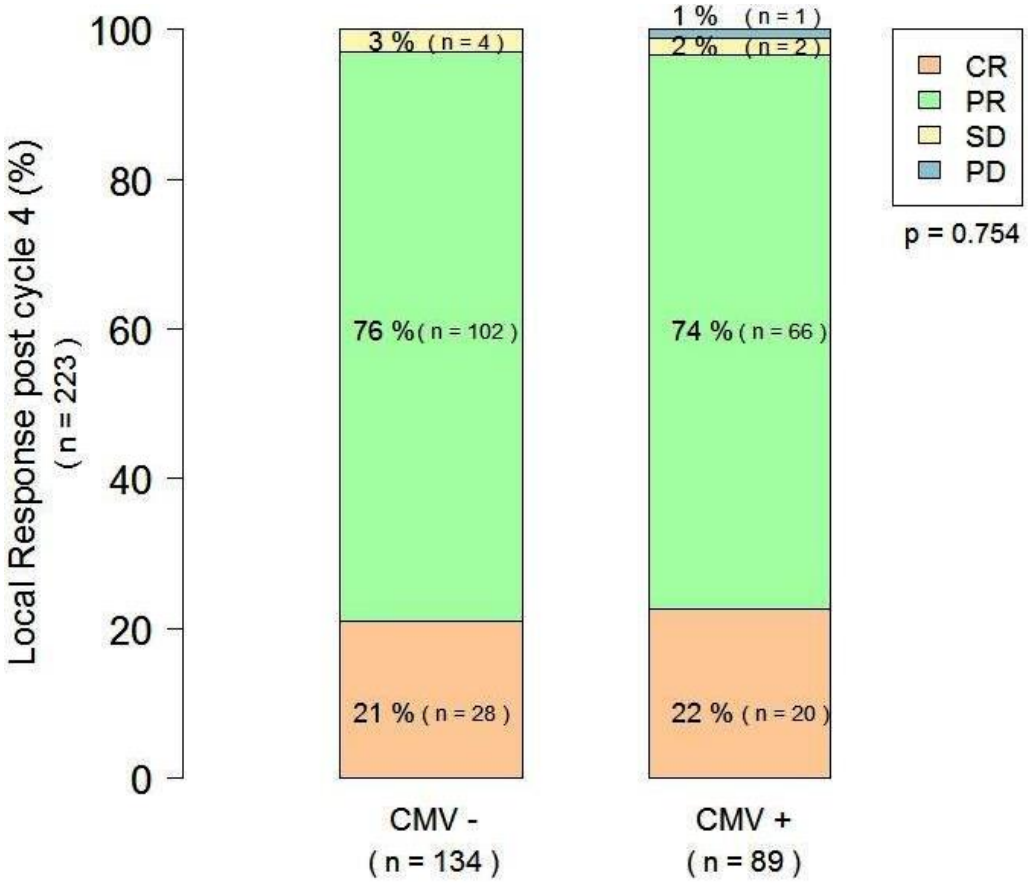

Figure 6-8: Barplot to show relationship between CMV status and post cycle 4 (overall trial cohort).

CR= Complete Remission, PR= Partial Remission, SD= Stable Disease, Progressive Disease (PD).

The stacked bar chart in Figure 6-9 shows that after 8 cycles of therapy, among the 115 CMV- negative FL patients, 49 (43%) had a (CR), 64 (56%) a PR and 2 (2%) PD, whereas among the 69 CMV- positive, 36 (52%) had a CR, 31 (45%) a PR and 2 (3%) PD.

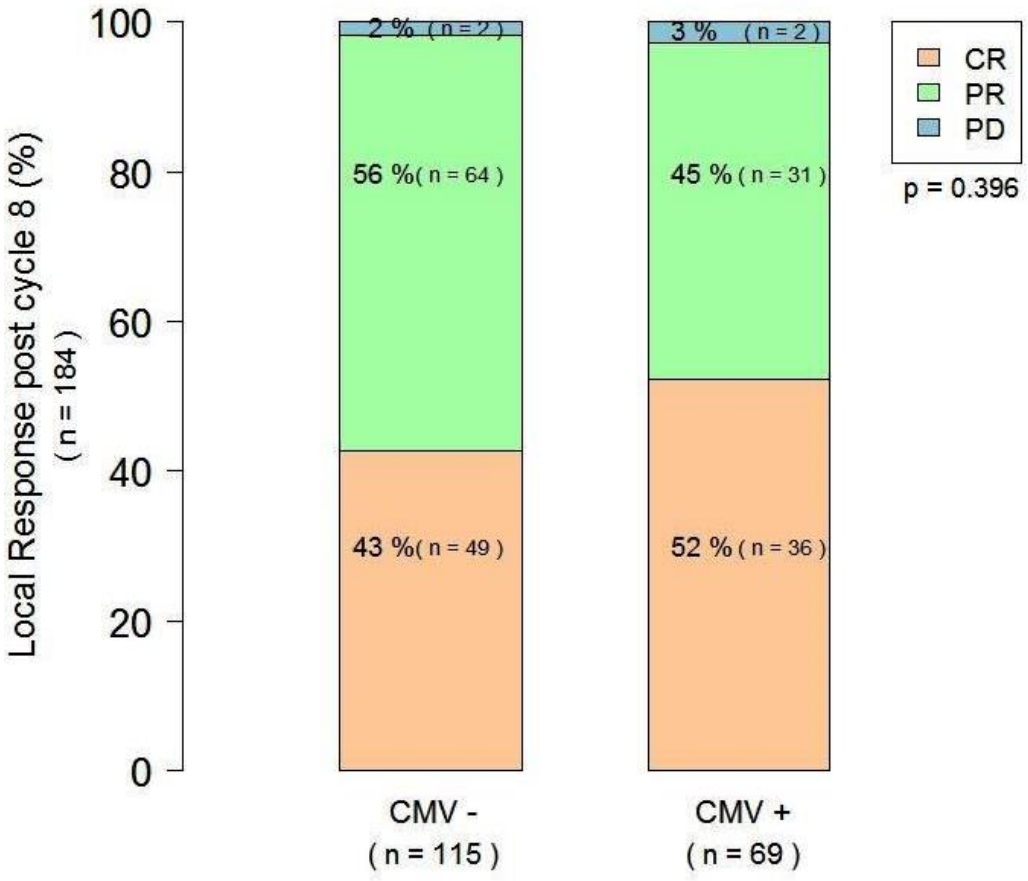

Figure 6-9: Barplot to show relationship between CMV status and post cycle 8 (overall trial cohort).

CR= Complete Remission, PR= Partial Remission, and Progressive Disease (PD).

From the summary line graph in Figure 6-10, there may be a small trend towards higher CR rates after 8 cycles of therapy in the CMV-positive group but the effect is much less striking than in the other 2 cohorts.

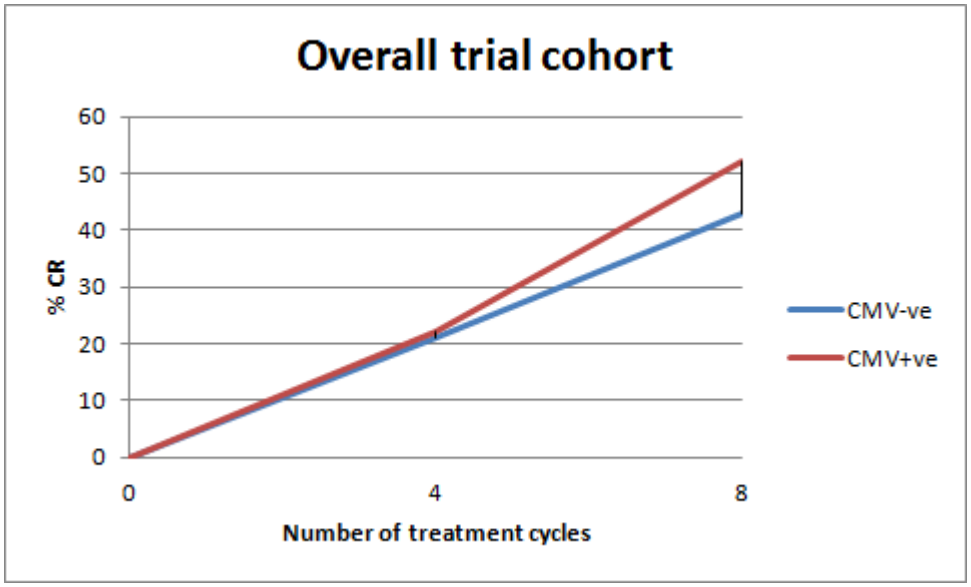

Figure 6-10: The line graph shows the proportion of CMV+ve and CMV-ve patients who achieved a CR at both time points (Overall trial cohort).

CMV-ve denotes CMV- negative, while CMV+ve denotes CMV- positive.

The discrepancy between results obtained in FACS/cytokine, IF and overall trial cohorts can potentially be explained by the fact that a higher proportion of CMV-positive patients in the overall trial cohort have high-risk FLIPI scores compared to the CMV-negative group in the overall Cohort (70% vs 61%). This could bias the CMV-positive group towards worse treatment outcome and potentially negate any favourable effect on treatment outcome associated with CMV positivity. In contrast, FLIPI scores in in FACS/cytokine and IF cohorts were well balanced between CMV-positive versus CMV-negative groups.

Overall, in a logistic regression models for the probability of having a Complete Response, both the time of assessment (post cycle 4 and 8) and the patient's CMV status are included as covariates in the model. There is a significant difference between post cycles 4 and 8 (p=0.002871). The probability of being CR increases from post cycle 4 to post cycle 8. Also, there is some evidence that being CMV-

positive increases the chances of a CR but this is not statistically significant (p=0.103).

6.5 Effect of CMV status on infection and febrile neutropenia

Following correlating CMV infection status with the response to treatment, we investigated relationship of such infection to the treatment toxicities in each of the two cohorts presented above and the entire cohort of patients recruited into the PACIFICO trial with clinical information available. CMV-positive and CMV-negative patients who experienced any form of toxicity were compared for the occurrence of infections and febrile neutropenia (reported as "neutropenic fever" or "neutropenic sepsis"). Toxicity was analysed as the percentage of affected patients, or the total number of infection or febrile neutropenia episodes reported within each group.

6.5.1 The number of patients experiencing AEs/SAEs

6.5.1.1 In the FACS/cytokine cohort

40 of the 42 patients in this cohort with information about toxicity available were included in this analysis. 20 of these patients were CMV-negative whereas 20 were CMV-positive.

Figures 6-11A-E show the proportion of patients in each of these groups who experienced infection and/or febrile neutropenia. There was no observed difference between CMV-negative and CMV-positive groups in the percentage of patients experiencing infection (Fig 6-11A), grade 3+ infection (Fig 6-11B), febrile neutropenia (Fig 6-11C), infection and/or febrile neutropenia (Fig 6-11D), or grade 3+ infection and/or febrile neutropenia (Fig 6-11E).

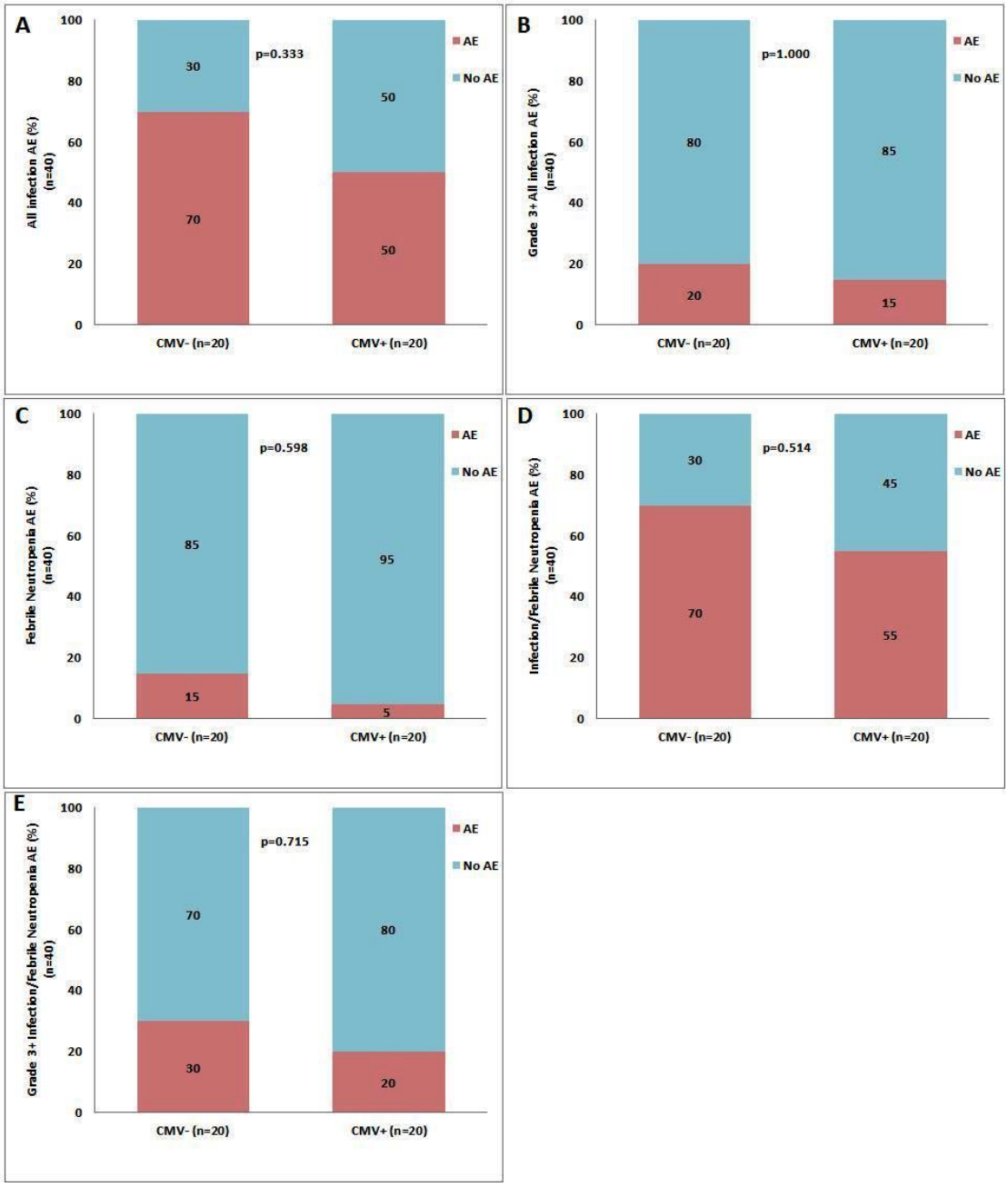

Figure 6-11: Comparison of incidence of infection and/or febrile neutropenia between CMV-positive and negative patients in the FACS/cytokine cohort.

Bar plot illustrating the proportion of patients in the FACS/cytokine cohort with infection and/or febrile neutropenia that were statistically compared between these two groups. The figure in each bar represents the % of cases.

6.5.1.2 In the IF cohort

In this cohort, the relevant information was not available from 9 of the 42 patients by the time of analysis. Therefore, results from 33 patients with 17 being CMV-negative and 16 CMV-positive are reported here.

Figure 6-12 shows the proportion of patients in each of these groups who experienced infection and/or febrile neutropenia. Similar to the findings above, there was no observed difference between the two groups in the percentage of patients experiencing infection (Fig 6-12A), grade 3+ infection (Fig 6-12B), febrile neutropenia (Fig 6-12C), infection and/or febrile neutropenia (Fig 6-12D), or grade 3+ infection and/or febrile neutropenia (Fig 6-12E).

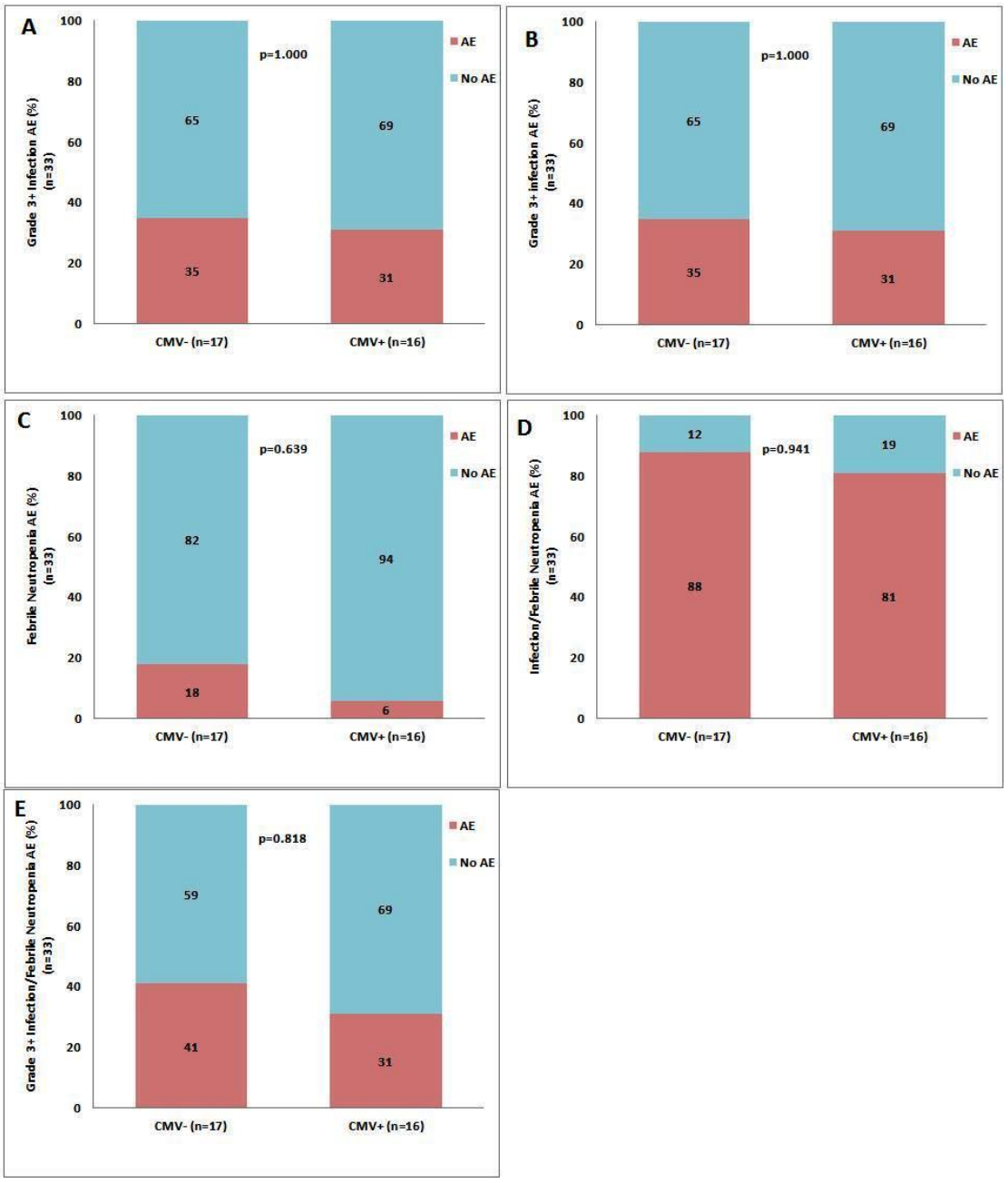

Figure 6-12: Comparison of incidence of infection and/or febrile neutropenia between CMV-positive and negative patients in the IF cohort.

The bar plot illustrates the proportion of patients in the IF cohort with infection and/or febrile neutropenia that were statistically compared between these two groups. The figure in each bar represents the % of cases.

6.5.1.3 In the overall trial cohort

209 of the 269 patients in the overall trial cohort experienced some form of toxicity. 128 of these patients were CMV-negative whereas 81 were CMV-positive.

Figure 6-13A-E shows the proportion of the number of AEs/SAEs in each of these groups that were due to infection and/or febrile neutropenia. A significantly higher proportion of CMV-positive patients experienced infections compared to CMV-negative patients (74% vs 59%, P=0.033, see Fig 13A). The same was true of infections and/or febrile neutropenia (78% vs 62%, P=0.031, see Fig 6-13D). There was no observed difference between CMV-negative and CMV-positive groups in the percentage of patients experiencing grade 3+ infection (Fig 6-13B), febrile neutropenia (Fig 6-13C or grade 3+ infection and/or febrile neutropenia (Fig 6-13E).

The summary of the percentage of patients who experienced specific toxicities in the three patient cohorts is presented in Table 6-2.

