IMMUNE MEMORY AND ACTIVATION MARKERS IN SYSTEMIC LUPUS ERYTHEMATOSUS

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Abstract

Systemic Lupus Erythematosus (SLE) is a chronic inflammatory autoimmune disease characterized by the loss of tolerance to self-antigens, immune complex deposition, tissue inflammation and destruction. SLE manifestations, prevalence and severity vary among different populations and ethnicities. Even today, the pathogenesis of SLE remains unclear. This complex autoimmune disease is more common in Asians (46.7/100000) than in Caucasians (20.7/100000). Female preponderance in SLE, especially during childbearing years is of an overall female: male ratio of about 9:1. With advances in SLE management via various therapeutic agents, the survival rate of the SLE population has increased compared to those of early days. But complications arising from current available treatment and therapy have propagated as they usually involve the use of toxic immunosuppressive drugs. More patients are getting serious and lethal infections due to the use of these not patient-specific drugs.

A previous study of samples from populations of mainly European ancestry found that transcriptional profiling of purified CD8+ T lymphocytes identifies two distinct prognostic subgroups in SLE, termed v8.1 and v8.2. It was found that more subjects in group v8.1 have shorter time to first flare, increased flare rate, and had increased expression of IL7R and Bcl2. These subgroups raise the prospect of individualized therapy and suggest new potential therapeutic targets in SLE. The purpose of my study was to investigate these and other relevant biomarkers in Asian lupus patients by flow cytometry to potentially allow individualized therapy to reduce the disease severe manifestations.

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Abbreviations

ACA	anti-cardiolipin antibodies
ACR	American College of Rheumatology
ANA	Antinuclear Antibody
ANCA	Anti-neutrophils Cytoplasmic Antibodies
anti-dsDNA	anti- double stranded DNA
anti-Jo1	antinuclear antibodies directed against histidine-tRNA ligase
anti-Scl-70	Anti-topoisomerase antibodies
A647	Alexa Fluor 647
ARA	American Rheumatism Association
APC	Antigen Presenting Cells
APC	Allophycocynin
BILAG	British Isles Lupus Assessment Group
BSA	Bovine Serum Albumin
C3	Complement Component 3
C4	Complement Component 4
CMV	Cytomegavirus
CRP	C-reactive Protein
Cr	Creatinine
EBV	Epstein Barr Virus
ENA4	Extractable Nuclear Antigens 4
ER	endoplasmic reticulum

ESR	Erythrocyte Sedimentation Rate
FACS	Flow Activated Cell Sorting
FITC	Fluorescein isothiocyanate
FMO	Fluorescence Minus One
FoxP3	Forkhead Box P3
HEV	high endothelial venules
IL	Interleukin
IL7R	Interleukin 7 receptor
LAC	Lupus Anticoagulant
MFI	Mean Fluorescence Intensity
MHC	major histocompatibility complex
MMF	Mycophenolate mofetil
NUH	National University Hospital
PB	Pacific blue
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate buffered saline
PC7	Phycoerythrin Cyanin 7
PE	R-Phycoerythrin
PFA	Paraformaldehyde
RA	Rheumatoid Arthritis
RBC	Red Blood Cells
RNA	Ribonuclei acid
SGH	Singapore General Hospital
SLE	Systemic Lupus Erythematosus
SLEDAI	SLE Disease Activity Index

TAP	transporter associated with antigen processing
Tc	cytotoxic T cells
Tcm	Central memory T cells
Tem	Effector Memory T cells
Temra	Revertant Memory T cells
Th	helper T cells
Tn	Naïve T cells
UK	United Kingdom
WBC	White Blood Cells

CHAPTER 1

INTRODUCTION

1.1 GENERAL INTRODUCTION

Systemic Lupus Erythematosus, SLE, is a chronic potentially fatal and debilitating autoimmune disease characterized by the loss of immune tolerance to self antigens, leading to the activation and expansion of autoreactive lymphocytes. The subsequent production of inflammatory mediators and autoantibodies ultimately causes damage to multiple organs. The hallmark of SLE is widespread inflammation, which may affect virtually any organ in the body, from skin and mucosal lesions to severe injuries in the central nervous system and kidney (Avihingsanon and Hirankarn 2010). Its severity in patients, mainly young women in their child-bearing years, can range from mild cutaneous involvement to devastating organ damage that can lead to death. This disease is heterogeneous; its diagnosis is easily confused with many other disorders.

Although Lupus is normally considered a twentieth century disease, initial descriptions probably go as far back as thirteenth century. The physician Rogerius likened the facial rash and skin ulceration to the bites and scratches made by a wolf's attack, from which some think lupus (Latin for wolf) derives its name. In 1948, Hargraves and colleagues discovered the LE cell (a neutrophil or macrophage that has phagocytized the denatured nuclear material of an injured cell, hematoxylin body). Although characteristic features of lupus Erythematosus are also found in other related connective tissue disorders. The LE cell phenomenon was the specific test for the diagnosis of SLE and led to the discovery of other immunological markers. In 1952, immunosuppressive drugs were first introduced to treat Lupus. Derivatives of cortisone, prednisone, and anti-malarial drugs like plaquenil and quinacrine were introduced. To incorporate new immunologic knowledge and improve disease diagnostic

classification, the American Rheumatism Association (ARA), which has now become the American College of Rheumatology (ACR), established in 1982, revised the 1971 preliminary criteria for the classification of SLE (Passas*, et al* 1985) (Tan*, et al* 1982).

The prognosis of SLE has improved over the past 4 decades, including 20-year survival rates. Things have gradually improved and now that SLE patients can live longer. Many patients manage to have a fair quality of life or are able to work full time and even have children. The effect of excessive active lupus disease has reduced but the complications and adverse reactions arising from prolonged therapy with potentially toxic drugs further aggravate these patients. Immunosuppressive drugs such as corticosteroid, prednisone, azathioprine, cyclophosphamide and sodium methotrexate are cytotoxic medications widely used in treating SLE. A common side effect of such drugs is immunodeficiency as the majority of them act non-selectively, resulting in increased susceptibility to infections and decreased cancer immunosurveillance. Zonana-Nacach and colleagues demonstrated that cumulative prednisone dose was significantly associated with osteoporotic fractures, symptomatic coronary artery disease and cataracts (Zonana-Nacach*, et al* 2000). Yet despite these significant advances, the scarcity of novel therapies continues. In light of this, an individualized therapy is needed to reduce the disease manifestations and the side effects of immunosuppressive drugs by the selective use.

The etiology of SLE remains unknown. SLE disease activity can fluctuate greatly with most patients suffering disease flares alternating with prolonged durations of remission. The immune system is broadly compromised in patients with SLE such that deregulation of a single element leads to altered behavior of the whole system. Immune system molecular and

cellular aberrations, as well as heritable or genetic, hormonal and environmental factors interplay in the manifestations and presentations of the disease.

All studies have shown that SLE has a particularly female preponderance, particularly in their childbearing years. Among children, it occurs three times more commonly in females than in males. Of SLE patients who experience onset of their disease between puberty and 40 years of age, the female to male ratio is 9:1. Only 10-15% develop the disease after age 50 when the female to male ratio approaches 4:1. This indicates that female hormones are likely to play a crucial role in the development of the disease.

There is a wide disparity of lupus prevalence rate among different regions, ethnic influence between geographical populations further confounds patient morbidity and mortality. Studies performed in the UK suggest that SLE is more prevalent among Asian (40-48.8/100,000) and Afro-Caribbean (207/100,000) compared with Caucasian Americans or British (20/100,000). In addition, clinical manifestations presented vary within ethnicity. Among Asian patients, musculoskeletal and cutaneous involvements are the two most common features, meanwhile leukopenia is the most common hematologic abnormality observed. Less common manifestations presented are discoid rash (less than 20% of Asian patients), serositis and neurologic features (less than 40% of Asian patients). Renal involvement, often a cause of significant morbidity, is common at onset and throughout the course of disease for more than 50% of Asian patients. Nephritis is also a concern, as it affects more than half of Asian patients with lupus. Nephritis is found in 10-40% of Caucasian populations (Spanish, Puerto Rican, European and American), meanwhile it is reported that Asian patients are 40-70% nephritis positive. The reported prevalence of renal disease involvement in lupus patients was

27.9% (Cervera*, et al* 2006) in Caucasians and 64-69.3% in Asians (Boey*, et al* 1988, Lee*, et al* 1993). The Asian cohort was reported to have predominantly proliferative glomerulonephritis on renal biopsy, leading to an increased risk of end-stage renal disease (Shayakul*, et al* 1995, Williams*, et al* 2003).

The above statistics suggest that the Asian cohort, comparing to Caucasians, has a higher prevalence of major organ involvement and present more severe and lethal SLE manifestations. As such, we expect to see a different and more severe prognostic pattern in Asian cohort by using flow cytometry to investigate the T memory subsets in CD4 and CD8 T lymphocytes.

1.2 Innate and Adaptive Immunity

SLE is a long-term autoimmune disorder, in which the immune system produces an inappropriate or abnormal response against its own cells, tissues and organs, resulting in inflammation and damage. As such, it is important to understand how the immune system works. The physiologic function of the immune system is to protect the body from infection. The system is divided into two principal branches: the innate immune system and the adaptive immune system. Innate immunity, also called natural or native immunity, provides the initial defense against infections. Based on the broad recognition of molecular patterns, it is nonspecific as to the type of organism it fights and is ready to be mobilized upon the first signs of infection. The main components of innate immunity consist of:

a) Physical and chemical barriers such as skin, mucosal epithelia and antimicrobial chemicals produced at epithelial surfaces.

b) Blood protein, including complements and other mediators of inflammation
c) Phagocytes (macrophages, neutrophils) and NK cells.
d) Cytokines which regulate and coordinate activities of cells of innate immunity.

In contrast to innate immunity, adaptive immunity (also called acquired immunity or specific immunity) develops later and involves lymphocytes and their products. It launches attacks specific to the invading pathogen and "remembers" antigens it has encountered and responds more vigorously and efficiently to repeated exposure to the same antigen. There are two types of adaptive immune responses, termed humoral and cell-mediated immunity.

1.2.1 Humoral Immunity

The humoral response begins with the activation phase, when a dendritic cell engulfs an extracellular antigen or microbe, by phagocytosis. Inside the cell, the new vesicle is now called phagosome. The phagosome then fuses with the lysosome, which contains digestive enzymes to degrade the endocytosed particle into fragments, in a phenomenon called antigen processing. Within the antigen presenting cell, the fragments then combine with class II MHC proteins. The complex is then displayed on the cell's plasma membrane in the process known as antigen presentation. Macrophages, dendritic cells and B cells are considered antigen presenting cells (APC). A helper T cell (Th) participates in the next stage of the humoral immune response. This Th cell has T cell receptors that can bind the class II MHC proteins presented antigen complex. This binding triggers the APC to release IL1, which activates the Th cell. The activated Th cell now releases its own cytokines, which stimulate

the Th cell to proliferate to form a clone of Th cells, all with the same T cell receptors specific for the antigenic determinant of the original processed antigen.

The B cell has membrane IgM receptors that are weakly specific for the same antigen as originally engulfed by the APC. An IgM receptor binds to the antigen and the cell engulfs the complex by receptor-mediated endocytosis. The B cell now behaves like an APC, processing and then presenting the antigen on class II MHC on the cell surface. The internalized vesicle fuses with a lysosome, which contains digestive enzymes. The enzymes then digest the antigen, processing it into fragments which are later attached to class II MHC molecules and displayed on the surface of the B cell. A Th cell from the clone of Th cells can now bind to the antigen displayed on the B cell. The T cell receptor specifically recognizes the antigen on the class II MHC protein. Upon binding, the Th cell releases cytokines that stimulate the B cell to divide and create a clone of identical cells. Engagement of CD40 on the B cell to CD40L on the Th cell leads to immunoglobulin class switching.

The resulting B cells develop into either long-lived memory cells or into antibody-secreting plasma cells. Plasma cells have an extensive endoplasmic reticulum and numerous ribosomes. Plasma cells are essentially antibody factories, they produce and secrete antibodies of the specificity identical to that of the surface receptors on the parent B cell. Like the surface IgM receptors on the parent B cell, the antibodies secreted by the plasma cells can bind to and inactivate the original antigen.