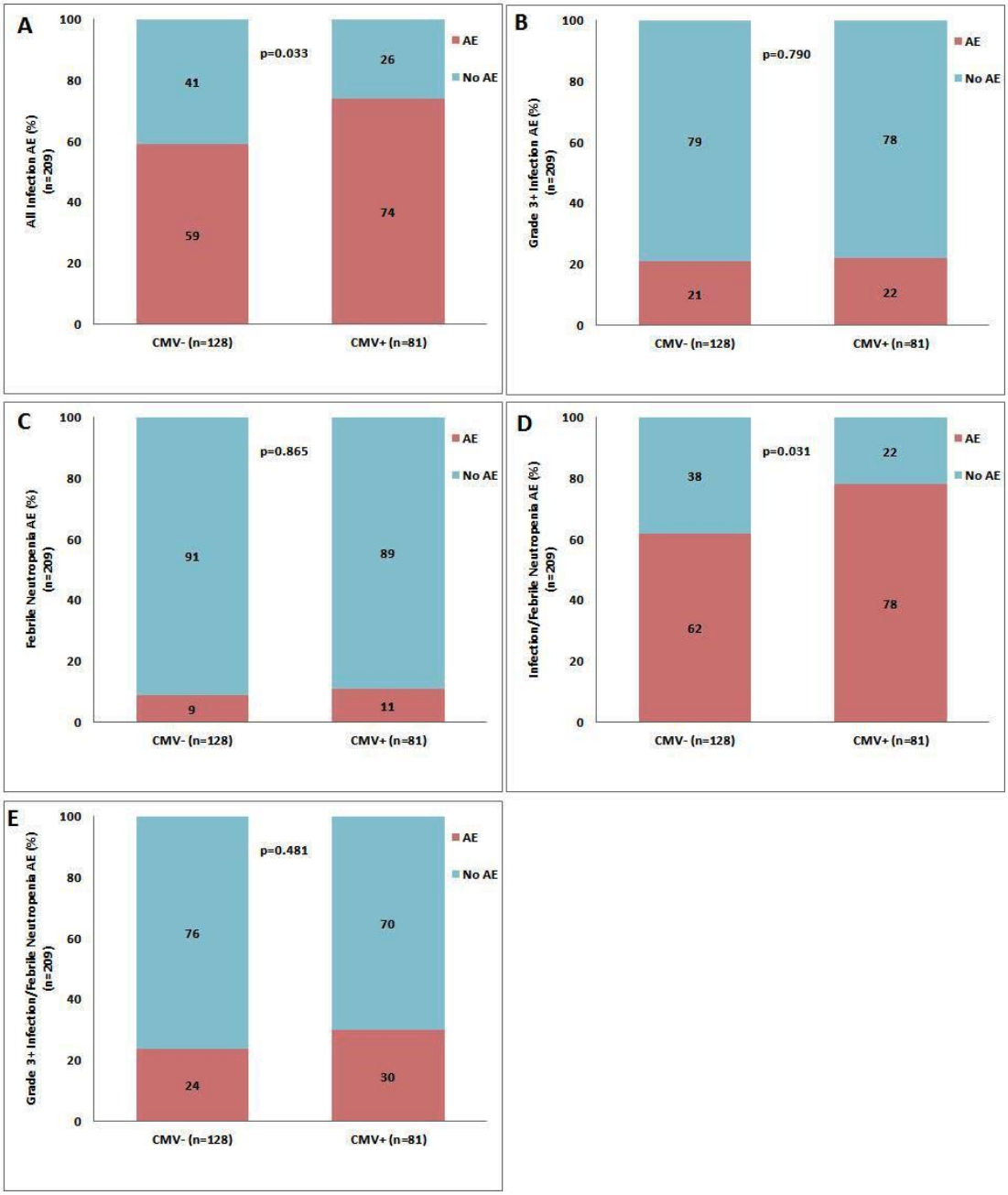

Figure 6-13: Comparison of incidence of infection and/or febrile neutropenia between CMV-positive and negative patients in the overall trial cohort.

The bar plot illustrates the proportion of patients in the overall trial cohort with infection and/or febrile neutropenia that were statistically compared between these two groups. The figure in each bar represents the % of cases.

Table 6-2: A summary of percentage of patients who experienced specific toxicities in the three cohorts

Percentage of patients experiencing specific toxicities		Cohort 1 (FACS/cytokine) 37 patients experiencing any toxicity			Cohort 2 (IF) 33 patients experiencing any toxicity			Cohort 3 (overall trial) 209 patients experiencing any toxicity		
		CMV- N=20	CMV+ N=20	P value	CMV- N=17	CMV+ N=16	P value	CMV- N=128	CMV+ N=81	P value
Infection (%)	Any grade	70	50	0.333	82	81	1.00	59	74	0.033
	Grade 3+	20	15	1	35	31	1.00	21	22	0.79
Febrile neutropenia (%)*	Any grade**	15	5	0.598	18	6	0.639	9	11	0.865
Infection or febrile neutropenia (%)	Any grade	70	55	0.514	88	81	0.941	62	78	0.031
	Grade 3+	30	20	0.715	41	31	0.818	24	30	0.481

*including neutropenic fever and neutropenic sepsis

** Febrile neutropenia is by definition grade 3+

6.5.2 Analysis of the number of reported AEs/SAEs

With the findings made above, we further compared the adverse effects quantified as the number of episodes experienced by the patients.

6.5.2.1In FACS/cytokine cohort

A total of 532 AEs/SAEs were recorded in the FACS/cytokine cohort. Among them, 342 occurred in CMV-negative and 190 in CMV-positive patients.

Figure 6-14 shows the percentage of the total number of AEs/SAEs in each of the two groups, including infection and/or febrile neutropenia. There was no observed difference between CMV-negative and CMV-positive groups in the percentage of AEs/SAEs that resulted from infection (Fig 6-14A), grade 3+ infection (Fig 6-14B), febrile neutropenia (Fig 6-14C), infection and/or febrile neutropenia (Fig 6-14D), or grade 3+ infection and/or febrile neutropenia (Fig 6-14E).

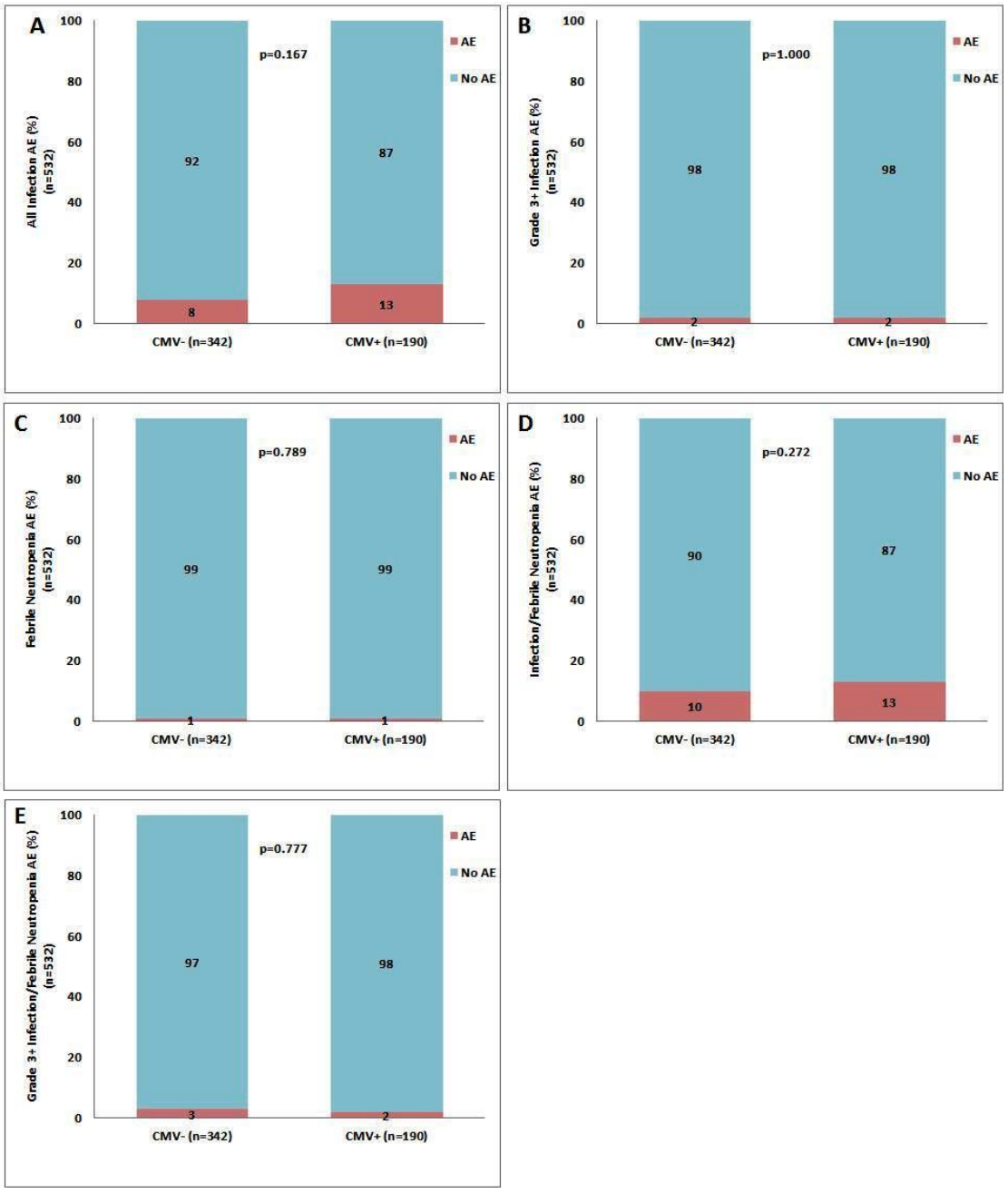

Figure 6-14: Comparing AE/SAE episodes between CMV-positive and –negative patients in the FACS/cytokine cohort.

Figures in each bar represent the % of the episodes.

6.5.2.2 The immunofluorescence (IF) cohort

A total of 486 AEs/SAEs were reported in the IF cohort. Amongst these, 323 occurred in CMV-negative patients and 163 in CMV-positive patients.

Figures 6-15A-E show the proportion of the number of AEs/SAEs in each of these groups that were due to infection and/or febrile neutropenia. Figure 6-15A and Figure 6-15D show that CMV-positive FL patients experienced statistically more episodes of infection (P=0.017) and infection and/or febrile neutropenia (P=0.037) compared to the CMV- negative group. No difference was observed between CMV-negative and CMV-positive groups in the percentage of AEs/SAEs that resulted from grade 3+ infection (Fig 6-15B), febrile neutropenia (Fig 6-15C) or grade 3+ infection and/or febrile neutropenia (Fig 6-15E).

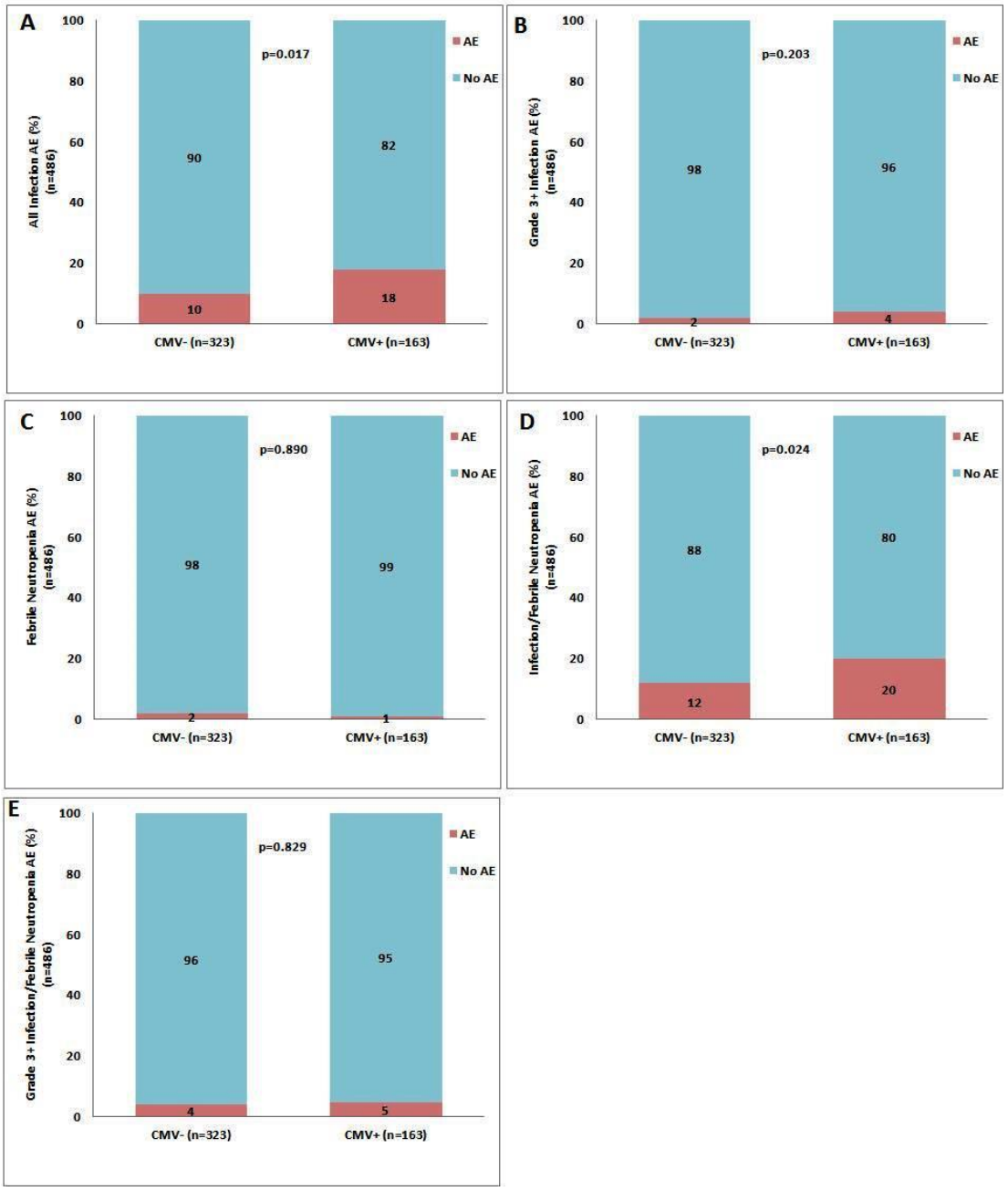

Figure 6-15: Comparing AE/SAE episodes between CMV-positive and –negative patients in the IF cohort.

Figures in each bar represent the % of the episodes.

6.5.2.3 The overall trial cohort

A total of 2544 AEs/SAEs were reported in the overall cohort. Out of this, 1690 occurred in CMV-negative patients and 854 in CMV-positive patients.

The CMV-positive patients experienced a significantly higher percentage of reported number of infections compared to CMV-negative patients (17% vs 12%, P=0.001, see Fig 6-16A). Also, CMV- positive had a significantly higher proportion of the percentage number of infections and/or febrile neutropenia AEs compared to CMV-negative patients (19% vs 13%, P=0.001, see Fig 6-16D). In addition, a significantly higher proportion of grade 3+ infection and/or febrile neutropenia was recorded by the CMV- positive FL patients compared to CMV-negative patients (6% vs 4%, P=0.021, see Fig 6-16E).

There was no observed difference between CMV-negative and CMV-positive groups in the percentage of patients experiencing grade 3+ infection (Fig 6-16B) or febrile neutropenia (Fig 6-16C).

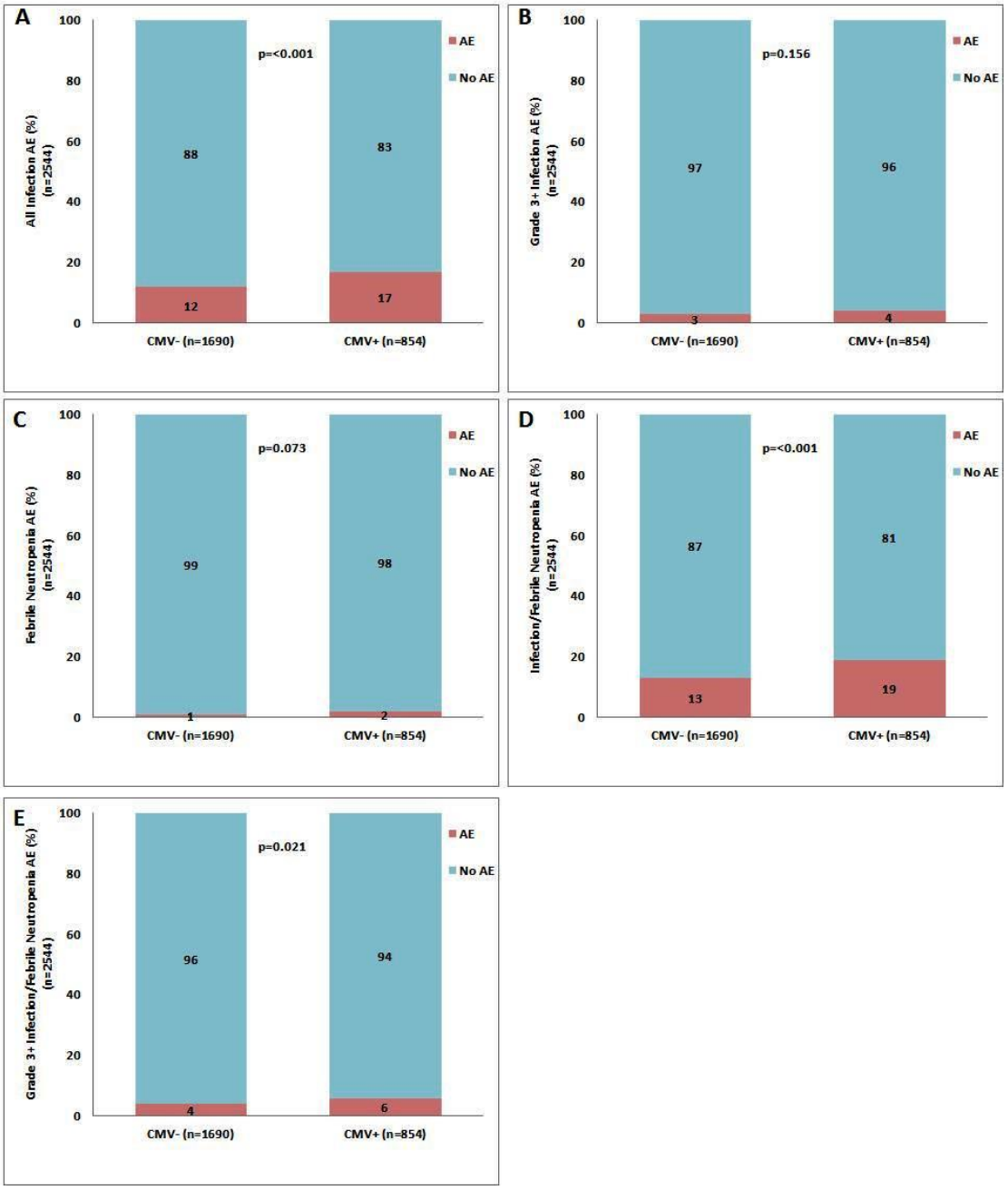

Figure 6-16: Comparing AE/SAE episodes between CMV-positive and –negative patients in the overall PACIFICO trial cohort.

Figures in each bar represent the % of the episodes.

Table 6-3: A summary of percentage episodes of specific toxicities reported in the three cohorts.

Percentage of reported AEs/SAEs due to specific toxicities		Cohort 1 (FACS/cytokine) 532 AEs/SAEs			Cohort 2 (IF) 486 AEs/SAEs			Cohort 3 (overall trial) 2544 AEs/SAEs		
		CMV- N=342	CMV+ N=190	P value	CMV- N=323	CMV+ N=163	P value	CMV- N=1690	CMV+ N=854	P value
Infection (%)	Any grade	8	13	0.167	10	18	0.017	12	17	<0.001
	Grade 3+	2	2	1	2	4	0.3	3	4	0.156
Febrile neutropenia (%)*	Any grade**	1	1	0.789	2	1	0.89	1	2	0.073
Infection or febrile neutropenia (%)	Any grade	10	13	0.272	12	20	0.037	13	19	<0.001
	Grade 3+	4	2	0.338	4	5	0.702	4	6	0.021

*includes "neutropenic fever" and "neutropenic sepsis"

** Febrile neutropenia is by definition grade 3+

6.6 Discussions

This Chapter focuses on the evaluation of the relationship between the available clinical endpoint data of the trial patients and CMV status. Results of the two cohorts studied suggests that CMV infection in FL associates with better and more rapid response to rituximab-containing chemo-immunotherapy in the FACS/cytokine and IF cohorts. This association will be tested in the entire trial cohort when data of clinical response of all patients is verified. Regarding toxicities of the treatment, the clinical data of all patients in the trial were verified and available. The proportion of patients experiencing infection or (infection and/or febrile neutropenia) was significantly higher in the CMV-positive group in the whole trial cohort, despite similar between the two groups in the two small cohorts studied for FACS/cytokine and IF profiles. Also, the percentage of the total number of AEs/SAEs episodes due to infection or (infection and/or febrile neutropenia) was significantly higher in the CMV-positive group in the overall trial cohort and IF cohort. In the overall trial cohort, the effect is particularly striking and extends to grade 3+ infection and/or febrile neutropenia. These observations suggest that CMV infection in FL associated with increased

episodes of infection and adverse events in the course of rituximab-containing chemo-immunotherapy, especially as observed in overall PACIFICO trial cohort.