By the end of the humoral response, the immune system has activated specific B cells, which produce and release large amounts of specific antibodies. Of the millions of different B cells produced by the immune system, only those that can recognize the invader with the highest specificity survive. This specificity prevents the body from making all types of antibodies possible, which would very likely harm the body, in addition to being energetically costly.

Antibodies defend the body in a number of ways. For example, if the antigen is a toxin or a virus, the binding of the antibodies to the antigens isolates the antigen, preventing them from contacting and harming cells of the body. Additionally, antigens which are coated with antibodies are easily recognized by macrophages, engulfed and digested. Antibodies also stimulate the complement system, which consists of a group of proteins that can permeabilized the cell wall of bacteria, thereby killing them and generate proinflammatory molecules such as C3a and C5a which are anaphylatoxins.

1.2.2 CD8 T Cell-Mediated Immunity

In the CD8 T cell-mediated immune response, immune cells kill endogenous pathogens like stromal cells that are cancerous or have been infected with viruses. This reaction depends on the lethal talents of the cytotoxic T cells (Tc). Tc cells contain perforin molecules which are released onto target cells and make holes in the membranes and thereby kill them. This cell-mediated immune response occurs in two stages. In the first, called the priming phase, Tc cells that have specific T cell receptors are activated and triggered to proliferate repeatedly.

In the second stage which is called the effector phase, these activated Tc cells encounter target cells in the periphery and eliminate them.

The cell- mediated immune response begins when an antigen, such as a virus, enters a cell. Note that tumor cells can also stimulate the same cellular immune sequence. During the infection, some of the viral proteins are degraded by the proteosomes into peptide fragments. These peptides are then transported to the endoplasmic reticulum (ER) by transporter associated with antigen processing (TAP). In the ER, the peptides are attached to class I Major Histocompatibility Complex (MHC) proteins, they are bound to the extracellular part of the class 1 MHC molecule. These complexes of antigens and class I MHC proteins are then inserted into the plasma membrane of cells and presented on the cell surface. A cytotoxic T cell (Tc) which has T cell receptors specific for the displayed antigen binds to the complexes of antigens and class I MHC proteins. This proliferates and forms clones of Tc cells, each with T cell receptors specific for the same antigenic determinant.

In the effector phase, these Tc cells can now encounter and kill other infected stromal cells. Infected body cells present the viral antigens on their class I MHC proteins. Tc cells from the activation phase, each with receptors specific for the viral antigen, bind to these class 1 MHC:peptide complexes. Upon binding, a Tc cell is activated to release perforin molecules which generate holes in its plasma membrane, causing the cell to lyse (**Figure 1.1(a)**).

Naïve antigen-specific CD8+ T cells have limited recirculation pattern which does not allow them directly eliminate transformed or infected cells. These naïve T cells recirculate

throughout the secondary lymphoid compartment, migrating between lymph nodes, blood and spleen. To become effector cytotoxic T lymphocytes, naïve CD8+ T cells depend on professional Antigen Presenting Cells (APC) to capture pathogen from site of infection, transport them to the draining lymph nodes and scan the antigens presented by these APCs (which are mainly Dendritic Cells, DCs). The co-stimulatory molecules expressed by these DCs activate these naïve CD8+ T cells, causing them to proliferate and differentiate, and are then able to enter peripheral tissues to fight the invading pathogen. When APCs are not directly infected, they need to acquire exogenous antigens from the infectious agent and present them on MHC class I molecules, by a mechanism known as cross-presentation (**Figure 1.1 (c)**).

1.2.3 Helper T cells

Helper T cells are a subset of CD4 T lymphocytes that provide help to other effector cells such as B lymphocytes and cytotoxic T lymphocytes. Four types of Th cells have been identified so far: Th1, Th2, follicular helper T cells and Th17.

Like all T cells, Th cells arise in the thymus where they express CD4 and CD8. When these cells lose CD8, they are called mature Th cells. Once they are presented with both an antigen and appropriate cytokines, they start to proliferate and become activated (**Figure1.1 (b)**). They are dependent on the type of antigen-presenting cells, cytokines and transcription factors to determine which type of Th cells they become.

When dendritic cells, DCs, present antigen to the Th cell's receptor and secrete IL-12, IL-18 and IFN-γ, Th1 cells are produced. The paracrine stimulation by these cytokines causes the Th1 to secrete their own lymphokines like TNF-β (lymphotoxin) and IFN-γ. These lymphokines stimulate macrophages to kill engulfed bacteria, recruit other lymphocytes to the site of inflammation, and stimulate B cell class switching. Transcription factor T-bet plays a critical role in Th cells commitment to become Th1 as it regulates the genes needed for Th1 function.

Th2 cells are produced when APCs present antigens to TCR with the paracrine stimulant interleukin 4 (IL-4). Th2 cells express GATA-3 and secrete IL-4, IL-5 and IL-13. IL-4 plays a major role in stimulating B cell class-switching and promotes IgE antibody production by B cells. IL-4 also blocks IFN-γ receptors from entering the immunological synapse on pre Th cells, thus preventing them from entering the Th1 pathway. Besides, IL-4 also acts as a positive feedback lymphokine to promote more Th cells to enter Th2 pathway. Meanwhile IL-5 attracts and activates Eosinophils. IL-13 recruits and activates basophils, and also promotes the synthesis of IgE antibodies.