By implication, the elevated end-stage effector T cell types observed in Chapter 3 and the reduced serum cytokine levels seen in Chapter 4 can be said to contribute to the increased infection episodes observed among the CMV- positive FL patients. Similarly, the more impressive chemo-immunotherapy response rate reported among CMV-positive patients compared to the CMV- negative can be attributed to the high expression levels of CD21 and FOXP3 reported in the tumour microenvironment (Chapter 5). Undoubtedly, leucocytes constitute the primary cellular source of cytokines, and since end stage T cells are poor secretors of cytokines (Rothe et al., 2016), then the presence of CMV infection can grossly impair immune responses to antigenic challenge leading to increased infection rate among the CMV- positive patients. These data, therefore, support the hypothesis that CMV infection alters immune function in two ways: (1) Alteration of lymphoma microenvironment with modulation of therapy response (opposed by worse FLIPI score in the overall trial cohort). The findings are in keeping with enhanced antibody-dependent cell mediated cytotoxicity (ADCC) (mechanism of action of rituximab) due to the increased FDCs demonstrated in CMV- positive cases of Chapter 5 (2) Acceleration of immune senescence and T cell exhaustion with increased susceptibility to infection.

The treatment regimens for both arms of the PACIFICO trial (R-FC & R-CVP) contain human anti-CD20-IgG1 monoclonal antibody, rituximab, in combination with the chemotherapy regimens, and afterwards, it is given as a single agent for maintenance therapy to avoid or slow the return of cancer following CR (Witkowska and Smolewski, 2016). The mechanisms by which rituximab function to cause the destruction of pan-B-cell (more than 90% of B-cell NHL) including the FL cells is by

the immune antibody-antigen binding that leads to cell-mediated cytotoxicity, direct signalling of apoptosis and complement activation (Smith, 2003). Therefore, some of the most notable explanations for the actions of rituximab may include antibody-dependent cell mediated cytotoxicity (ADCC), complement dependent cytotoxicity (CDC) as well as induction of apoptosis and direct growth arrest (Seyfizadeh et al., 2016, Weiner, 2010, Witkowska and Smolewski, 2016, Zhou et al., 2008).

In the ADCC pathway, the target (FL) cells are coated with the anti-CD20 antibodies which trigger the accessory effector immune cells in the FL microenvironment to induce ADCC process that facilitate the lysing of the rituximab-coated target cells (Hogarth and Pietersz, 2012, Weiner, 2010). Consequently, the result suggests that the significantly elevated FDCs (as identified by CD21+ marker) observed in the FFPE tissue sections of the CMV-positive patients could be responsible for potentiating the action of rituximab and resulting to the higher proportion of CR rate compared to the CMV- negative patients. Although FDCs are known to play a promoting role in lymphomagenesis, growth, histologic transformation and survival of FL B cells (Lossos and Gascoyne, 2011), FDCs help in shaping immune responses and are crucial for the removal of cell debris from GCs and in preventing autoimmunity (Aguzzi et al., 2014). Also, the binding of rituximab to FL cells will activate the activities of regulatory T cells (FOXP3+ cells) which happen to be elevated at borderline significance in CMV-positive patients compared to CMV- negative. Although it is not clear whether FOXP3 is an anti- or pro- tumourigenic molecule, functional evidence strongly suggest that FOXP3 serves as tumour suppressive transcription factor in cancer development (Zuo et al., 2007, Tao et al., 2012). Therefore, by the ADCC pathway, the potential anticancer activity of FOXP3 will

confer a superior response advantage to the CMV- positive FL patients on rituximab – chemotherapy regime than the negative.

Even through some authors have reported that FDCs (CD21+) can block apoptosis and enhance cell survival (Mongini et al., 2003), rituximab can act through the activities of FOXP3 that represses cell proliferation and induces apoptosis by activating the apoptotic signalling pathway to enhance response to treatment (Ma et al., 2013, Darmochwal-Kolarz et al., 2012).

The increased incidence and episodes of infection AEs reported among the CMV-positive FL patients (in the overall PACIFICO trial cohort) can be attributed to the predominance of exhausted T cells over functionally competent T cells reported in Chapter 3 of the thesis. This is due to the decreased long-lasting memory potential, limited effector function and poor cytokine production capacity of the terminally differentiated T cells, which can cause a profound impairment of the immune functions and thereby increasing patients susceptibility to infections (Rothe et al., 2016, Wherry, 2011). Furthermore, the low levels of circulating cytokines across pro- and anti-inflammatory categories, as well as chemokines and haematopoietic growth factors among the CMV-positive patients, may account for the heighten proportion and rate of infection AEs among the patients. Other reasons for the increased risk of infection among CMV- positive FL patients may include, though not statistically significant, low levels of naïve and central memory T to fight novel infections and mount strong response against secondary infections compared to CMV- negative population.

It is anticipated that understanding the mechanisms by which CMV modulates the activity of rituximab-containing chemotherapy in FL treatment might provide

headway to researching into drug discovery and improved quality of life of FL patients. Furthermore, if data is confirmed by a prospective study, routine CMV serostatus of FL patients may be recommended. This step will help in promoting precision medicine and to checkmate incidences and rates of toxicities in FL patients, especially during immuno-chemotherapy. In this case, the CMV- positive FL patients might benefit from enhanced clinical monitoring against AEs/SAEs in the course of therapy and for inclusion on other protocols aimed at slowing down the rate of T cell exhaustion and immune senescence among patients. Furthermore, it is believed that carrying out a survival analysis at the close of the trial relating primary endpoints data with CMV infection status may reveal a clearer picture of the role of CMV infection in FL biology.

In conclusion, CMV infection among FL patients has is associated with elevated frequencies of circulating end-stage T cell types and NK cells, decreased serum cytokine levels and high expression of CD21+ cells in the FL microenvironment. Also, CMV infection associated with more episodes of infection AEs but higher CR rate. The next chapter (Chapter 7) focuses on exploring the relationship between CMV status and the proportion of end stage T cells, median serum cytokine levels and intensities of surface markers in the FL microenvironment versus AEs and response status. This approach will give ideas on how increased or decreased values of these parameters relate to AEs and response status.

Chapter 7 : Relating laboratory findings with treatment efficacy

7.1 Introduction

The data presented in Chapters 3-5 indicate that CMV infection is associated with an altered profile of blood lymphocytes (reduced CD4: CD8 ratio, increased effector and effector memory-3 cells within the CD4+ and CD8+ populations, increased NKT cells, decreased CD4+ and increased LAG3-CD4 cells), reduced serum cytokines (IL-1β, IL-4, IL-6, IL-8, IL-9, IL-17, FGF-basic, GM-CSF, MIP1α, MIP1β) and increased accessory cells expressing PD-1, FOXP3 and CD21 in affected lymph nodes. Furthermore, in Chapter 6, I showed that CMV infection is associated with increased infections and possibly an improved response to chemo-immunotherapy. In this Chapter, I sought to establish the relationship between the CMV-dependent biological variables listed above and the clinical associations of CMV. In doing so, I hoped to be able to shed light on the mechanisms through which CMV exerts its clinical effects in FL. Unfortunately, it was not possible within the time frame available to relate CMV-dependent biological variables to infection or febrile neutropenia. Since these were the only toxicities associated with CMV, the analysis was confined to elucidating the relationship between CMV and treatment efficacy (CR rates after 4 and 8 cycles).

7.2 Approaches

To achieve the aim of this chapter, we planned to compare the clinical outcomes (response to treatment) between patients with different levels of those laboratory variables differentially expressed between CMV-positive and -negative patients, including the 10 cytokines identified in Chapter 4, the 3 microenvironmental markers presented in Chapter 5, and the 8 cell types elucidated in Chapter 3. However, for each of these variables the data was continuously distributed rather than categorical

and it was not possible to identify a natural cut-off separating "high-level" from "low-level" cases. The median level of each laboratory variable of the 42 patients studied was therefore applied to divide cases into two groups. Figure 7-1 provides an example of how this approach was applied to serum cytokine levels. For each variable, patient groups defined as "high level" (greater than the median value) or "low-level" (lower than the median value) were compared for clinical endpoints using the statistical analyses described in Chapter 2.

Although data on treatment response after 4 and 8 cycles was available for most patients, it was missing in 5 and 7 patients, respectively, among the cohort of 42 patients used to examine lymphocyte subsets (Figures 7-2 & 7-3) and serum cytokines (Figure 7-6 & 7-7), and in 4 and 15 patients, respectively, among the cohort of 42 patients used to examine accessory cells in affected lymph nodes (Figures 7-10 & 7-11). To validate the results and avoid underestimation of any difference between patients due to the limited sample size, a reciprocal statistical analysis was performed in which patients were grouped by whether or not they achieved a CR and compared across groups for levels of the CMV-dependent laboratory variables.

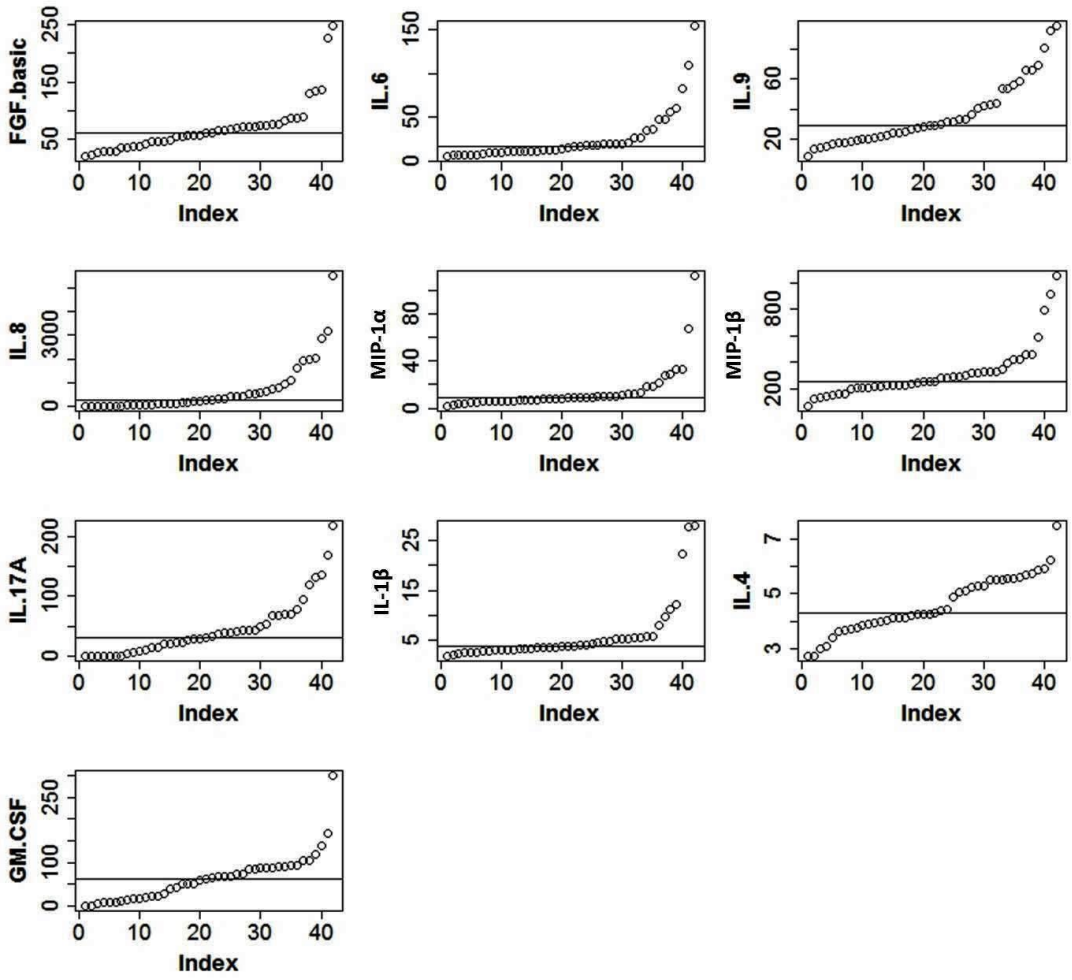

Figure 7-1: Expression levels of cytokines in the studied cohort of patients with FL.

The y-axis represents the levels of cytokines (pg/ml) found to be differentially expressed in CMV-positive versus CMV-negative cases. For each cytokine, the data have been arranged in rank order. The horizontal line in each chart represents the median level of individual cytokines.

7.3 Results

7.3.1 Relationship between treatment response and blood lymphocyte subsets

Following the approaches outlined above, patients were divided into high- and low-level groups for each lymphocyte subset found to be differentially expressed between

CMV-positive and CMV-negative patients (NKT, pE2.CD4, E.CD4, E.CD8, EM3.CD8, CD4 & LAG3-CD4). Interestingly, patients with high proportion of E.CD4 cells (CD4+CD45RA+CCR7-CD27-CD28-) had a higher CR rate after 8 cycles of treatment compared to those with a low proportion (77.78% versus 41.18%, p = 0.041, Figure 7-3C), although no significant difference was found after 4 cycles. Also, patients expressing a high proportion of E.CD8 cells tended to have a higher CR rate after 8 cycles of treatment compared to patients that had low E.CD8 cells, although this observation failed to reach statistical significance (p=0.094, Figure 7-3G). No difference in CR rates was observed between patient groups defined by high versus low proportions of other lymphocyte subsets.

Consistent with the finding above, patients who achieved a CR after 8 cycles had a higher proportion of E.CD4 cells compared to those who did not achieve a CR at this time point (Figure 7-5). Similarly patients who achieved a CR after 4 cycles tended to have a higher proportion of pE2.CD4 cells, although this observation just failed to reach statistical significance (Figure 7-4).

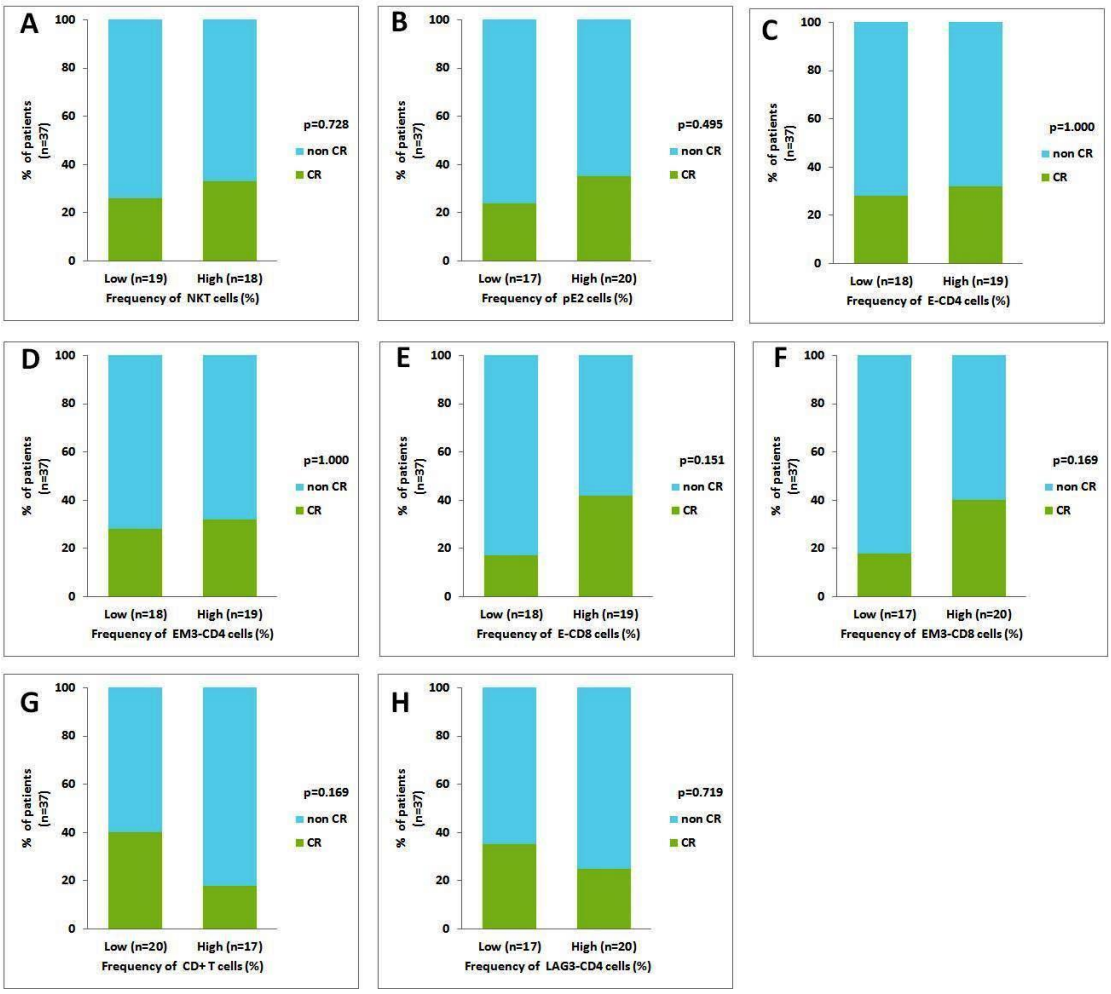

Figure 7-2: Comparison of treatment response post cycle 4 between patients expressing low and high frequencies of lymphocyte subsets.

Patients were grouped based whether they expressed low or high frequency of lymphocyte subsets. The y-axis of the stacked bar charts depicts the percentage of patients that achieved CR and non CR at post treatment cycle 4.

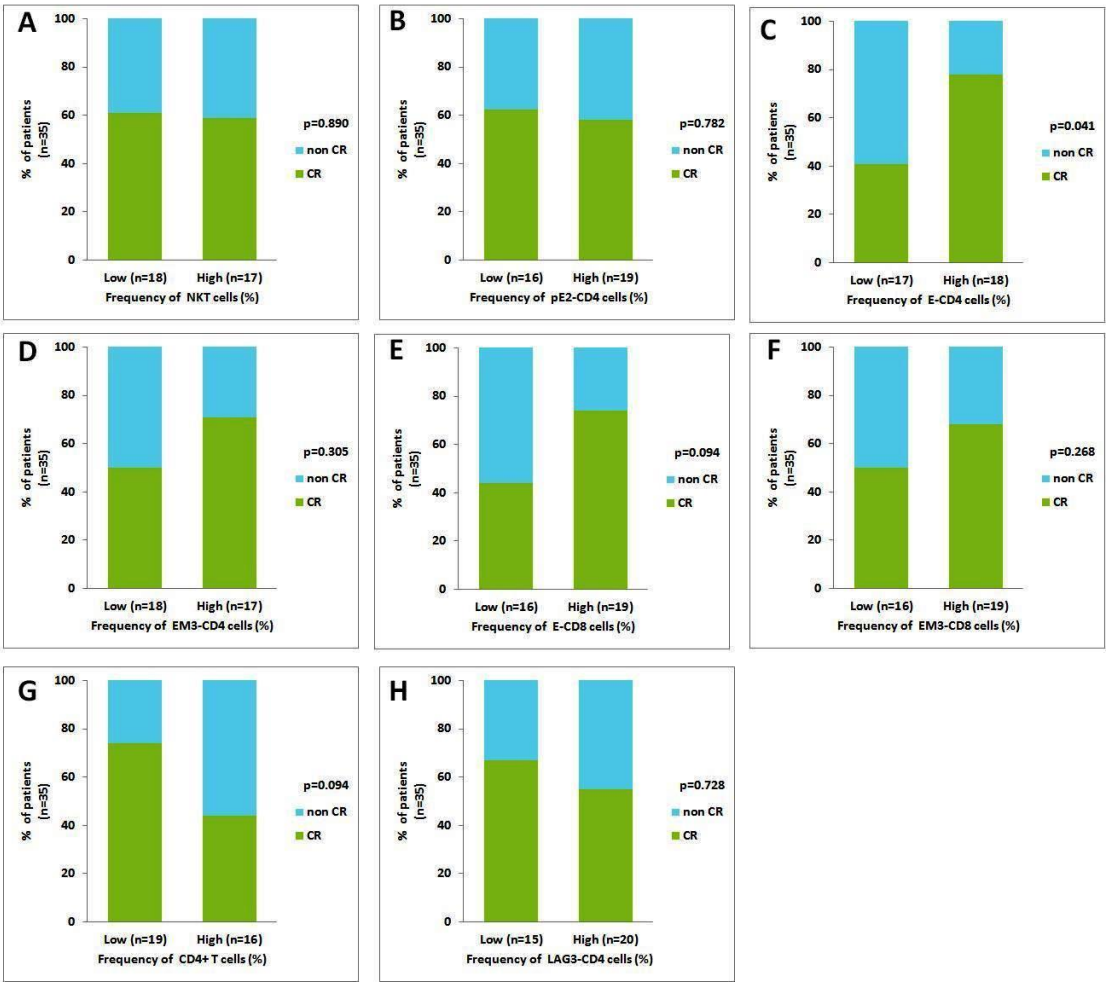

Figure 7-3: Comparison of treatment response post cycle 8 between patients expressing low and high frequencies of lymphocyte subsets.

Patients were grouped based whether they expressed low or high frequency of lymphocyte subsets. The y-axis of the stacked bar charts depicts the percentage of patients that achieved CR and non CR at post treatment cycle 8.