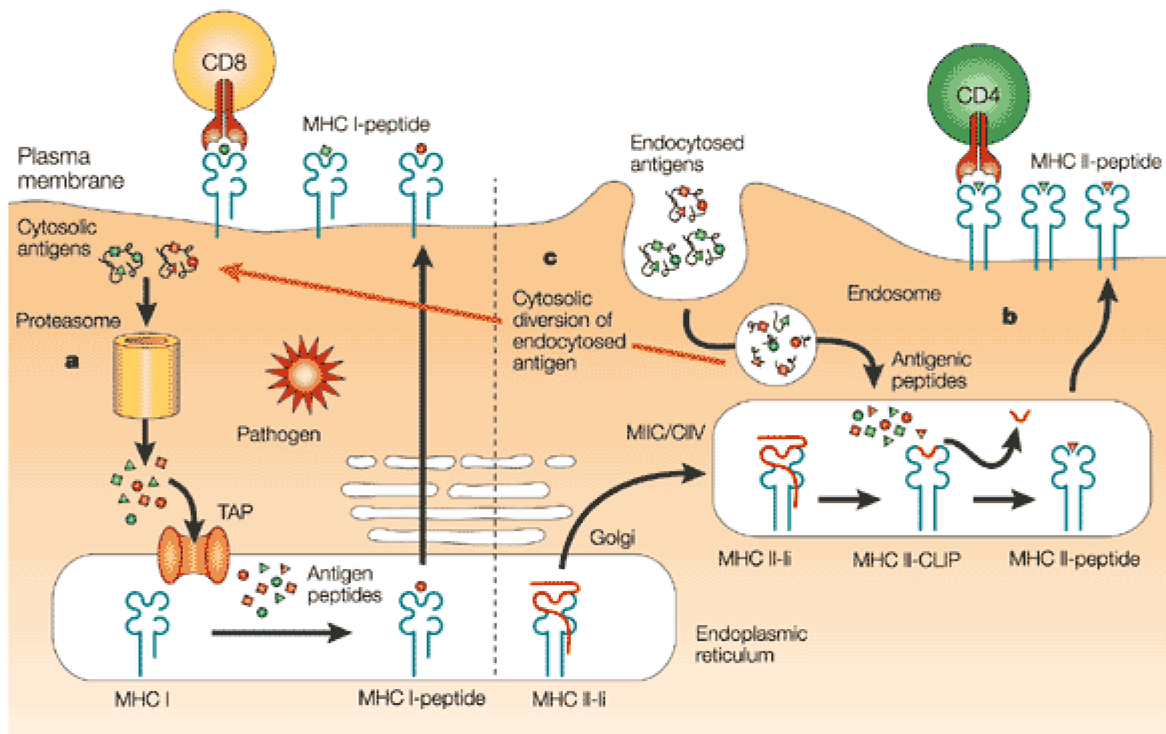

Figure 1.1 shows the schematic diagram of MHC class II protein presentation (a), MHC class I peptide presentation (b) and Cross Presentation by APCs (c). (William R. Heath & Francis R. Carbone 2001)

1.3 Memory T cells

Memory is the hallmark of the acquired immune system. Antigenic stimulation of naïve T cells is a requirement to generate memory T cells. Naïve T cells migrate to secondary lymphoid organs in search of antigen presented by antigen presenting cells (Butcher and Picker 1996). Upon exposure to a foreign antigen, primed antigen-specific T lymphocytes proliferate vigorously and exponentially, differentiating into effector cells which can travel to the inflamed tissues (Mackay 1993). The vast majority of these effector T cells undergo

apoptosis as the immune response progresses. They fail to survive as they fail to acquire the cardinal features of memory cells (**Figure1.2**). Expression of anti-apoptotic molecules and responsiveness to homeostatic cytokines are the key properties acquired progressively as the strength of antigenic stimulation is increased (Gett*, et al* 2003) . The few T cells that survive persist as long-lived circulating memory cells that can confer a more rapid protection upon secondary stimulation (Ahmed and Gray 1996, Dutton*, et al* 1998, Sprent and Surh 2002).

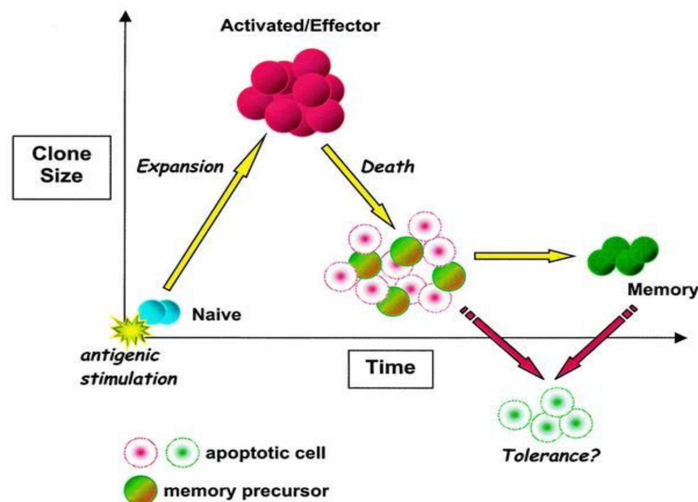

Figure 1.2 Antigenic stimulation triggers T naïve cells to proliferate and differentiate into effector cells. Majority of the effector T cells undergo apoptosis after the antigen clearance but a small proportion of them survive as long-lived T memory cells. Immunogenic tolerance is define as the demise of these antigen-specific memory T cells (*Lakkis and Sayegh 2003*).

Memory T cells have several inherent advantages over their naïve counterparts:

1. The Memory T cell response to foreign antigens is faster and greater in magnitude than for naïve T cells (Bachmann*, et al* 1999, Garcia*, et al* 1999, Rogers*, et al* 2000, Veiga-Fernandes*, et al* 2000).
2. Antigenic-specific memory T lymphocytes can persist for a lifetime in the absence of antigen and MHC molecules (Freitas and Rocha 1999, Goldrath and Bevan 1999,

Hammarlund*, et al* 2003, Mullbacher 1994, Murali-Krishna*, et al* 1999, Swain*, et al* 1999).

3. Memory T cells circulate through both secondary lymphoid tissues and peripheral non-lymphoid tissues (Chalasani*, et al* 2002, Masopust*, et al* 2001, Reinhardt*, et al* 2001). Memory T cells can directly encounter foreign antigens and mount an immune response within non-lymphoid tissues. This enables them to detect and destroy foreign pathogens.

Memory T cells are heterogeneous in terms of both their homing capacity and effector function. CD45 isoforms has been widely used to define naïve and memory T cells. Naïve T cells are held to be CD45RA positive and memory T cells to be CD45RA negative. CD62L, a homing receptor which is also called L-selectin, is required for cell extravasation through high endothelial venules (HEV) and migration to T cell areas of secondary lymphoid organs. In humans, co-expression of CD62L and CD45RA (marker of naïve T cells) distinguish 4 subsets of memory T cells: T naïve (CD45RA+CD62L+), T central memory (CD45RA-CD62L+), T effector memory (CD45RA-CD62L-) and T revertant memory (CD45RA+CD62L-).

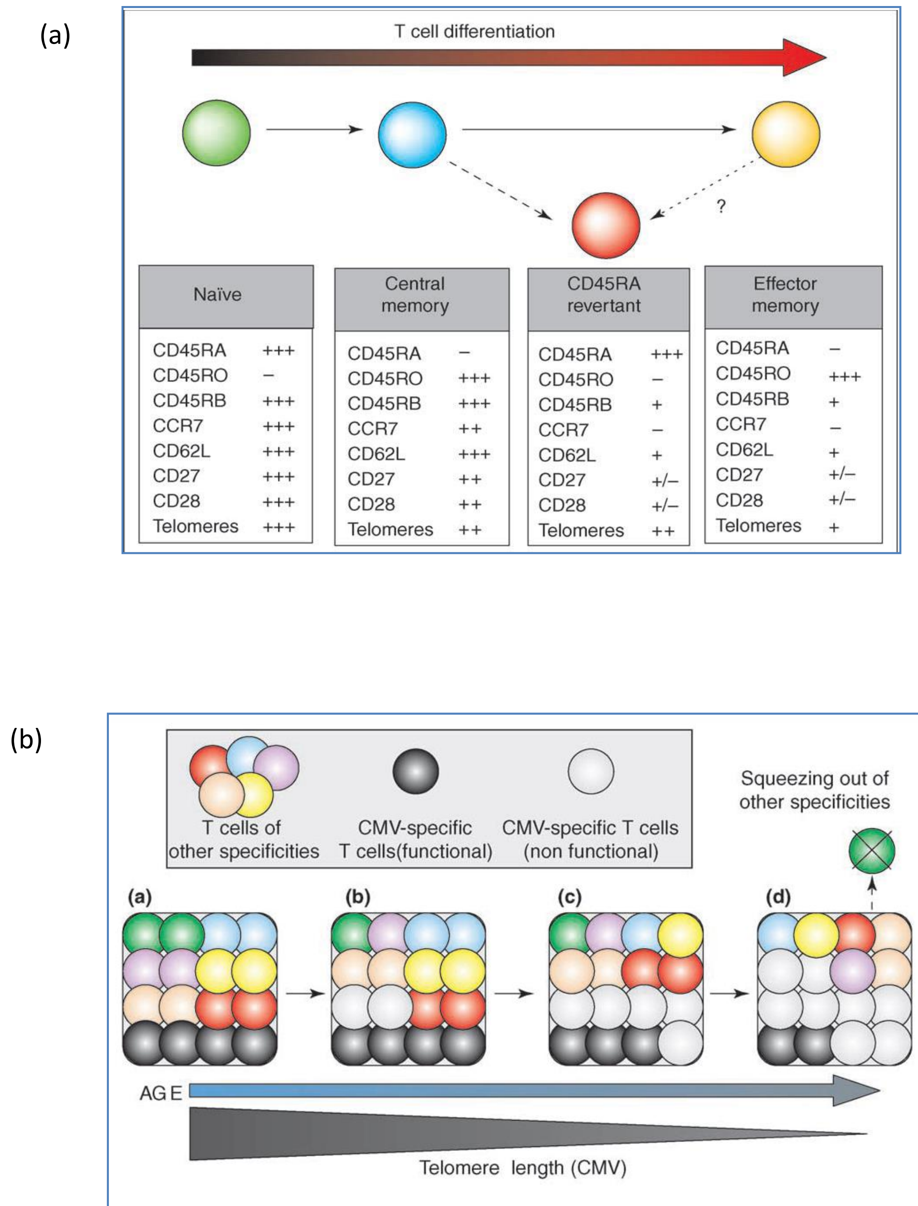

Figure 1.3 T cell differentiation and biological space competition in CMV-specific T cell pool. (a)Schematic of T cell differentiation (b) Effect of vigorous CMV-specific T cell differentiation and accumulation of non-functional CMV-specific cells minimize the available space for other specific T cells *(Akbar and Fletcher 2005*).

T revertant memory cells, which include CMV-and EBV-specific cells, are an intriguing subset that re-expresses CD45RA. They have higher frequencies among CD8+ than CD4+ T cells. Under the influence of IL-15, they can be induced to proliferate, are not an end stage subset and are resistant to apoptosis (Dunne*, et al* 2005, Dunne*, et al* 2002, Geginat*, et al* 2003). Dunne and colleagues were the first to address the functional significance of the reversion of memory CD8+ T cells to the CD45RA phenotype (Dunne*, et al* 2005), they also show that these cells do not need to proliferate for effector function. Revertant T memory cells were found to have similar telomere length to the T cm cells, to function poorly and are increased in elderly subjects. Accumulation of this non-functional population reduces the immunological space for T cells of other specifities, which are lost through competition (Almanzar*, et al* 2005) (**Figure 1.3**). In elderly subjects, this population is highly differentiated and drives the immune pool to replicative senescence.

During the secondary immune response, memory T cells proliferate and differentiate into effector T cells much more vigorously and rapidly than naïve T cells. Lakkis and Sayegh reported that upon antigenic restimulation, virus-specific CD8+ memory T cells take an average of 12 hours to multiply and differentiate into cytotoxic T lymphocytes, as opposed to several days for their naïve counterparts (Lakkis and Sayegh 2003). Furthermore, the number of effector T cells generated during a recall response is fivefold more than a primary immune response (Opferman*, et al* 1999). But what are the factors which cause naïve T cells to differentiate into either effector or memory cells upon primary antigenic stimulation?

Lanzavecchia and Sallusto demonstrated that strength of antigenic and cytokine stimulation drives progressive T cell differentiation (Lanzavecchia and Sallusto 2002). Proliferating T

cells receive different levels of stimulation and thus reach different levels of differentiation (**Figure 1.4**). The magnitude of the signals that they receive is an integration of TCR, co-stimulatory molecules and cytokine receptors signals. At increasing magnitude of antigenic stimulation, responding T cells gradually acquire the capacity to respond to homeostatic cytokines, anti-apoptotic molecules and effector functions and tissue homing receptors, meanwhile losing the lymph node homing marker, proliferative potential and activating their IL-2 producing capacity (Lanzavecchia and Sallusto 2005).