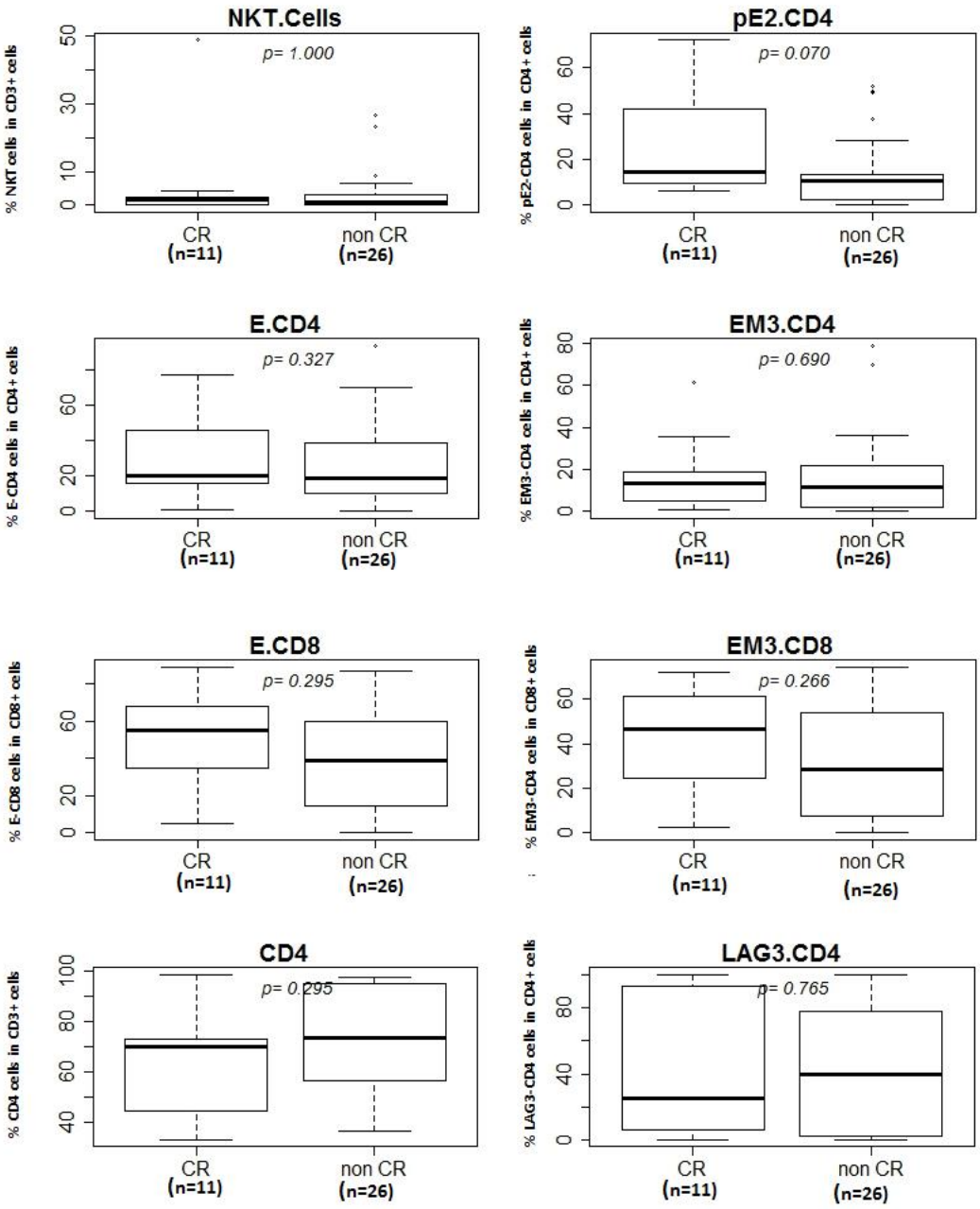

Figure 7-4: Comparison of lymphocyte subsets between patients achieving and not achieving CR after 4 cycles of treatment.

The y-axis on the boxplots represents the percent of the cell subtypes analysed. The response status represented by complete remission (CR) and non-complete remission (non CR) are presented on the x-axis.

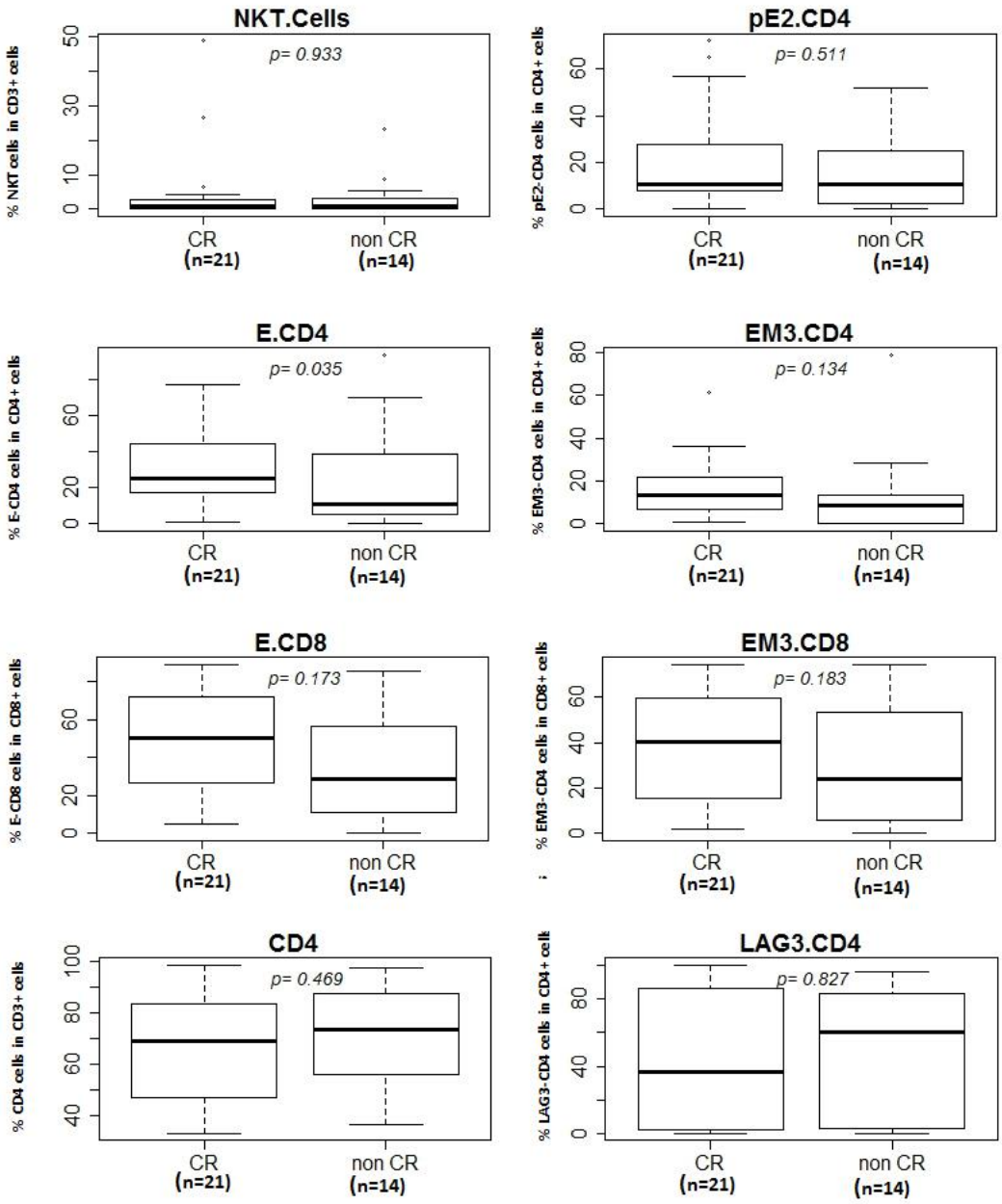

Figure 7-5: Comparison of lymphocyte subsets between patients achieving and not achieving CR after 8 cycles of treatment.

The y-axis on the boxplots represents the percent of the cell subtypes analysed. The response status represented by complete remission (CR) and non-complete remission (non CR) are presented on the x-axis.

7.3.2 Relationship between treatment response and serum cytokines

Next, we divided the cohort of patients into two groups using the median level of each of the 10 cytokines identified in Chapter 4. As shown in Figures 7-6 & 7-7, Chi square test or Fisher's exact test found no significant difference in CR rates between patients with low versus high levels of any of these 10 cytokines.

The patients were then grouped by outcome, and cytokine levels were compared between patients who did or did not achieve a CR after 4 cycles (Figure 7-8) or 8 cycles (Figure 7-9) of treatment using the Mann-Whitney U test. Again, no significant difference was observed.

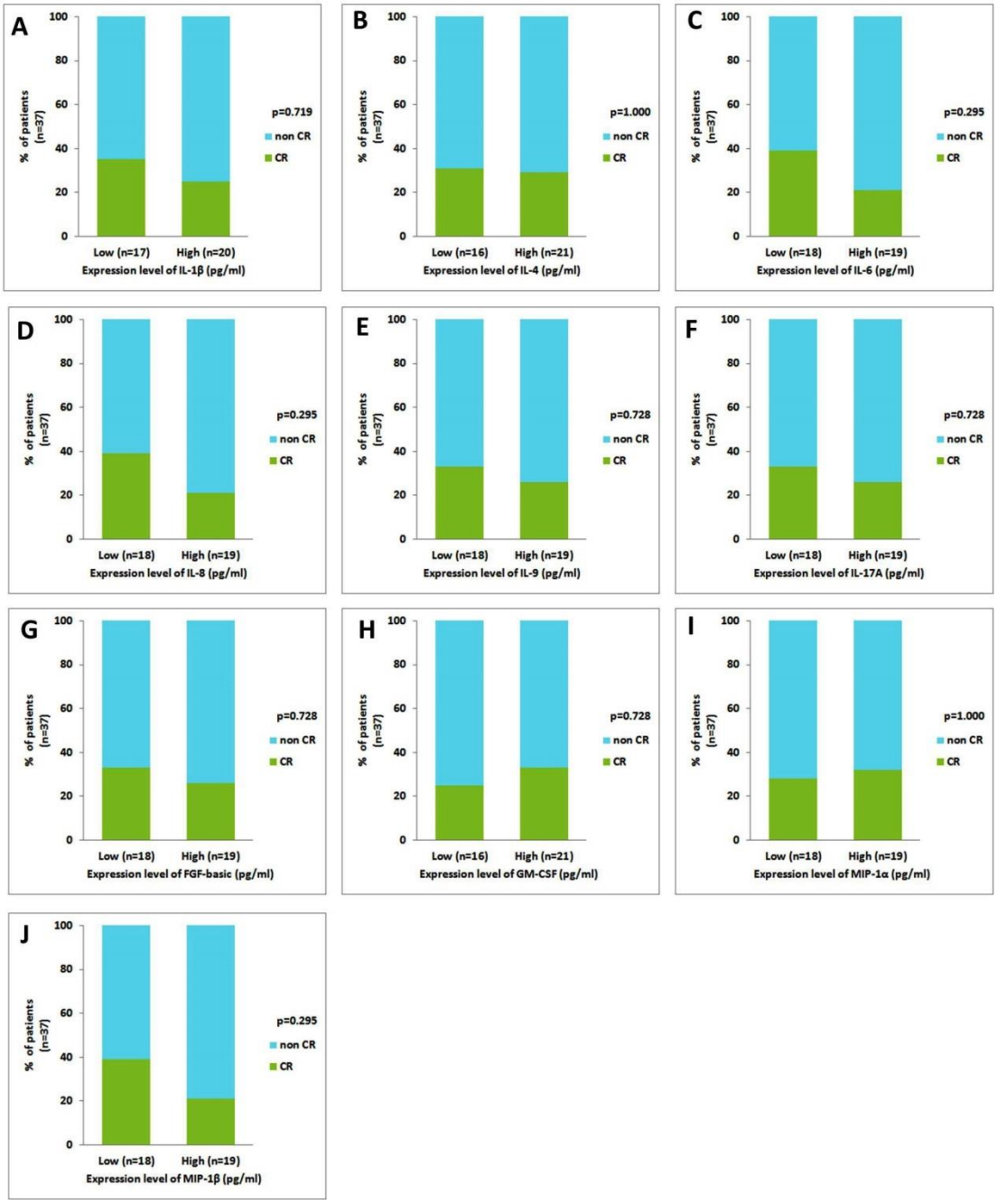

Figure 7-6: Comparison of treatment response post cycle 4 between patients expressing low and high levels of cytokines.

Patients were grouped based on whether they expressed low or high levels of serum cytokines. The y-axis of the stacked bar charts depicts the percentage of patients that achieved CR and non CR at post treatment cycle 4.

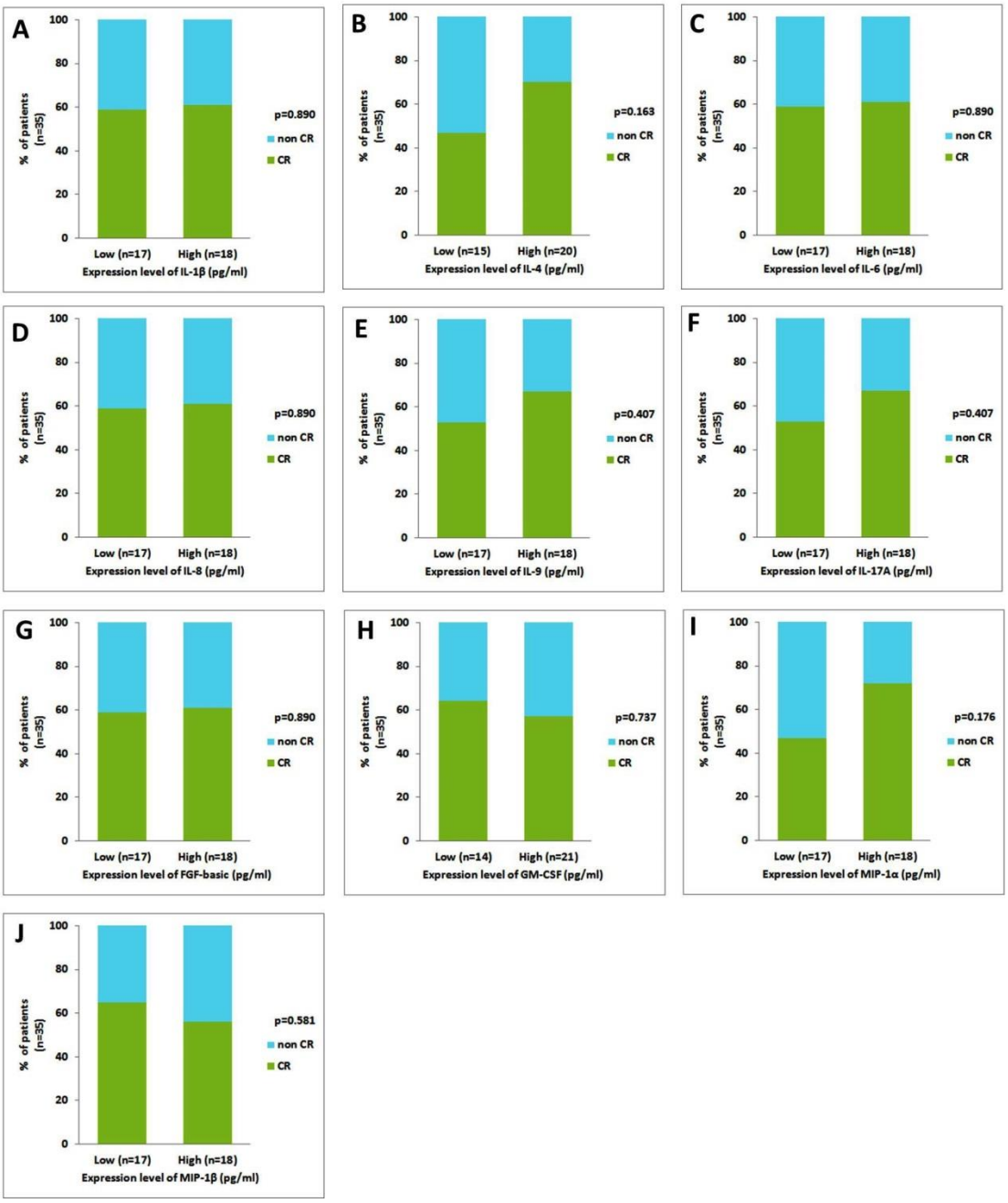

Figure 7-7: Comparison of treatment response post cycle 8 between patients expressing low and high levels of cytokines.

Patients were grouped based whether they express low or high levels of serum cytokines. The y-axis of the stacked bar charts depicts the percentage of patients that achieved CR and non CR at post treatment cycle 8**.**

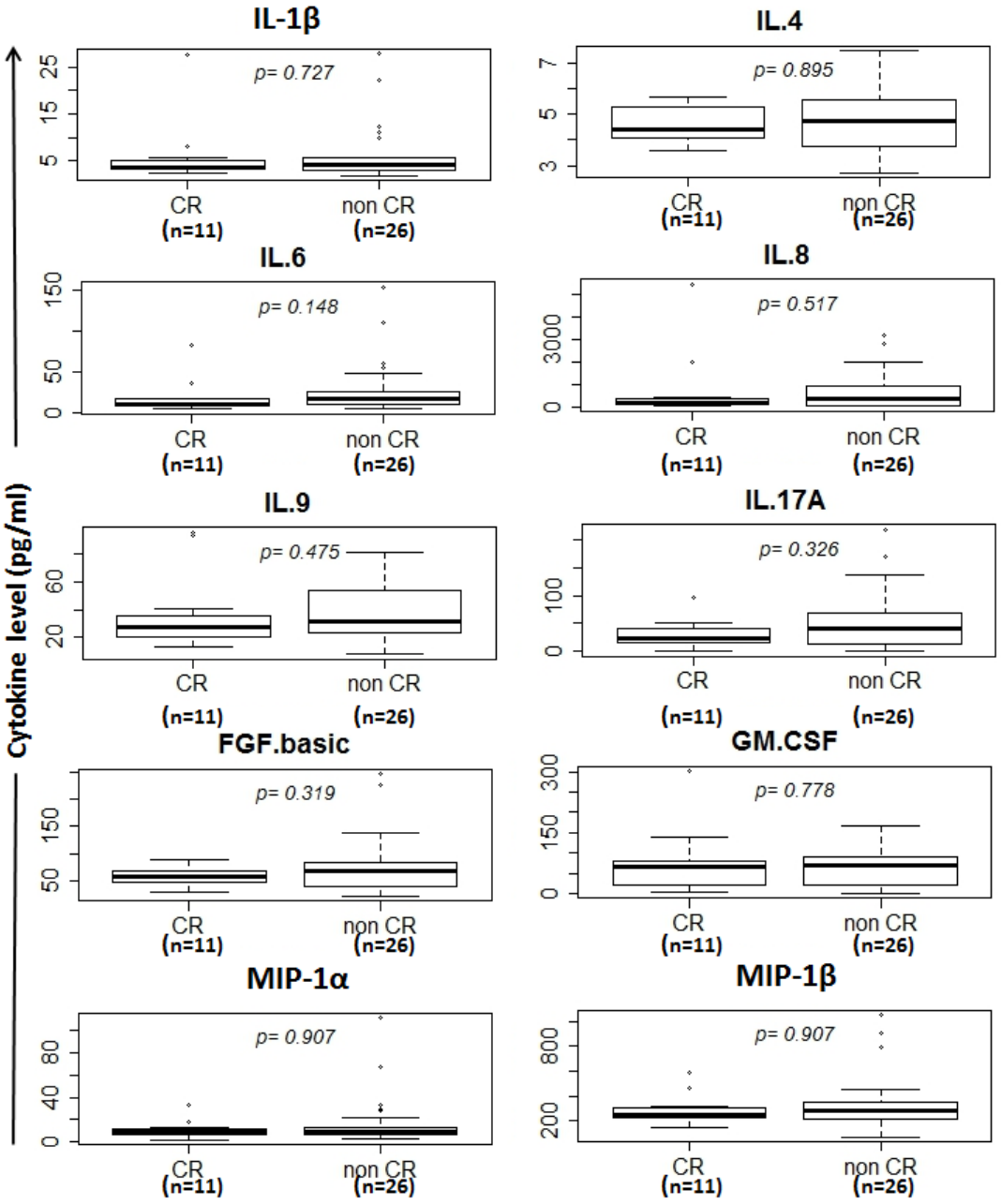

Figure 7-8: The cytokine levels in patients with and without CR after 4 cycles of treatment.

Patients were grouped based on whether or not they achieved a CR after completion of 4-cycle treatment. The y-axis of the boxplots depicts expression levels of cytokines (pg/ml).

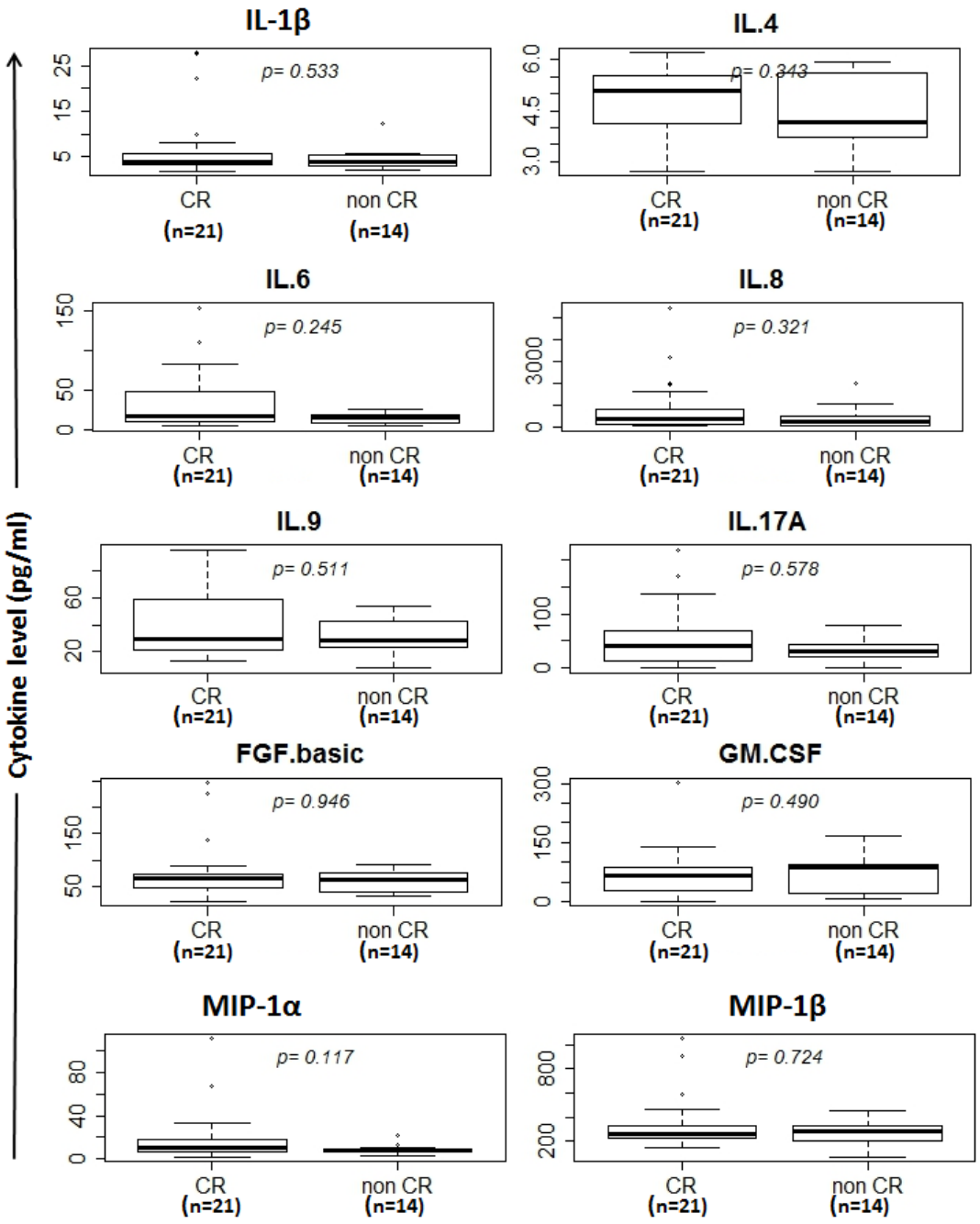

Figure 7-9: The cytokine levels in patients with and without CR after 8 cycles of treatment.

Patients were grouped based on whether or not they achieved a CR after completion of 8-cycle treatment. The y-axis of the boxplots depicts expression levels of cytokines (pg/ml).

7.3.3 Relationship between treatment response and accessory cells

Next, we used the median intensity of the 3 differentially expressed microenvironmental protein markers (PD1, FOXP3 and CD21) to divide the patients into two groups. The Chi-square test or Fisher's exact test did not reveal any significant difference between the groups in the CR rate after either 4 or 8 cycles of treatment (Figures 7-10 & 7-11). Similarly, no significant difference in intensity of any of the three markers was found between cases achieving and not achieving CR (Figures 7-12 and 7-13).

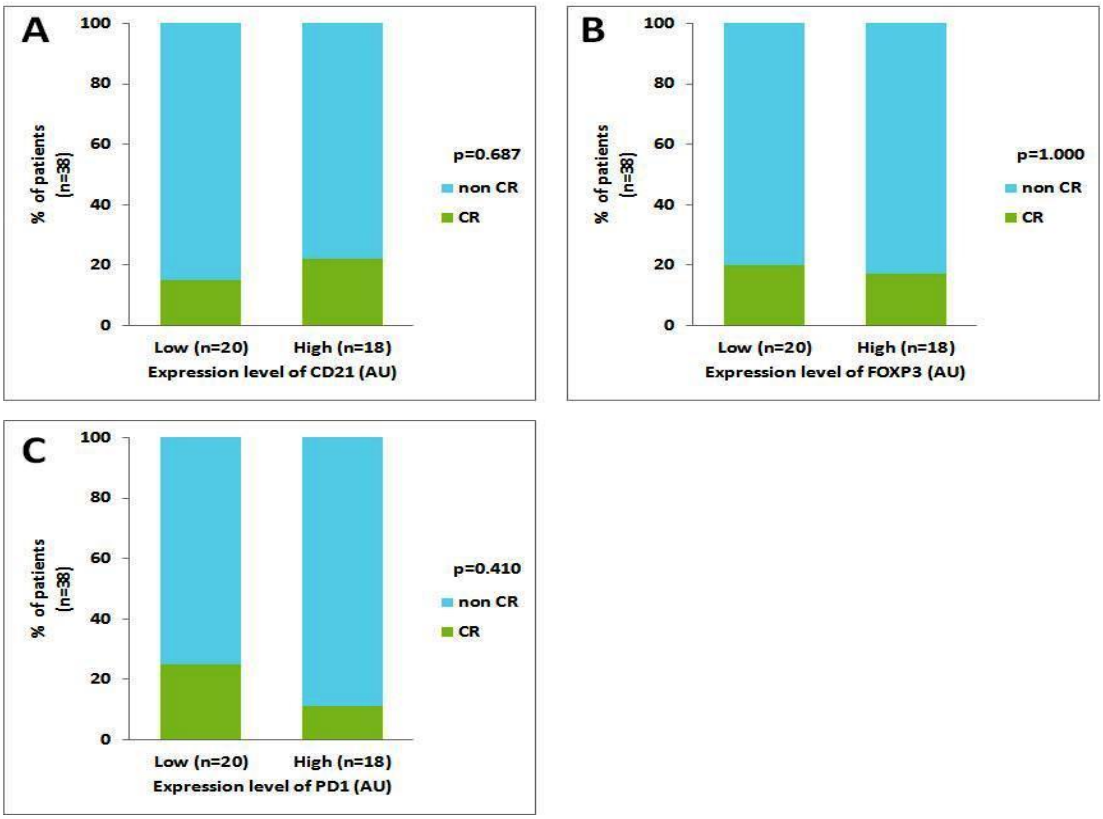

Figure 7-10: Treatment response post cycle 4 between patients expressing low and high intensities of immune cell markers in the FL microenvironment.

Patients were grouped based whether they express low or high frequencies of cell markers in the FL microenvironment. The y-axis of the stacked bar charts depicts the percentage of patients that achieved CR and non CR at post treatment cycle 4**.**

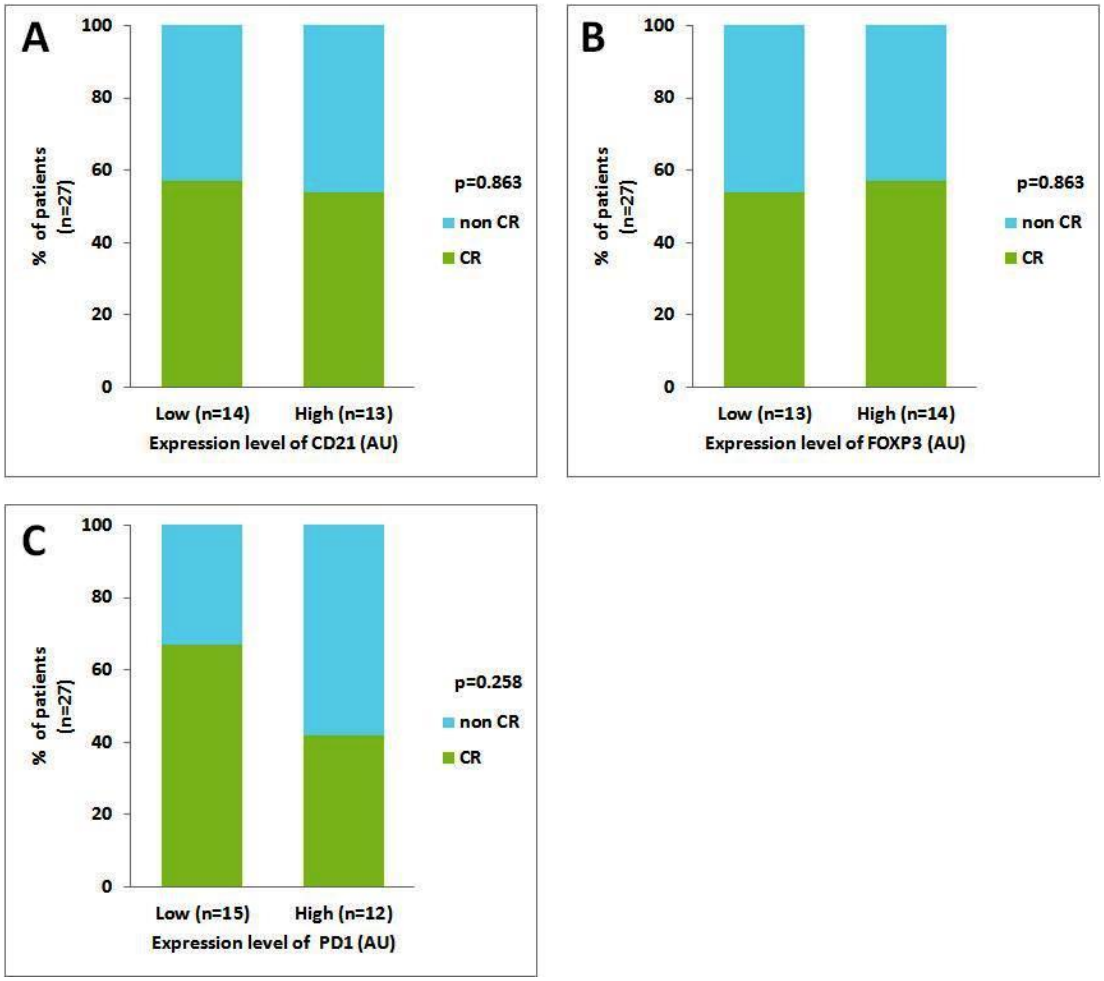

Figure 7-11: Comparison of treatment response post cycle 8 between patients expressing low and high intensities of immune cell markers in the FL microenvironment.

Patients were grouped based whether they express low or high frequencies of cell markers in the FL microenvironment. The y-axis of the stacked bar charts depicts the percentage of patients that achieved CR and non CR at post treatment cycle 8.

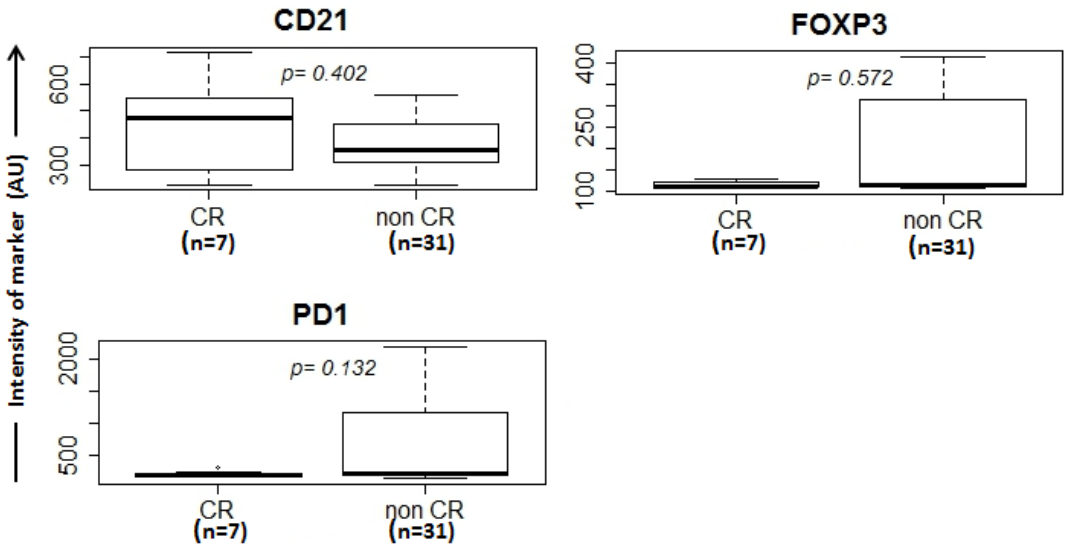

Figure 7-12: Comparison of intensity of protein markers in microenvironment between cases with and without CR post cycle 4 of treatment.

The y-axis on the boxplots represents the fluorescence intensities of the protein markers in the FL microenvironment.

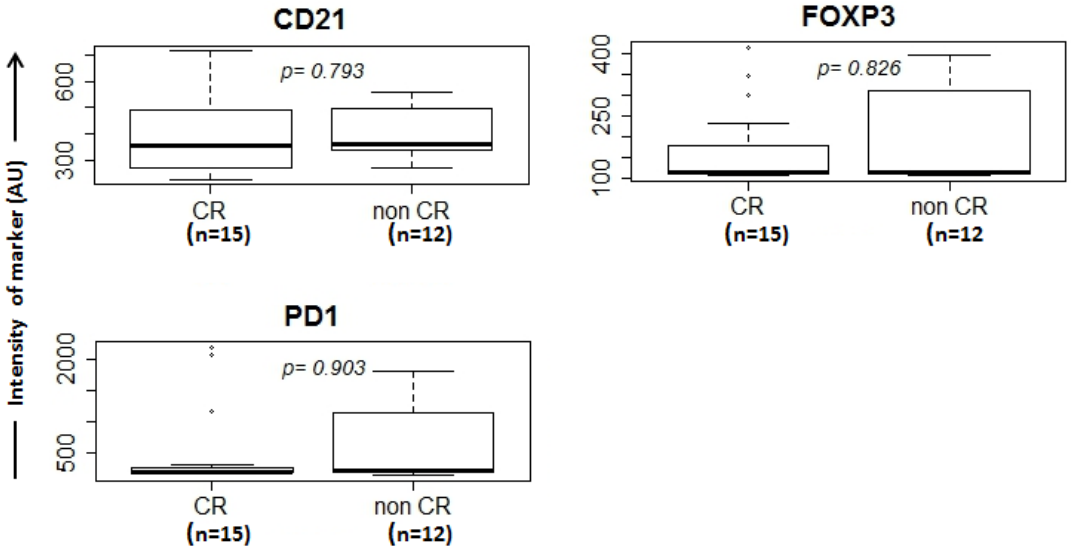

Figure 7-13: Comparison of intensity of protein markers in microenvironment between cases with and without CR post cycle 8 of treatment.

The y-axis on the boxplots represents the intensity of the protein markers in the FL microenvironment. A summary of the relationship between CMV-dependent biological variables and treatment efficacy is presented in Table 7-1.

Table 7-1: Summary of the relationship between CMV-dependent biological variables and treatment efficacy

CMV-dependent biological variable		CR after 4 cycles		CR after 8 cycles	
		Analysis 1	Analysis 2	Analysis 1	Analysis 2
Blood lymphocytes (Chapter 3)	NKT	NS	NS	NS	NS
	pE2-CD4	NS	0.070	NS	NS
	E-CD4	NS	NS	0.041	0.035
	EM3-CD4	NS	NS	NS	NS
	E-CD8	NS	NS	0.094	NS
	EM3- CD8	NS	NS	NS	NS
	CD4	NS	NS	0.094	NS
	LAG3-CD4	NS	NS	NS	NS
Serum cytokines (Chapter 4)	IL-1β	NS	NS	NS	NS
	IL-4	NS	NS	NS	NS
	IL-6	NS	NS	NS	NS
	IL-8	NS	NS	NS	NS
	IL-9	NS	NS	NS	NS
	IL-17	NS	NS	NS	NS
	FGF-basic	NS	NS	NS	NS
	GM-CSF	NS	NS	NS	NS
	MIP-1α	NS	NS	NS	NS
	MIP-1β	NS	NS	NS	NS
Tissue accessory cells (Chapter 5)	PD-1	NS	NS	NS	NS
	FOXP3	NS	NS	NS	NS
	CD21	NS	NS	NS	NS

Analysis 1 compares CR rates in groups of patients with high or low expression of CMV-dependent biological variables (using median values as a cut-off). Analysis 2 compares the expression of CMV-dependent biological variables in groups of patients who achieve or do not achieve a CR. NS = Not significant (P>0.1).

7.4 Discussions

The aim of this Chapter was to relate treatment responses to CMV-dependent biological variables with the aim of shedding further light on the mechanisms underlying the possible beneficial effect of CMV infection on response to chemoimmunotherapy as reported in Chapter 6. The key positive finding was that

patients with a high proportion of E.CD4+ T cells in the blood (> median value) were significantly more likely to achieve a CR after 8 cycles of treatment (~80% vs. ~40%). The reciprocal was also true, i.e. patients achieving a CR after 8 cycles of treatment had significantly higher numbers of E.CD4+ T cells in the blood compared to those who did not achieve a CR. Patients with a high number of E.CD8+ T cells in the blood also tended to have higher CR rates after 8 cycles of treatment, but this observation failed to reach statistical significance. There was also a non-significant trend for patients with high PE2.CD4+ T cells in the blood to have higher CR rates after 4 cycles of treatment. In contrast, patients with a low total number of CD4+ T cells (as seen in CMV positive) tended to have higher CR rates after 8 cycles of treatment, but again, this observation failed to attain statistical significance (p=0.094). No association was observed between CR rates after 4 of 8 cycles of treatment and levels of individual cytokines in the serum or number of accessory cells in affected lymph nodes expressing PD-1, FOXP3 or CD21.

Our data reveal that patients with higher proportions of PE2.CD4 (p=0.070), E.CD4 (p=0.041 & p=0.035) and E.CD8 (p=0.094) as well as low proportion of CD4+ T cells (p=0.094) responded better to the rituximab-chemotherapy. In the case of E.CD4 cells, these differences were identified in a two-way statistical approach that involved firstly grouping the patients based on the quantity of each of the laboratory variables and then on the basis of clinical outcome (CR). This reciprocal approach to statistical analysis should increase the level of confidence that the findings are real rather than spurious.

The intriguing observation is that similar elevated levels of these T cell subtypes were reported among the CMV-positive patients (Chapter 3), and that this group of patients also showed greater sensitivity to rituximab-containing chemotherapy compared with

the CMV negative group (Chapter 6). Taken together, these observations are in keeping with the idea that E.CD4 and E.CD8 cells might mediate some of the cytotoxicity of chemo-immunotherapy in FL, and that the superior CR rates observed in CMV-positive patients is due to the greater number of E.CD4 and E.CD8 cells that accumulate in these patients.

My results provide the first triangulation between CMV infections, accumulation of effector T cells and enhanced sensitivity to rituximab-containing therapy in FL. Thus, although CMV infection is known to promote the accumulation of these circulating effector and pre-effector T cells in healthy individuals, this phenomenon has not been previously documented in FL. Furthermore, although effector cells have been shown to promote the antitumor activity of antibody-based therapy in FL (Golubovskaya and Wu, 2016, Chames et al., 2009), there are no prior reports of CMV enhancing the effectiveness of such treatment.

Rituximab, the therapeutic mAb which forms part of the treatment regimen for FL and a broad variety of other B-cell malignancies, targets the CD20 antigen on B cells (Kosmas et al., 2002, Weiner, 2010). Mechanisms by which rituximab exerts its therapeutic effects include complement (C1q) binding, induction of B-cell (follicular lymphoma cell) depletion via FcR-mediated antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC), inhibition of cell proliferation, direct induction of apoptosis, and disruption of anti-apoptotic pathways with sensitisation of malignant B cells to chemotherapy (Kosmas et al., 2002, Boross and Leusen, 2012).

The killing of FL cells (antibody-coated target cell) through ADCC mechanism requires effector cells, especially the cytotoxic effector cells expressing particular Fc

receptors (FcRs) that will release cytotoxic granules or by the expression of cell death-inducing molecules (Romána et al., 2013). Although it is argued that resting CD4+ T-cells lack low-affinity FcRs, however, activated CD4+ T-cells express substantial amounts of FcγRIIIa, which were initially observed in small numbers in circulating T cells (Chauhan, 2016, Sandor and Lynch, 1993). Therefore, one may argue that the high effector CD4+ and CD8+ T cell types reported among the CMV-positive patients (Chapter 3) might contribute to the CR rate observed in Chapter 6 which is further confirmed in this Chapter (Chapter 7). Following massive depletion of FL (B) cells in the course of rituximab -chemotherapy regimen, effector T cells, though short-lived, are capable of homing to opsonized antigens (FL cells) and eliminating them at a rate that correlates with the number of effector T cells (Golubovskaya and Wu, 2016). This phenomenon should theoretically lead to a better CR rate among patients with higher numbers of effector T cells prior to commencement of the treatment, as is seen in the CMV-positive cohort.