After antigen clearance, activated T cells are selected for their capacity to survive in the presence of cytokines. Those that fail to acquire the cardinal features of memory cells which are defined by expression of anti-apoptotic molecules and responsiveness to homeostatic cytokines die by neglect. Whereas the fit cells home to appropriate tissues and survive as Tcm or Tem cells.

Tcm home to lymph nodes and have limited effector function but upon secondary challenge they proliferate and become effector cells. Tcm are involved in the secondary response and long term protection, they might behave as memory stem cells capable of self-renewal while continuously generating effector cells that contribute to maintain the Tem pool (Lanzavecchia and Sallusto 2002). By contrast Tem are involved in immediate defense, have limited proliferation capacity, home to peripheral tissues and rapidly produce effector cytokines upon antigenic stimulation. Newly generated memory T cells have to compete with pre-existing cells for survival which depends on intrinsic properties (expression of anti-apoptotic molecules and cytokine receptors) and available space (Di Rosa and Santoni 2003). It was reported that CD4+ Tem proliferate (4.7%) faster than Tcm (1.5%) and T naïve cells (0.2%)

(Macallan*, et al* 2004). This suggests that these memory T cells, particularly Tem have rapid turnover rate and require continuous replenishment.

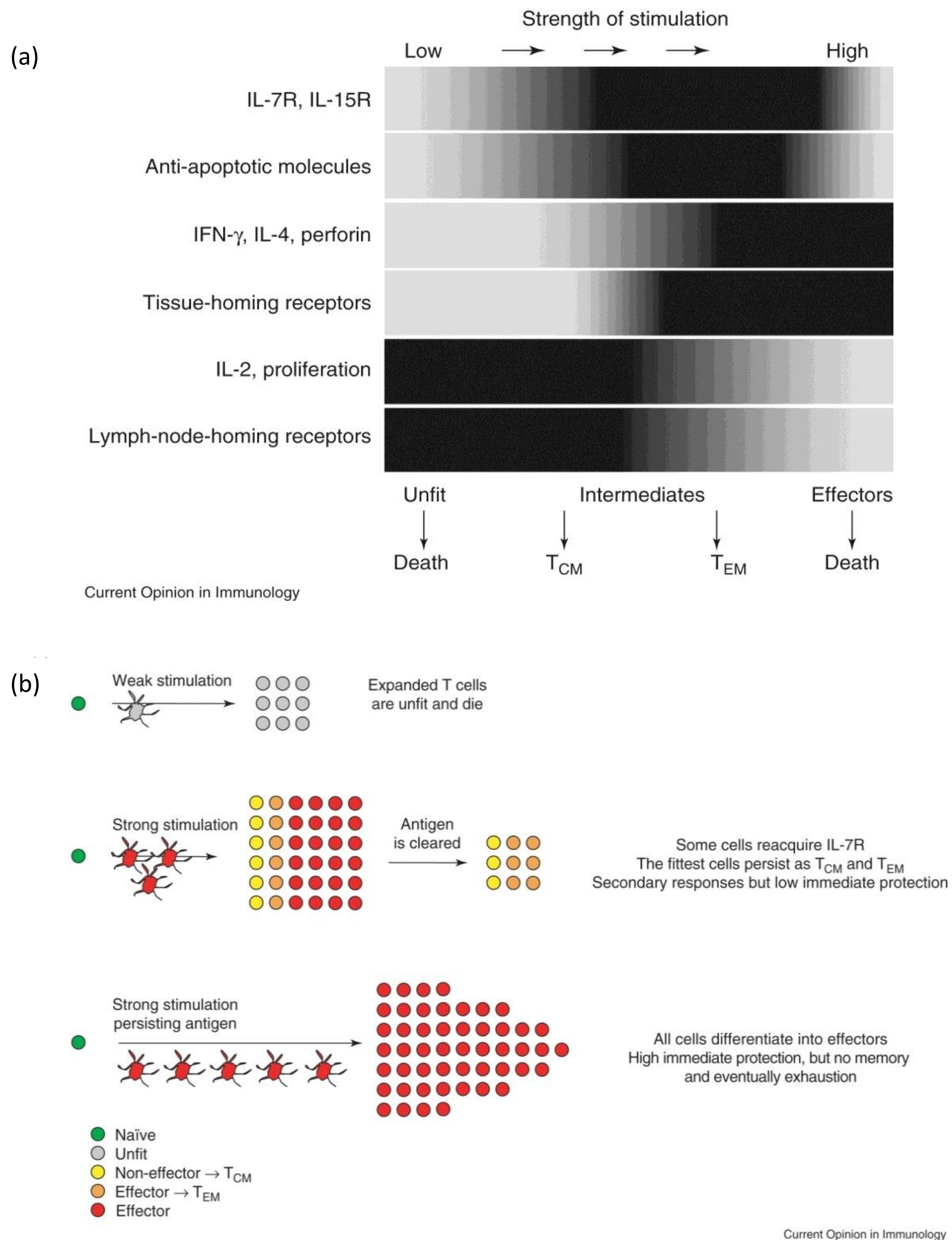

Figure 1.4 Effect of signal magnitude and antigenic stimulation signals to cell proliferation. (a) Different signal magnitude leading to progressive T cell differentiation. (b) Responses of different levels of antigenic stimulation signals which drive different levels of specific T cell proliferation and differentiation *(Lanzavecchia and Sallusto 2005*).

1.4 Aims and Objectives

McKinney and colleagues studied two autoimmune diseases, ANCA-associated vasculitis and SLE in a UK population in which they identified gene-expression patterns based biomarkers that facilitate the clinical diagnosis of these patients. Transcriptional profiling of purified CD8+ T lymphocytes predicts two distinct prognostic subgroups in SLE, termed v8.1/ v4.1 and v8.2/ 4.2 (Lyons*, et al* 2010, McKinney*, et al* 2010).