Regarding the potential value of passive immunotherapy through ADCC and CDC, studies have suggested that because the maximum clinical benefit of rituximab is achieved months after the commencement of therapy, the adaptive arm of immunity is involved in the overall realisation of an enduring benefit of rituximab therapy (Cartron et al., 2004, Weiner et al., 2010). Evidence suggests that induction of adaptive immune responses in the course of antibody therapy is achieved when DCs present peptides from engulfed apoptotic cells on MHC class I molecules to elicit antigen-specific CD8+ T cell responses (Weiner et al., 2010). These understandings provide a possible explanation for the higher CR rates observed post cycle 8 compared to post cycle 4 (see Table 7.1) and the impact of the effector CD4 and CD8 T cell functions in clearing FL cells.

To understand the mechanism by which rituximab can influence the functional properties of T cells, a group reported that rituximab efficiently depletes all the CD20 expressing T cells (CD20 T cells), which account for about 1.6% to 2.4% of all T cells (Wilk et al., 2009). The study concluded that CD20 T-cell population contributes largely to disease severity, and that depletion of this T-cell subset can serve an additional mechanism by which the monoclonal CD20 antibodies therapy can effectively work in the treatment of other diseases such as RA.

Of course, mechanisms other than ADCC and CDC might explain the better treatment responses observed among patients with elevated PE2.CD4+, E.CD4+ and E.CD8+ T cells, as seen in the CMV-positive group, although it is difficult to speculate on what these mechanisms might be.

No association was found between CR rates and serum cytokines or number of accessory cells expressing PD-1, FOXP3 or CD21. This negative finding may reflect physiological variation in serum cytokine levels. Thus, even in healthy individuals, levels of serum cytokines have been reported to vary widely, with even more variation seen in pathological conditions (Kleiner et al., 2013). For instance, some studies have linked higher levels of pro-inflammatory cytokines (e.g. IL-1 or TNF-α) to worse disease outcomes, and curtailing the biological activities of these cytokines can reduce the impact of illness (Dinarello, 2000, Fitzgerald et al., 2005). Alternatively, the lack of association between serum cytokine levels and CR rate may simply reflect the small sample size. This speculation also applies to the lack of association observed between CR rates and PD-1, FOXP3 or CD21-expressing accessory cells in the tumour microenvironment. Thus, others have shown that T-cell subtypes, proteins and cells in the tumour microenvironment are associated with survival in FL and may

influence response to treatment and the risk of transformation in this disease (Yang and Ansell, 2012a).

Finally, my findings are likely to be confounded by other variables that I have not studied but which might modulate response to treatment in FL such as the genetic and cellular factors (KIR genotyping, HLA genotyping, and KIR ligand assignment) that drive immune responses in mAb therapy (Du et al., 2014). Also, genetic polymorphisms in the gene for C1q have been linked to variations in rituximab efficacy in humans, single-nucleotide polymorphisms (SNP) and copy number variations of the FCGR2A and FCGR3A genes that play roles in the efficacy of rituximab therapy (Moriya et al., 2014, Rezvani and Maloney, 2011) may impact on response. Such variables could dilute and potentially mask any effects due to the variables of interest. The identification of laboratory variables that may associate with clinical outcome but were not studied in this Chapter could form the basis for a future research project.

In summary, the data presented in this Chapter suggest that CMV increases sensitivity to chemo-immunotherapy in FL due to the secondary accumulation of effector T cells which mediate the therapeutic effects of rituximab.

Chapter 8 General Discussion & Conclusions

The hypothesis addressed in this thesis is that CMV infection alters the immune system in FL in two ways: first, by expediting immune senescence, thereby leading to increased susceptibility to heterologous infection; and second, by altering the lymphoma microenvironment in such a way as to alter the response to chemoimmunotherapy. My experimental approach involved comparing CMV-positive and CMV-negative patients with FL who entered the PACIFICO trial for circulating lymphocyte subsets (Chapter 3), blood cytokine levels (Chapter 4), accessory cells at sites of tissue involvement (Chapter 5), and clinical outcome (Chapter 6). Furthermore, in an attempt to elucidate pathogenetic mechanisms responsible for the effects of CMV on clinical outcome, the latter was related to laboratory variables found to be associated with CMV serostatus. The present Chapter attempts to integrate my experimental findings and, in doing so, provide a unifying explanation for the effects of CMV infection in FL.

In considering the extent to which my findings support the hypothesis underlying my thesis, the first point to make is that CMV status was indeed associated with a number of the biological variables examined. These include blood lymphocyte subsets, serum cytokine profile, and the number of specific accessory cells at sites of tissue involvement. The existence of such differences indicates that hypothesis has the potential to be correct.

The salient findings were as follows: Flow cytometric analysis of stored PBMCs of FL patients in Chapter 3 revealed higher percentages of end-stage T cell subtypes (pE2.CD4 ($p=0.048$), E.CD4 ($p=0.030$), EM3.CD4 ($p=0.019$), E.CD8 ($p=0.035$) & EM3.CD8 ($p=0.005$)), as well as NKT cells ($p=032$), in CMV-positive patients

compared to the CMV-negative counterparts. In Chapter 4, the Luminex assay show significantly decreased levels of multiple cytokines in the serum of CMV-positive patients compared to the CMV-negative group. These included IL-6 (p=0.029), IL-8 (p=0.037), IL-9 (p=0.006), IL-17A (p=0.020), FGF-basic (0.028), MIP-1α (p=0.039) and MIP-1β (p=0.029). Furthermore, there were more accessory cells expressing CD21 (p=0.043), FOXP3 (p=0.069) and PD-1 (0.097) in the lymphoma microenvironment of CMV-positive cases compared to CMV-negative patients, suggesting an accumulation of FDCs, Tregs and exhausted T cells, respectively. At the clinical level, I showed in Chapter 6 that CMV infection was associated with a better response to chemo-immunotherapy in the cohort used for flow cytometry/cytokine profiling (p= 0.019), and with an increased risk of infection in the cohort used for tissue immunofluorescence (p=0.017), as well in the much larger overall patient cohort (p<0.001). In Chapter 7, I showed that response to chemoimmunotherapy was associated with increased circulating end-stage T cells (p=0.041 for E.CD4; p=0.094 for E.CD8) but not with any of the other CMV-dependent biological variables examined. Figure 8-1 summarises these associations. In summary, I have produced evidence that CMV infection in FL results in (1) the accumulation of circulating end-stage T cells, (2) a reduction in multiple serum cytokines, (3) increased FDCs, Tregs and exhausted T cells in the lymphoma microenvironment, (4) increased infection and (5) better therapeutic response to chemo-immunotherapy, the latter being associated with increased circulating end-stage T cells but not with reduced serum cytokines or increased FDCs, Tregs and exhausted T cells in tissues.

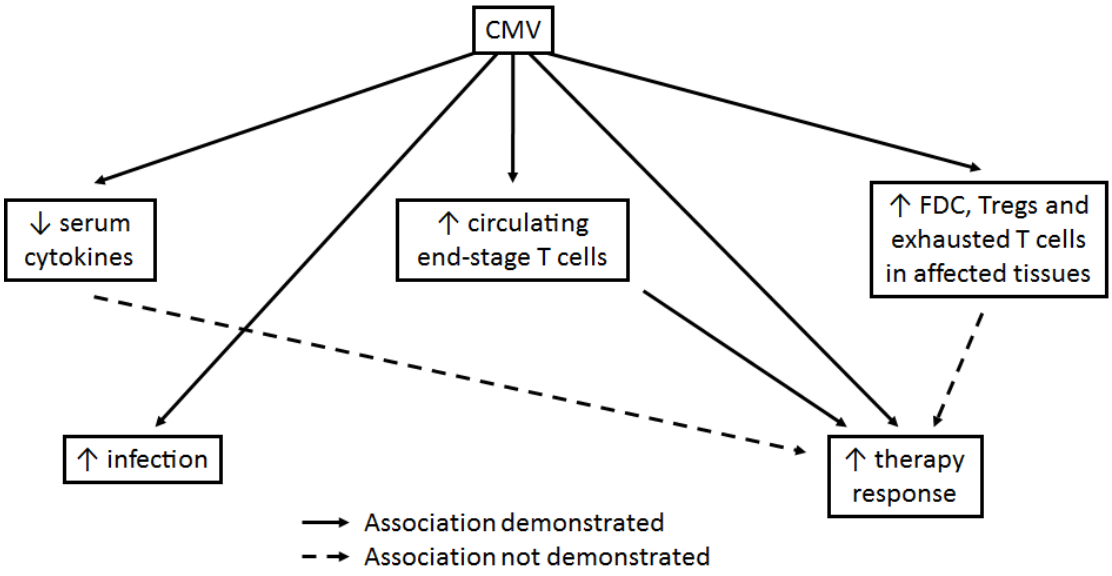

Figure 8-1: Associations observed between CMV infection, alterations in the immune system and clinical outcome in patients with FL

The observed association between CMV positivity and the accumulation of circulating end-stage T cells strongly supports the hypothesis that, in patients with FL, CMV accelerates the general process of immune senescence as has been suggested in healthy individuals (Solana et al., 2012, Tsoukas, 2012).

Since many cytokines are produced by T cells, and since one of the features of end-stage T cells is their inability to produce cytokines, it is reasonable to assume that the reduction in serum cytokines observed in CMV-positive FL patients is secondary to alterations in lymphocyte subsets. The observed association between CMV positivity and infection following chemoimmunotherapy indicates that CMV infection is immunosuppressive in this context and implicates accelerated immune senescence as an underlying mechanism. Unfortunately it was not possible within the time frame available to look for a direct relationship between infection and either blood lymphocyte subsets or serum cytokines to confirm the relationship between accelerated immune senescence and susceptibility to infection.

As outlined above, CMV status was also associated with alterations in the number of accessory cells in the lymph node microenvironment with increased numbers of FDCs (CD21+), Tregs (FOXP3+) and exhausted T cells (PD-1+) detected in CMV-positive patients. The functional relationship between these CMV-dependent tissue alterations and CMV-dependent alterations in blood lymphocyte subsets is unclear as it was not possible to apply the same level of cellular characterisation to FFPE biopsy material as was possible for cryopreserved PBMCs. Nevertheless, it is noteworthy that one of the histological associations of CMV in FL tissues was an increase in exhausted (PD-1+) T cells. Although these cells are phenotypically distinct from the TEMRA (T effector memory re-expressing CD45RA) cells that accumulate in the circulating blood of CMV-positive FL patients, they resemble TEMRA cells in being functionally incompetent. Consequently, it appears that CMV infection in FL results in the accumulation of a multiple types of functionally incompetent T cells both in the blood and also at sites of tissue involvement.

The FL microenvironment in CMV-positive patients was associated not only with an increase in exhausted T cells but also with higher numbers of Tregs and FDCs which, in the case of FDCs, reached statistical significance. It is therefore logical to speculate that one or more of these cell types might be responsible for the better response to chemoimmunotherapy that was observed in CMV-positive patients. The latter observation was apparent in both of the smaller patient cohorts (where other variables that could confound outcome were well balanced between CMV-positive and CMV-negative cases) and reached statistical significance in the FACS/cytokine cohort.

Given the effect of CMV on both therapy response and accessory cells at sites of tissue involvement, it was surprising that no association was detected between the

latter two variables. Specifically, response to chemo-immunotherapy was not associated with the number of exhausted T cells, Tregs or FDCs in the FL microenvironment. This could indicate that these cells were not responsible for the effect of CMV on therapy response, or that one or more of these cell types was responsible for the effect of CMV on therapy response but that a statistically significant association was overlooked due to small sample size. Interestingly, the only CMV-dependent biological variable found to be statistically associated with therapy response was the proportion of circulating end-stage T cells. Thus, twice as many patients with a high (greater than the median) E.CD4/E.CD8 count achieved a CR after 8 cycles of chemoimmunotherapy compared to patients with a low E.CD4/E.CD8 count ($p=0.041$ and $p=0.094$, respectively). The explanation for this observation is unclear. One possibility is that the accumulation of circulating TEMRA cells is simply a marker of CMV infection and that these cells do not mediate the effect of CMV on therapy response. This model is supported by the fact that TEMRA cells lack homing receptors such as CCR7 and CD62L (Willinger et al., 2005) and should therefore be poorly equipped to migrate into the FL microenvironment. Alternatively, it is possible that TEMRA cells do accumulate in the FL microenvironment and modulate therapy response but were undetected by the immunofluorescence staining for CD4 and CD8 that was reported in Chapter 5 as their accumulation does not affect the total number of CD4+ or CD8+ T cells in affected tissues.

The above conclusions and deductions are based on data which (as is the case with all datasets) has both strengths and limitations. The fact that all the data generated in this thesis is derived from samples obtained as part of a clinical trial should be regarded as a major strength. In fact, due to the relatively low incidence of FL, it would be

difficult for a single treatment centre to accumulate a sufficient number of samples for the studies performed in this thesis. Under these circumstances, centralised sample collection performed as part of a multicentre trial provides the only realistic opportunity to perform such research. Furthermore, since trial entry is tightly governed by strict entry criteria, and since clinical data collection is performed to a high standard, patients recruited into trials are extremely well defined in terms of pre-treatment characteristics, treatment administered and treatment outcome. On the negative side, trial populations may not be entirely representative of the overall population with a given disease in terms of age (older patients are typically under-represented) and co-morbidity (less fit patients are typically unrepresented). An additional problem with trial samples is that clinical outcome data cannot be released until the primary results of the trial are published, as there is a risk that the primary results might be deduced from the released data. For this reason, it was not possible to analyse the two treatment arms of the PACIFICO trial separately. This is unfortunate as the R-FC arm is likely to be more T cell-immunosuppressive than R-CVP. It therefore follows that patients who received R-FC are likely to be more sensitive to the effects of CMV compared to those who received R-CVP. Furthermore, it was not possible to relate pre-treatment characteristics (including CMV status) to long-term therapy response (progression-free survival) as the number of deaths/progressions is currently too small for analysis to be meaningful. Consequently, the analysis was confined to short-term response (CR rate).

Another limitation in the data was the need, for practical reasons, to confine the FACS/cytokine and IF analysis to a cohort compromising 42 patients. The actual number of samples for which a complete dataset was available was even smaller as trial data collection is ongoing. These two factors reduced the statistical power of

comparisons between different patients groups within these cohorts and in doing so increased the risk of overlooking true associations between the variables examined. On the other hand, the process of selecting samples for the FACS/cytokine and IF cohorts ensured that CMV-positive and CMV-negative patients were well balanced for prognostic factors that could bias the results. Such patient selection should increase the reliability of comparisons made between CMV-positive and CMV-negative patients. However, despite showing that CMV seropositivity is associated with a better therapy response in the two smaller, well-balanced cohorts, failure to show any association between CMV status and therapy response in the larger, unselected cohort means that CMV serostatus is unsuitable as a clinically useful predictive biomarker for therapy response. In contrast, the strong association observed between CMV serostatus and infection in the larger, unselected cohort means that CMV serostatus has potential value as a clinically useful predictive biomarker for infection.

The sophistication of the analytical techniques employed and the large amount of data produced by these techniques should be regarded as one of the strengths of this thesis. Furthermore, the data should be reliable since all assays were validated before being applied to the trial samples. Indirect validation of the assays is provided by the fact that some of the observed differences between CMV-positive and CMV-negative cases include some previously reported effects of CMV in healthy individuals. It would have been interesting to quantify CMV-specific T cells using CMV-specific tetramers and assess lymphocyte function by measuring activation following stimulation with recall antigens (Gratama et al., 2001, Gratama and Kern, 2004), and the lack of such data should be regarded as a limitation. A further limitation is the lack of longitudinal data to show the combined effect of CMV and

chemoimmunotherapy on circulating lymphocytes and serum cytokines. Although sequential samples were available for some patients, it was simply not possible to perform these analyses within the time frame available.

Although some of the associations that I have demonstrated between biological variables are strong, others are weaker – just bordering on statistical significance. Furthermore, causal relationships have not been proven between any of the variables, although many can be strongly inferred from the application of existing knowledge and basic principles. Although factors other than CMV such as age, gender, and other pathophysiological conditions may cause variation in the numbers and types of circulating immune cells (Koch et al., 2008b), the differences between CMV-positive and CMV-negative patients observed in my study are likely to be due to CMV itself since these other factors were well balanced between the CMV-positive and CMV-negative groups that were compared with one another.

One important limitation of my study is that it did not formally establish whether the end-stage lymphocytes that accumulated in CMV-positive patients with FL were exclusively CMV-reactive or whether they included non-CMV-reactive lymphocytes. In the absence of co-staining with CMV-specific tetramers, it was not possible to formally address this question. However, the average proportion of CMV-reactive circulating memory T cells in healthy CMV-seropositive individuals is ~10% (Naeger et al., 2010). In contrast, in my study, circulating end-stage T cells were 2-3 folds higher in CMV-positive cases (Table 3-3). Consequently, it seems very likely that the end-stage T cells that accumulate in CMV-positive patients with FL include not only CMV-specific T cells but also T cells with other specificity.

Having considered the strengths and limitations of the data presented in this thesis, it is important to discuss the extent to which my data support or contradict the existing literature and suggest possible explanations for any discrepancies.

The concept that CMV infection results in the accumulation of terminally differentiated lymphocyte subtypes in the blood is in agreement with previous reports of both healthy individuals (Welzl et al., 2014, Ohlin and Söderberg-Nauclér, 2015) and cancer patients (P Chou and B Effros, 2013, Myklebust et al., 2013). The end-stage lymphocytes found to accumulate in CMV-positive individuals in these studies included T cells lacking the costimulatory receptors CD27 and CD28 as well as the lymph node-homing receptors CCR7 and CD62L (Pawelec et al., 2005) (Moro-García, Alonso-Arias et al. 2015). Other studies that used blood and bone marrow samples spanning from young (mean age, 29 years; range, 20–39 years) to old (mean age, 80 years; range, 71–91 years) healthy donors demonstrated a correlation between CMV infection and increased end-stage (short-lived) CD45RA+ CD27− CD4+ and CD8+ T cells (Libri et al., 2011, Smith et al., 2016). By using multiple linear regression analysis, these authors demonstrated that increases in $CD45RA^{-}$ $CD27^{-}$ (effector memory) and CD45RA+ $CD27^{-}$ CD4+ (effector) T cell phenotypes are due mainly to CMV infection. Furthermore, Griffiths in his PhD thesis linked CMV seropositivity in healthy blood donors to increased CD45RA+ memory T cells in the peripheral blood (Griffiths, 2011). These cellular phenotypes are all features of immune senescence and are indicative of repeated antigenic stimulation and impaired immune functionality that can potentially lead to increased susceptibility to recurrent infections (Pawelec et al., 2005, Alonso Arias et al., 2013, Smith et al., 2016). The concept that the end-stage T cells that accumulate in CMV-positive patients are functionally incompetent is further supported by a study reporting a significant

correlation between accumulated end stage T lymphocytes, especially enriched CD8+ TEMRA cells, and delayed bone fracture healing (Reinke et al., 2013).

My study also showed a significant increase in circulating NKT cells amongst CMV-positive patients compared to those who were CMV negative. NKT cells are known to exhibit either protective or injurious effects through the secretion of cytokines that promote either immune tolerance or inflammation (Savage et al., 2016). Although the exact mechanisms are not clear, the increase in the NKT cells in CMV-positive cases might reflect a compensatory response of the innate immune system to heterologous infections caused by the reduction in the number of CD4+ T cells and the skewing of T cells towards a senescent phenotype.

In a study of healthy individuals, Schrier et al found an association between CMV infection and circulating T cells expressing PD-1, a marker of T-cell "exhaustion" (Schrier et al., 1986). In my study, CMV infection was associated with increased PD-1 expression in affected lymph nodes but not in circulating T cells. Since PD-1 influences the apoptosis of stimulated T-cells and minimises their capacity to produce cytokines (Sester et al., 2008). Thus may help to explain the reduced serum cytokine levels observed among CMV-positive FL patients.

Surprisingly, my study appears to be the first to directly demonstrate that CMV seropositivity is associated with a significant increase in infections. That this phenomenon has not been previously reported suggests that the effect may only be evident in the presence of additional immunosuppression such as that induced by chemo-immunotherapy. This key observation provides crucial new evidence to support the idea that CMV infection can cause clinically significant immunodeficiency by inducing the accumulation of terminally differentiated T cells.

On the other hand, my demonstration that CMV infection may enhance sensitivity to chemo-immunotherapy in FL (Chapter 6) via the accumulation of terminally differentiated E.CD4 and E.CD8 T cells (Chapter 7) suggests that the T cells that accumulate in CMV-positive patients are functionally active and capable of modulating the cytotoxicity of chemotherapy drugs or, more likely, rituximab via enhanced ADCC. This concept is in keeping with the report of Libri et al, which demonstrated the multifunctional capability of the CD45RA+ $CD27^-$ T-cell subtype (Libri et al., 2011). These latter cells possess cytotoxic function similar to those of $CD45RA^-$ CD27+ T-cell subsets, indicating that end-stage T cells are not completely functionally incompetent. A report by Henson et al also described CMV infection as one of the leading causes of elevated end-stage T cells (Henson et al., 2012). In their article, end-stage T cells were characterised by increased activation of senescence signalling pathways, decreased proliferative capacity, and higher sensitivity to apoptosis, yet were capable of multiple effector functions. The latter characteristic is in agreement with the report of Libri et al and potentially explains my own observations regarding CMV serostatus and therapy response.

The notion that the end-stage T cells that accumulate in CMV-positive indivuals are functionally active need to be reconciled with my observation that CMV sero-positivity is associated with increased infection following chemo-immunotherapy, which implies loss of T-cell function. The likely explanation for this apparent contradiction is that the effector T cells that accumulate in CMV-positive patients are mainly pathogen-specific and show poor response to novel pathogens (Pennock et al., 2013, Briceño et al., 2016).

We have reported significantly decreased levels of 7 serum cytokines in the CMV-positive patients in Chapter 4 as summarised in Table 8-1.

Table 8-1: Serum cytokines differentially expressed due to CMV infection

Cytokine	Produced by	Primary target	Hallmark
IL-6	Macrophages, T_H2, Adipocytes	T_H2 B cells	Proinflammatory/ T_H2
IL-8 (CXCL8)	Neutrophils, Macrophages, Epithelial cells	Endothelial cells	Chemotactic Angiogenesis
IL-9	T_H9 cells, NKT cells, Mast cells, T_H2 cells, Tregs, T_H17 cells	Promotes mast cell and T cell growth, stimulates mast cell accumulation in tissues enhances class switching to IgE in B cells and alters haematopoietic progenitor cell activity	pleiotropic in function, proinflammatory
IL-17A	Activated T cells	Chronic inflammatory diseases	Proinflammatory
FGF-basic	Adipocytes	Wound healing, Normal tissue and Tumour development	Angiogenesis
MIP-1α (CCL3)	Macrophages, DCs, Lymphocytes	Infection, inflammation	Proinflammatory & Chemotactic effects
MIP-1β (CCL4)	Macrophages, DCs, Lymphocytes	Infection, inflammation	Proinflammatory & Chemotactic effects

A brief summary of cytokines that are differentially expressed in CMV positive FL compared to the CMV negative (Kaplan et al., 2015, Rouvier et al., 1993) and providing summary sources, targets and actions. DCs= dendritic cells, NKT cells = Natural Killer T cells, MIP-1α = Macrophage Inflammatory Proteins-1 α, MIP-1β = Macrophage inflammatory protein-1-β.

Interestingly, serum levels of most cytokines and chemokines are generally low in healthy persons, although some variation has been observed due to ageing (Kleiner et al., 2013, Kim et al., 2011). By inference, one may be tempted to think that the lower levels of serum cytokines reported among CMV-positive patients confer some benefit to this group of patients. However, such an assumption would be overly simplistic owing to the considerable functional heterogeneity of cytokines.

The significantly reduced levels of serum cytokine observed in the CMV-positive patients in Chapter 4 was not unexpected, given that T cells are the primary source of

these cytokines and that the T cells that accumulate in CMV-positive patients are terminally differentiated and therefore poorly equipped to produce cytokines. Contrary to our data, other studies have reported elevated levels of serum cytokines (IL-6, IL-8 & TNF-α) among CMV-positive bone marrow transplant recipients (Humar et al., 1999). Unlike in our study cohort, the bone marrow transplant patients experienced CMV reactivation and severe inflammatory CMV disease which likely accounts for the increased serum cytokine levels. In contrast, a recent study that evaluated hand-grip strength in older subjects did not observe any strong correlations between CMV serostatus and serum cytokine (IL-1β, IL-6 or TNF) levels (Goldeck et al., 2016). From these observations, one may argue that in the absence of an acute inflammatory response, CMV infection does not increase cytokine levels but rather results in a reduced capacity for cytokine production in chronic diseases such as FL. It is also likely that reduced capacity for cytokine production is partly responsible for the increased infection that is observed among CMV-positive patients with FL following chemo-immunotherapy. Unfortunately, the mechanisms deployed by CMV in concert with relevant cellular sources of cytokines to downregulate the productions of these cytokines are not well studied. Due to the limited time frame, no experiments were conducted to understand the exact mechanisms.

The immunofluorescence Chapter reveals that CMV-positive patients show higher intensities of CD21+ (FDCs), FOXP3 (regulatory T cells) and PD1+ (exhausted T cells) expressing cells in the FL microenvironment compared to CMV negative patients. The significantly higher intensity of CD21-expressing cells among CMV-positive patients is interpreted as indicating a higher number of FDCs as the staining showed distinctive meshwork in most cases. FDCs are mainly found in the primary and secondary follicles of the B cell areas where they play a vital role in organising

lymphoid microarchitecture, antigen capturing, memory B-cell support, debris removal and prevention of autoimmunity (Kranich et al., 2008, Aguzzi and Krautler, 2010, Cyster, 2010). However, the elevated level of CD21-expressing cells and well as those of FOXP3 and PD1 in the CMV-positive patients did not show any association with the rituximab treatment efficacy. Going by the efficacy data in Chapter 7, it can be deduced that CD21, FOXP3 and PD1 expressing cells as seen in the FL microenvironment of CMV-positive patients might not hold any role in potentiating treatment efficacy in FL therapy. This implies that their activities may not have a direct beneficial impact on the actions of Rituximab such as ADCC, CDC as well as in the induction of apoptosis and direct growth arrest of cell circle.

Intriguingly, CMV infection and elevated end stage T cells (E.CD4 and E.CD8) have shown an association with enhanced response to rituximab-chemotherapy, especially at the post cycle 8. Ordinarily, with the increased number of senescent, exhausted and end-stage T cell subtypes, the higher CR rate seen among the CMV-positive patients seemed contradictory to expectation. The mechanism by which CMV infection modulates rituximab therapy resulting to enhance response in FL is not known. However, its activity has been linked to the accumulation of effector (E.CD4 and E.CD8) T cells which as a recent study investigating effector functional potentials of CMV-pp65-reactive T-cells including IL-2, TNF-α, IFN-γ, perforin, and CD107a expression revealed that CMV-specific CD4 + and CD8 + T cells manifest polyfunctionality including cytotoxic functions (Chiu et al., 2016). However, the mechanism by which these T cell subtypes accentuate response to rituximab chemotherapy is not known and will require investigation. Using a univariate analysis, a study that evaluated the impact of CMV infection on the clinical outcome of a prospective cohort of B-CLL patients reported a poor OS in the CMV-positive

group compared to the CMV negative, although in a multivariate modelling, no significant survival difference was observed between the groups (Parry et al., 2016a). Despite patients were recruited into PACIFICO trial based on stringent inclusion and exclusion criteria and those selected into this study were well balanced based on equal distribution of their clinical and laboratory features, a multivariate analysis will further confirm whether above cellular changes were independently or interrelated with each other and/or with other clinical and laboratory variables to contribute to the clinical outcome. Similar to our data, increased incidence infections have been reported in patients with lymphomas being treated with rituximab (Kelesidis et al., 2011, Looney et al., 2008). The significantly increased incidence and episodes of infections among the CMV-positive FL patients on rituximab –chemotherapy seen in our data suggests that the end stage T cells reported in Chapter 3 and the low cytokine levels in Chapter 4 is not able to provide a robust immunity against infection. This observation is in agreement with several reports that linked CMV infection with immune senescence that results in phenotypic alterations and functional decline in the T cell repertoire, culminating in increased vulnerability to infections (P Chou and B Effros, 2013).

Regarding the originality of this study, this is the first report of accumulated end-stage T cell types, significantly decreased serum cytokine levels and high intensities of CD21 expressing cells tumour microenvironment of CMV positive FL patients. Our data has demonstrated a perceived favourable role of CMV infection in rituximab-containing chemotherapy among FL patients. Furthermore, the study has observed a potential relationship between high frequencies of effector CD4+ and CD8+ T cells and CR in the course of FL immunochemotherapy. A prospective study on a large cohort of patients to elucidate the impacts of CMV infection on the clinical outcome

of patients with chronic lymphocytic leukaemia (B-CLL) showed that CMV-positive patients had significantly worse overall survival than the negative patients in the univariate analysis (Parry et al., 2016b). However, no evidence that CMV infection impacts on the clinical outcome of patients with B-CLL was observed in a multivariate analysis of the same cohort. Another study using seasonal influenza vaccination expectedly showed weak responses to the vaccine and an overall decreased immune response in the elderly regardless of CMV serostatus (Furman et al., 2015). Conversely, CMV-positive young adults showed an enhanced response to influenza vaccine, enhanced CD8+ T cell sensitivity, and raised levels of circulating IFN-γ compared to CMV negative (Furman et al., 2015). This perceived beneficial effect of CMV infection supports our data which shows enhanced response to rituximab therapy in the CMV positive patients. It is even speculated that the high global prevalence of latent CMV infection may be due to the beneficial effect it has on the host immunity (Furman et al., 2015). Although several researchers have demonstrated correlations between CMV serostatus and heterologous infection, we have shown increased incidence and episodes of specific infection AEs among CMV positive FL patients compared to CMV negative.

In conclusion, this study has demonstrated strong associations between CMV seropositivity and the following: increased circulating end-stage T cells, decreased levels of serum cytokines, increased FDCs in the FL microenvironment as well as increased incidence and episodes of infections/neutropenia AEs and better/more rapid response to rituximab-containing chemo-immunotherapy. These results support our hypothesis which states that CMV infection alters immune function in two ways: (1) Acceleration of immune senescence with increased vulnerability to heterologous infection and (2) Alteration of lymphoma microenvironment with the possibility of

modulating FL therapy response. These understandings can ultimately improve on the therapeutic strategies in FL management toward a combinatorial approach for direct cytotoxic and indirect immunomodulatory treatment regimens.

With these associations observed in this retrospective study using pre-trial (baseline) samples from FL patients , the next would be to prospectively assess the predictive value of CMV infection status in this disease. Also, repeating this study using post – therapy samples from the PACIFICO trial patients may provide additional insights into the impact of CMV infection in FL. The plan of future development, including study design and technical approaches, is presented in Chapter 9.

Chapter 9 Future Research

9.1 Introduction

As presented in previous chapters, the hypothesis of this study has been largely addressed by comparison of profiles of T cell subtypes, cytokine expression and tissue protein markers between CMV-positive and –negative patients with FL, and correlation of clinical outcomes with CMV infection and the study variables. However, due to limitations described in Chapter 8, the quality of this study can be further improved with the following work proposed for future.

9.2 Future work

9.2.1 Confirmation of results in an independent cohort of patients with a sample size estimated based on observations in this study

Validation of results is the first task in the future work. For this purpose, it is necessary to recalculate the sample size. Despite having been calculated in section 2.5, the samples size of 42 used in most comparisons in this study was based on results of a pilot experiment of cytokine expression in the two groups of patients. It might not be big enough to find all of real differences due to the β error. For example, an estimated difference of one was used in the calculation. But the actual difference for most of these cytokines was smaller than that (Table 4.1.). This might result in the statistical power smaller than the estimated 80%. As the initial analysis was exploratory in nature and covered a wide range of endpoints, a sample size on any single outcome is not possible. To replicate the finding of the analyses presented (FACS, Luminex, and IF) in the future, we would consider it clinically significance if the odds of an outcome was twice as likely in one CMV cohort compared to the other. Using an odds ratio 2, a power of 80% and an alpha level of 0.001 to account for the

multiple testing required, a sample size of 206 patients would be sufficient. This assumes a binomial distribution with outcomes assumed to occur around the centre of the distribution as this is the scenario that gives the maximum variance. Outcomes measured using a continuous scale or which are observed to be more/less likely will increase the power. In addition to a reliable validation of results, it is also important for increasing chances to identify real differences that were underestimated, e.g. those borderline differences recorded in the current study. Those borderline differences existed in T cell subtypes (e.g. CD4+ T cells (p=0.051), LAG3-CD4 (p=0.074), CD4: CD8 ratio (p=0.054)), cytokines (e.g. IL-1β (p=0.068), IL-4 (p=0.066) and GM-CSF (p=0.070)) and tissue markers (e.g. FOXP3 (p=0.069)).

9.2.2 Validation of cytokine data using an alternative method and stable serum samples

In Chapter 4, I found the levels of 7 out of the 27 cytokines significantly lower in CMV-positive patients in experiments with LUMINEX. Those should have been confirmed either using an alternative method or repeating with the same technique. But only IL-9 was confirmed using sELISA (see section 4.3.3). Confirmation of others was hampered by a lack of stabilised serum samples (see section 4.3.2). In a future study, therefore, the serum sample from each of individual patients will be stored at -80^{o}C in multiple aliquots after phlebotomy, so that each aliquot can be used only once in each of subsequent experiments. This can minimise the sample instability caused by repeated thawing and freezing, as found in section 4.3.2. Once the stable samples are available, the standard sELISA will be applied in the validation given its cost efficiency as compared to LUMINEX and other available methodologies, such as V-PLEX and multiplex immunoassay technologies for profiling the expression of multiple proteins from minute sample volumes (Jones et al., 2016, Sandberg et al., 2016).

9.2.3 Investigating the impact of CMV infection on the outcome of the two arms of therapies in PACIFICO trial

As noted in section 2.2, although the patient recruitment has been closed, the PACIFICO trial is still ongoing. The full treatment outcome will be not available untill2018. In Chapter 6, I found an increased treatment response rate together with a trend of higher incidence of adverse events in CMV-positive patients regardless of the treatment regimen designed for the trial. Once the trial is completed within two years, these clinical outcomes will be further compared with CMV infection status between patients treated with R-VCP and R-FC. This may address whether or not the impact of CMV infection is the same in patients treated with the two regimens. Furthermore, with the follow-up extended, a further analysis of the impact of CMV infection status on overall survival (OS) and progression-free survival (PFS) will be performed for the entire trial cohort and patients treated in each of the two therapy arms.

9.2.4 Evaluating the potential predictive value of CMV infection status in FL

In the current retrospective study, CMV infection has been demonstrated to associate with elevated end-stage T cell subtypes (Chapter 3), decreased serum cytokine (Chapter 4), and increased intensity of FDCs in the FL microenvironment (Chapter 5). It has also been shown to associate with high incidence and episodes of infection AEs/SAEs and a beneficially enhanced response to rituximab-containing chemotherapy (Chapter 6). We have also reported an association between effector CD4 and CD8 cell types with better response to rituximab-chemotherapy (Chapter 7). We have also planned future work to strengthen most of above findings FL. Once the future work is completed, the next is to test the most possible biomarker candidates in prospective studies. That will eventually confirm the clinical usefulness of these biomarkers in management of FL. To do so, the total number of patients to be

recruited will be determined by a calculation the frequency of CMV infection in FL and the level of each biomarker expressed in CMV-positive and negative groups as observed in the current study. If necessary, the prospective study will include multiple centres to shorten the study duration.

9.2.5 Analysis of PD-L1 in FFPE tissue biopsies

As reported in Table 5-2 and Figure 5-9, my study has revealed a marginal increase in the intensity of cells expressing PD1 in the FL microenvironment of the CMV-positive patients compared to the negative (median: 291.3 versus 187.9, p=0.097). It is worthy to recall that the prevalence, location and subtypes of intratumoral T cells can influence tumour immunity either negatively or positively (Yang et al., 2015). Meanwhile, aside from its involvement in the induction of T cell exhaustion, PD-1 has been shown to associate with negative regulation of T-cell receptor signalling, decrease proliferation as well as reduce cytokine secretion in T cells (Riella et al., 2012, Day et al., 2006). Diverse subsets of intratumoral T cells have shown to express PD1 in FL, for instance, exhausted T effector cells and follicular helper T (TFH) cells (Yang et al., 2014, Yang et al., 2012, Schaerli et al., 2000). The PD1/PD-L1 pathway has been explored for cancer immunotherapy due to the importance of this checkpoint to ensures that the immune system is only activated appropriately (Topalian et al., 2012, Chen and Mellman, 2013). This pathway is also susceptible to exploitation by tumour cells to elude detection and repress the immune response (Topalian et al., 2012). Considering the importance of PDL-1 in facilitating PD1 immune response, the determination of the intensity of PDL-1 in the FFPE tissue section from the PACIFICO trial patients by immunofluorescence technique may reveal a potential immune biomarker in FL management.

9.2.6 A study of post-treatment sequential samples

The PACIFICO clinical trial requires clinical follow-up of recruited patients and sequential sample collection. This trial design will support additional investigation of the interrelationships between exposure to the two arms of therapies in the presence or absence of CMV infection and subsequent outcome measures, including treatment efficacy and toxicities(Grossmann et al., 2006, Fraley and Hudson, 2014). This could potentially provide additional data on the impact of the two arms of immuno-chemotherapies, the R-VCP and R-FC on the circulating peripheral immune cells, serum cytokines and infiltrating cells in the tumour microenvironment of CMV-positive patients. This would present a clearer picture of the impact of CMV infection on the clinical behaviour to treatment of FL. Furthermore, the availability of study outcome data will further improve on the analysis of the impact of CMV infection (CMV status) and the treatment arm on overall survival (OS) or progression-free survival (PFS).

9.3 Conclusions

In conclusion, future work has been outlined above. It is planned based on limitations in the current study and possible developments in the near future. It is expected that completion of those plans will further improve the quality of this project and help us to better understand the biology and clinical relevance of CMV infection in FL.

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Appendices

Appendix 1: Standard Curves for cytokines

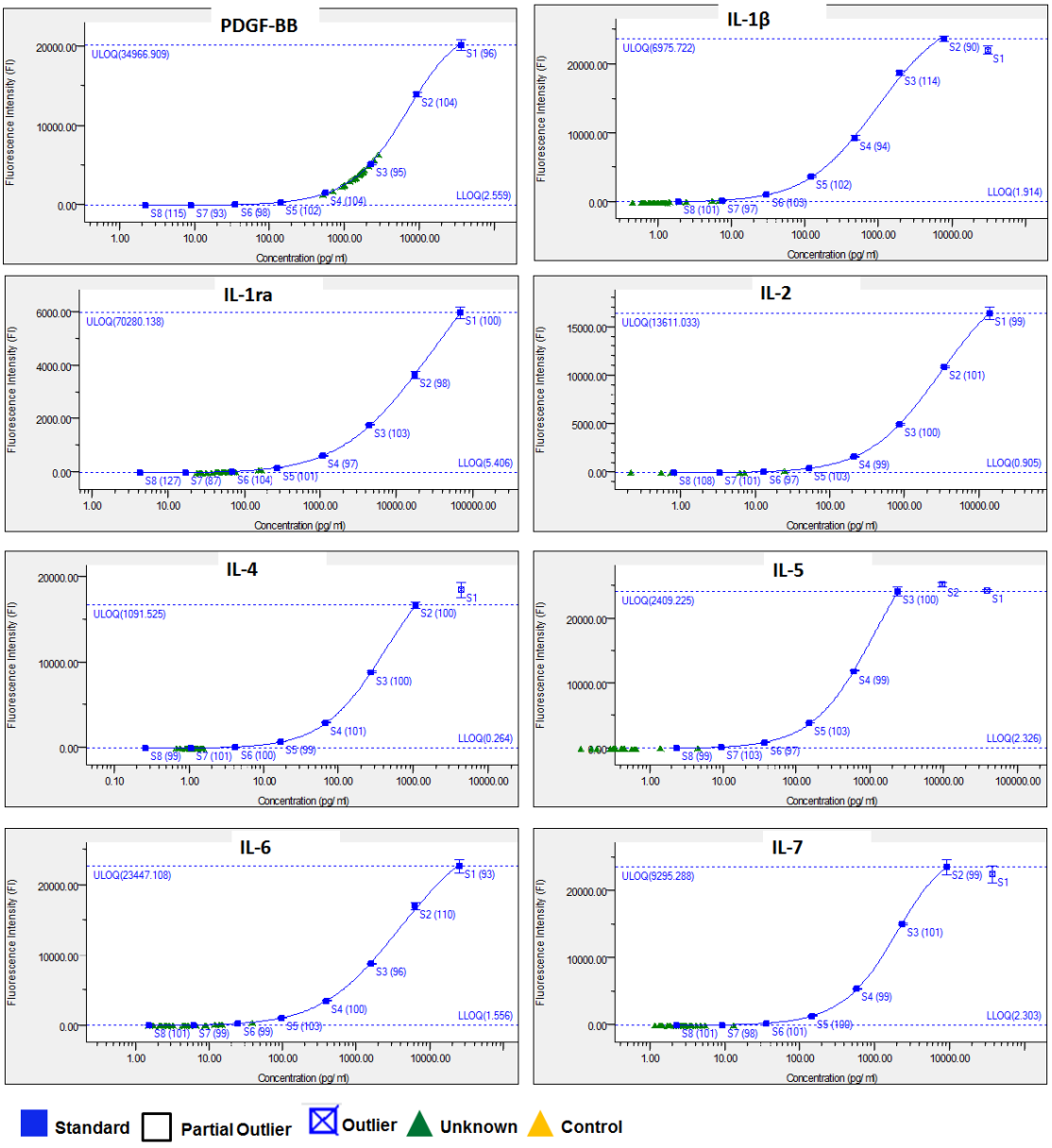

Figure 1A: The 8-point Bio-Plex Manager™ software version 6.0 derived standard curves used for the analysis of serum cytokine by Luminex assay.