It was found that subjects in the poor prognostic group v8.1/4.1 have a shorter time to first flare and increased flare rate per month. The subset of genes defining the poor prognostic group v8.1/4.1 is enriched with genes involved in IL7R pathway and TCR signaling and those that are expressed by memory T cells. The poor prognostic subgroup v8.1 is also associated with higher frequencies of T memory cells (Tcm and Tem), as shown in the Figure 1.5. These subgroups are also found in the normal healthy population. They also had increased expression of IL7R and Bcl2. Bcl-2 (B-cell lymphoma 2) is defined as the founding member of the Bcl-2 family of apoptosis regulator proteins encoded by the BCL2 gene. IL7R has been shown to play a critical role in the V(D)J recombination during lymphocyte development (McLeod*, et al* 2011). This protein is also found to control the accessibility of the TCR gamma locus by STAT5 and histone acetylation.

Patients in subgroup v8.1/4.1 may benefit from intensified maintenance therapy and follow-up. While 75-80% of patients in v8.2/4.2 may need less maintenance therapy reducing treatment-associated toxicity. By identifying these subgroups as prognostic indicators, SLE patients' severe manifestations could be predicted and raise the prospect of individualized toxic immunosuppressive therapy and may suggest new potential therapeutic targets in SLE.

In collaboration with Mckinney and colleagues, the purpose of my study is to investigate these and other relevant biomarkers in Asian lupus patients by flow cytometry to potentially allow individualized therapy to reduce the severe disease manifestations. Such study by flow cytometry as a mean to identify prognostic subgroup of SLE patients is novel and original. Besides investigating expression of Bcl2 and IL7R in T memory populations, expressions of CD25 and CXCR6 are also studied in the T memory populations of our Singapore cohort. Association of these prognostic groups with T regulatory cells, monocytes, neutrophils, plasma B and memory B cells were also investigated in my study.

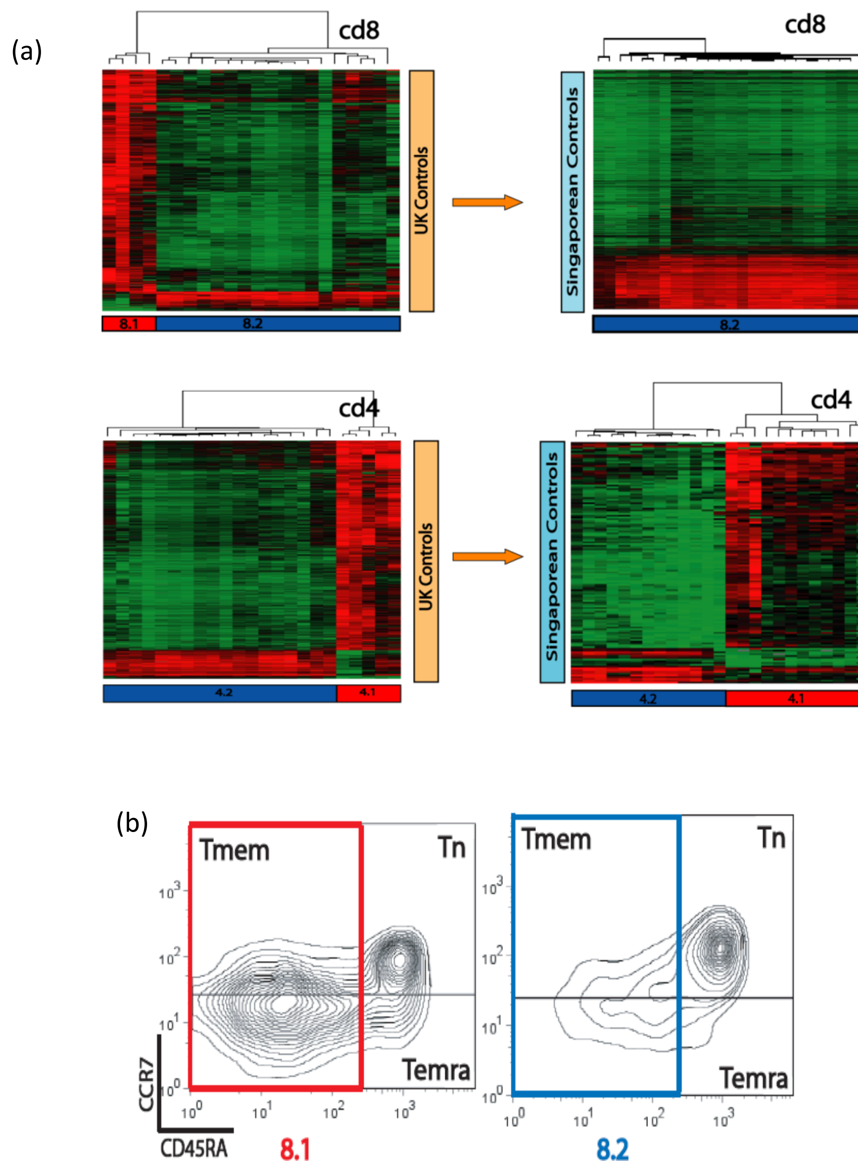

Figure 1.5 Miroarray and flow cytometry data profiles of healthy and SLE cohorts. (a) Microarray profiles performed in Cambridge of CD8 (a) and CD4 (b) T populations for Singaporean (n= 136) and UK (n=718) healthy cohorts. Unsupervised hierarchical clustering was performed using uncentered correlation distance metric with average linkage. Red bar signifies prognostic group v8.1/v4.1 meanwhile blue bar means prognostic group v8.2/v4.2. It is clearly shown here that Singaporean cohort has a homogenous distribution of v4.1 and v4.2 prognostic groups. Meanwhile UK cohort is more inclined to v4.2. (b) Poor prognostic group v8.1 is associated with higher frequencies of T memory cells (Tcm and Tem), as presented in the contour plot of CCR7 (homing marker) versus CD45RA *(McKinney, et al 2010*).

CHAPTER 2

MATERIALS AND METHODS

2.1 SLE Patient Recruitment

Local Systemic Lupus Erythematosus (SLE) patients were recruited from two hospitals in Singapore: Singapore General Hospital (SGH) and National University Hospital (NUH). Both hospitals receive nationwide referrals from general practitioners and specialists. Informed consent forms were disseminated to patients, with one-to-one explanation of the objectives and aims of the study.