In performing curve fitting, outliers are considered invalid. As such they are removed to generate a more realistic and accurate standard curve. This may result in an extended assay working range and allows quantitation of samples that might otherwise be considered out of range (OOR).

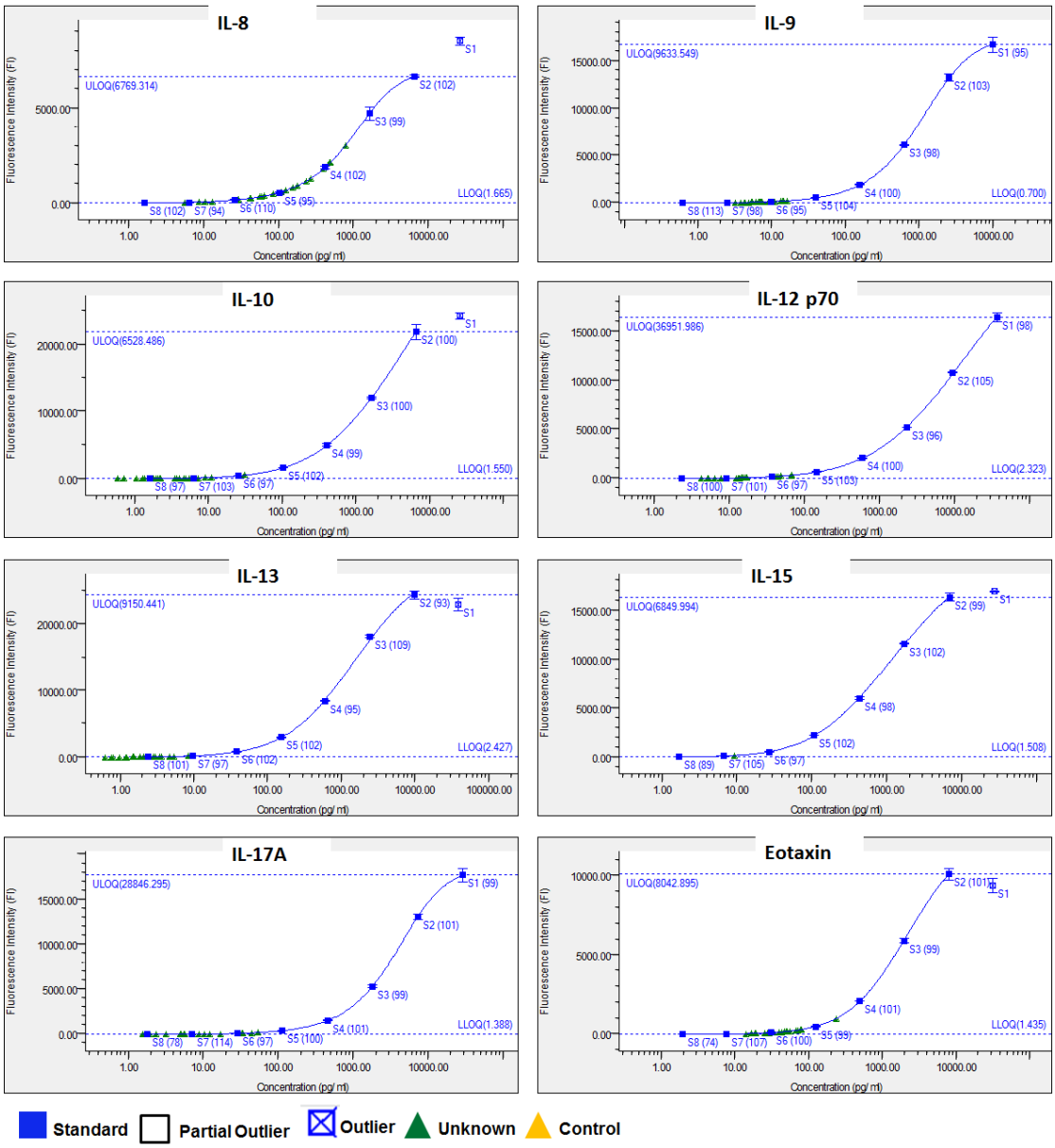

Figure 1B: The 8-point Bio-Plex Manager™ software version 6.0 derived standard curves used for the analysis of serum cytokine by Luminex assay.

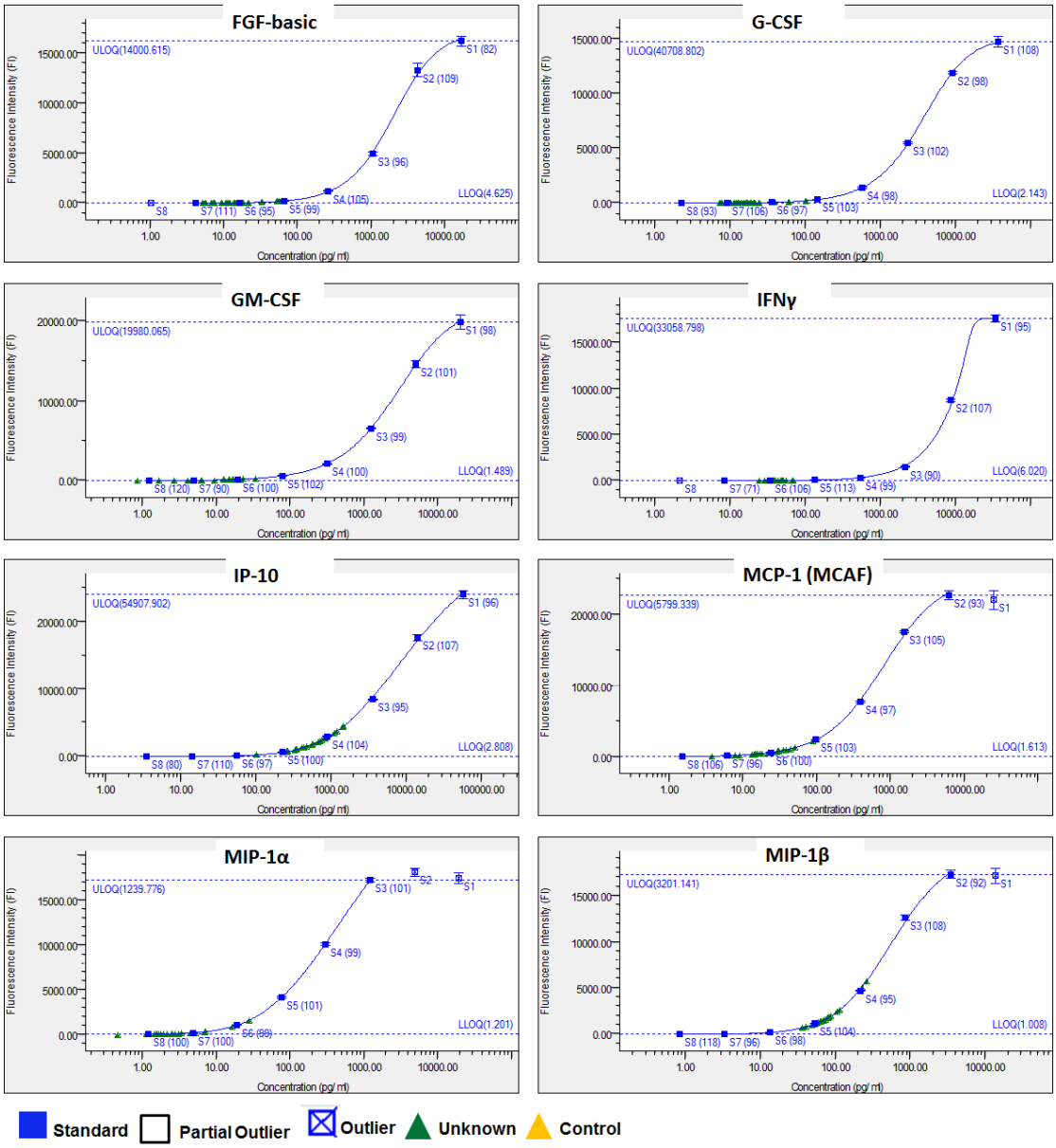

Figure 1C: The 8-point Bio-Plex Manager™ software version 6.0 derived standard curves used for the analysis of serum cytokine by Luminex assay.

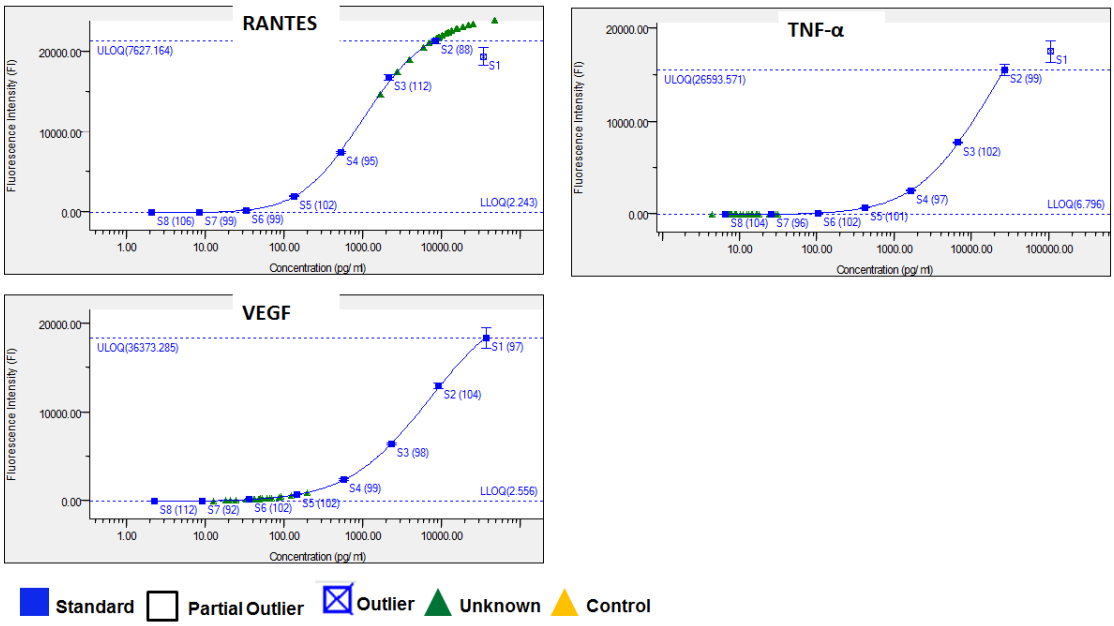

Figure 1D: The 8-point Bio-Plex Manager™ software version 6.0 derived standard curves used for the analysis of serum cytokine by Luminex assay.

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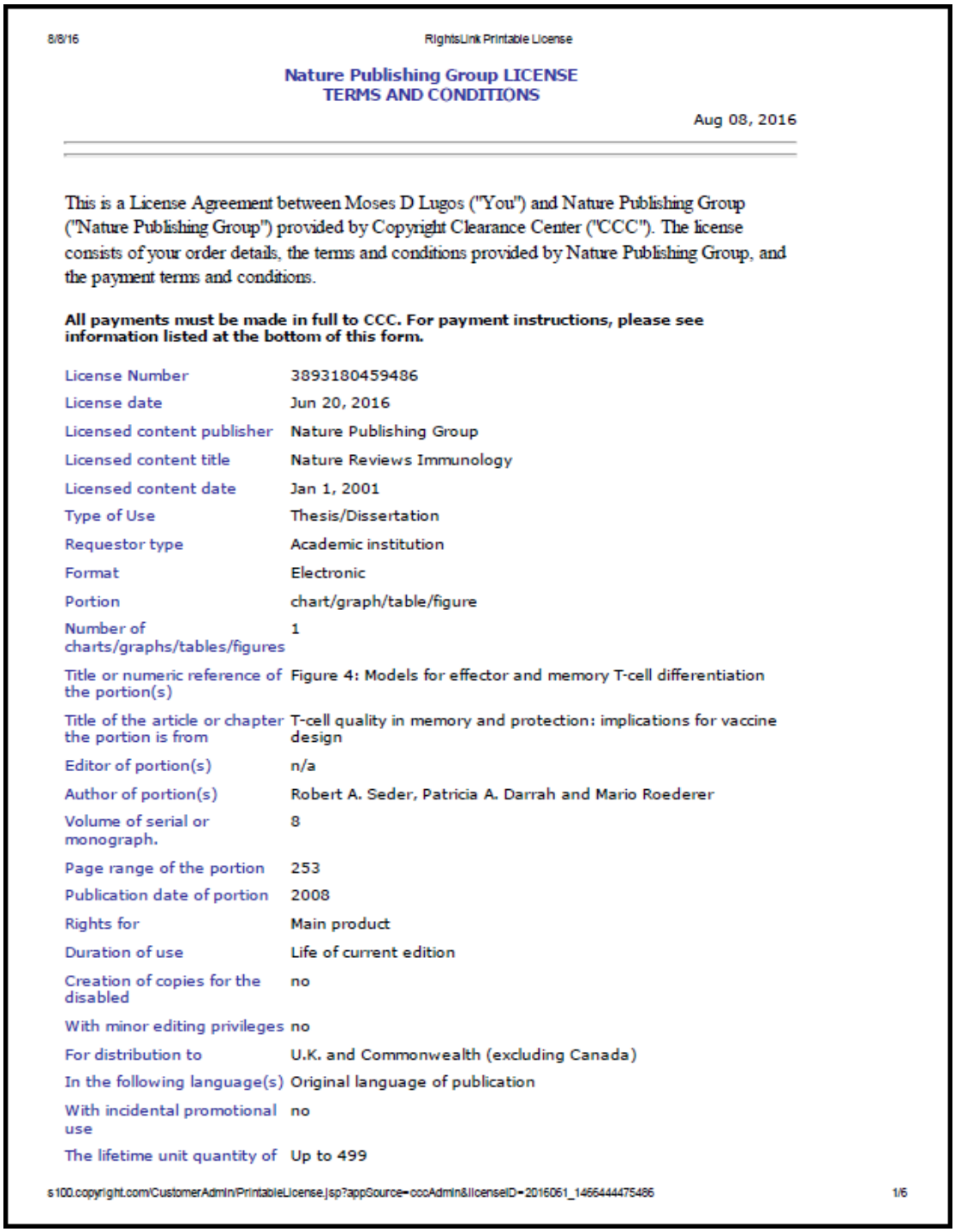

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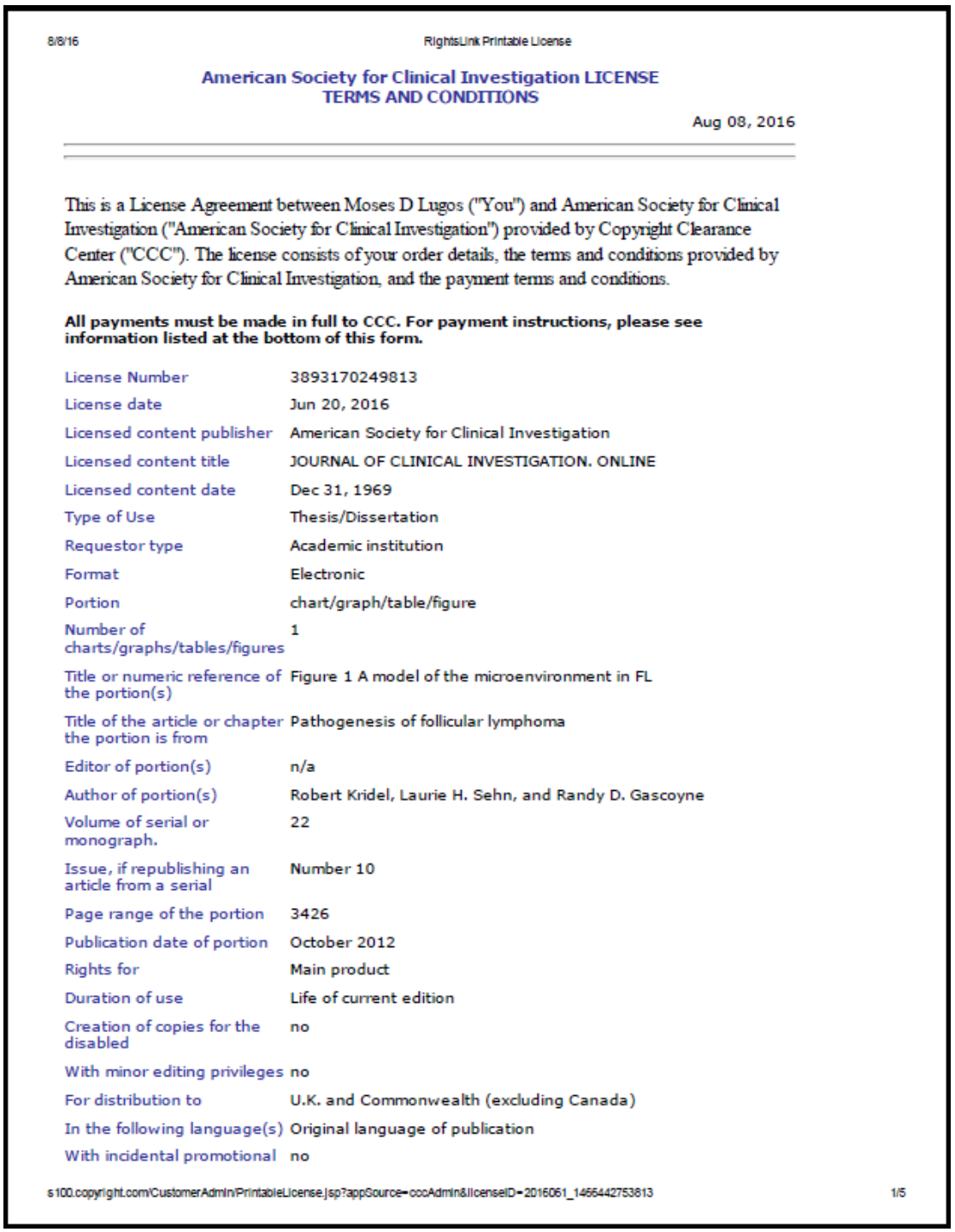

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Title of the article or chapter the portion is from	Pathogenesis of follicular lymphoma
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Author of portion(s)	Robert Kridel, Laurie H. Sehn, and Randy D. Gascoyne
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Figure 3: Copyright licence for Figure 5-10: A model of the FL microenvironment. The page 1 of 5 pages of copy right licence for reproduction Figure 5-10: A model of the FL microenvironment.

Appendix 3A: Uncontrolled copy of the PACIFICO Patient Consent Form-Page1

EudraCT Number: 2008-004759-31 PACIFICO

ON TRUST HEADED NOTE PAPER

PATIENT CONSENT FORM (please read carefully)

PACIFICO: Purine-Alkylator Combination In Follicular lymphoma Immuno-Chemotherapy for Older patients: a phase III comparison of first-line R-CVP versus R-FC lite

Please tick if you do not wish to provide this information ☐

Patient's NHS Number: _____ Patient's Date of Birth: __/___/____

Name of Researcher: _____

	Please initial
1. I confirm that I have read and understand the Patient Information Sheet, dated..... (version.....) for the above study and have had an opportunity to consider the information, ask questions and have had these answered satisfactorily.	☐
2. I understand that my participation in this study is voluntary and that I am free to withdraw at any time without giving a reason, without my medical care or legal rights being affected.	☐
3. I understand that sections of my medical notes may be looked at by responsible individuals involved in this research or from regulatory authorities where it is relevant to my taking part in research. I give permission for these individuals to have access to my records.	☐
4. I understand that my stored diagnostic biopsy samples will be transferred from my local hospital to the University of Liverpool for central pathological review.	☐
5. I give permission for samples of my blood to be transferred to the University of Liverpool and retained for research into follicular lymphoma and its treatment. I understand that these projects may be conducted both within and outside of the European Union.	☐
6. I give permission for part of my biopsy specimen to be retained at the University of Liverpool for research into follicular lymphoma and its treatment. I understand that the research may be conducted both within and outside of the European Union.	☐
7. I give permission for DNA to be extracted from my blood and biopsy samples and for it to be used for genetic research, relating to follicular lymphoma and its treatment.	☐
8. I give permission for a copy of my consent form to be sent to the Liverpool Cancer Trials Unit (where it will be kept in a secure location), to provide confirmation that consent was given.	☐
9. I agree to allow my General Practitioner and any other relevant medical practitioner to be informed of my involvement in the study.	☐
10. I understand that information held by the NHS and records maintained by the NHS Information Centre may be used to keep in touch with me and follow-up my health status.	☐
11. I agree for my data on NHS hospital admissions to be collected from electronic routine NHS health care records for the purpose of this study.	☐
12. I agree to take part in the above study.	☐

PACIFICO - Patient Consent Form
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Appendix 3B: Uncontrolled copy of the PACIFICO Patient Consent Form-Page2

Name of Patient	Date	Signature
Name of person taking consent (If different from researcher)	Date	Signature
Researcher	Date	Signature

1 copy is for patient, 1 for researcher; 1 to be kept in the hospital notes.