Patients who fulfilled at least four of the 1997 American College of Rheumatology (ACR) revised criteria for the classification of SLE (Table 2.1), serially or simultaneously, during any interval of observation were recruited in the study. A maximum of 50ml of peripheral blood was collected from each patient, depending on the patient's health conditions on the date of blood collection. Personal bio-data recorded include name, age, gender, ethnic group, date of birth, disease duration, renal biopsy classification, date of diagnosis and date of blood taken. Medications taken on the date of blood collection were recorded: Prednisolone, Azathiopine, Cyclophosphamide, Hydroxycholoquine, Methotrexate, MMF and Rituximab. Blood tests completed were Creatinine (Cr), C-reactive Protein (CRP) , Erythrocyte Sedimentation Rate (ESR), White Blood Cells (WBC), neutrophil count, lymphocyte count, C3 (Complement Component 3) and C4 (Complement Component 4). Presence of autoantibodies such as ANA (Antinuclear Antibody), anti-dsDNA (anti- double stranded DNA), Extractable Nuclear Antigens 4 (ENA4, which include anti-Ro, anti-La, anti-Sm and anti-RNP), anti-Scl-70 (Anti-topoisomerase antibodies, also referred as Anti-topo 1), anti-Jo 1 (antinuclear antibodies directed against histidine-tRNA ligase), ACA IgM (anti-cardiolipin antibodies directed against IgM), ACA IgG (anti-cardiolipin antibodies directed against IgG) ,

LAC (Lupus Anticoagulant) were included in the test too. Titres of ANA, anti-dsDNA, ACA IgM and ACA IgG for each patient were noted too. Clinical measure of disease activity was assessed using the British Isles Lupus Assessment Group (BILAG) score (**Table 2.2**). BILAG consists of 86 questions grouped under 8 headings including general, mucocutaneous, neurological, musculoskeletal, cardiovascular & respiratory, vasculitis, renal and haematological details.

In this study, a total of 25 subjects were investigated: 19 of them were SLE patients from Singapore General Hospital, 3 patients from National University Hospital and the remaining 6 were healthy donors.

	Criterion	Definition
1	Malar rash	Fixed erythema, flat or raised, over the malar eminences, tending to spare the nasolabial folds
2	Discoid rash	Erythematous raised patches with adherent keratotic scaling and follicular plugging; atrophic scarring may occur in older lesions
3	Photosensitivity	Skin rash as a result of unusual reaction to sunlight, by patient history or physician observation
4	Oral ulcers	Oral or nasopharyngeal ulceration, usually painless, observed by a physician.
5	Arthritis	Nonerosive arthritis involving 2 or more peripheral joints, characterized by tenderness, swelling or effusion.
6	Serositis	Pleuritis- convincing history of pleuritic pain or rub heard by a physician or evidence of pleural effusion OR Pericarditis- documented by ECG or rub or evidence of pericardial effusion.
7	Renal Disorder	Persistent proteinuria greater than 0.5 grams per day or greater than 3+ if quantitation not performed. OR Cellular casts- may be red cell, hemoglobin, granular, tubular or mixed.
8	Neurologic disorder	Seizures- in the absence of offending drugs or known metabolic derangements; e.g., uremia, ketoacidosis, or electrolyte imbalance OR Psychosis- in the absence of offending drugs or known metabolic derangements, e.g., uremia, ketoacidosis, or electrolyte imbalance.
9	Hematologic disorder	Hemolytic anemia- with reticulocytosis OR Leukopenia- less than 4000/mm^3 total on 2 or more occasions. OR Lymphopenia- less than 1500/mm^3 on 2 or more occasions. OR Thrombocytopenia- less than 100,000/mm^3 in the absence of offending drugs.
10	Immunologic disorder	Anti-DNA: Antibody to naïve DNA in abnormal titer OR Anti-Sm: presence of antibody to Sm nuclear antigen OR Positive finding of antiphospholipid antibodies on: an abnormal serum level of IgG or IgM anticardiolipin antibodies.A positive test result for lupus anticoagulant using a standard methodA false-positive test result for at least 6 months confirmed by Treponema pallidum immobilization or fluorescent treponemal antibody absorption test
11	Antinuclear antibody	An abnormal titer of antinuclear antibody by immunofluorescence or an equivalent assay at any point in time and in the absence of drugs.

Table 2.1 Guideline of 1997 Update of 1982 Revised Criteria for Classification of SLE.

Cat A	Denotes disease sufficiently **active** to merit treatment of the disease process. Relates to **acute or progressive/ recurrent** problems.
Cat B	Denotes awareness of a potential problem. Comprises acute lesions but are **less severe** than A, or **milder reversible** features.
Cat C	Conditions for which **symptomatic therapy** would be sufficient. No indication of new or change in immuno-suppression.
Cat D	Indicates previous involvement of a system that has **now resolved**.
Cat E	Indicates the **system has never been involved**.

Cat=Category

Table 2.2 Guideline of BILAG Score

Reagent	**Clone**	**Filter**	**Stain Index**
PE	RPA-T4	585/40	356.3
Alexa-Fluor 647	RPA-T4	660/20	313.1
APC	RPA-T4	660/20	279.2
PE-Cy7	RPA-T4	780/60	278.5
PE-Cy5	RPA-T4	695/40	222.1
PerCP-Cy5.5	RPA-T4	695/40	92.7
PE-Alexa Fluor 610	RPA-T4	610/20	80.4
Alexa Fluor 488	RPA-T4	530/30	75.4
FITC	RPA-T4	530/30	68.9
PerCP	RPA-T4	695/40	64.4
APC-Cy7	RPA-T4	780/60	42.2
Alexa Fluor 700	RPA-T4	720/45	39.9
Pacific Blue	RPA-T4	440/40	22.5
AmCyan	RPA-T4	525/50	20.2

Table 2.3 Stain index of various fluorochrome conjugates on a BD flow cytometer.

Courtesy from Becton Dickinson.

2.2 Polychromatic Flow Assay Design

Experimental design of multicolour flow assay was prepared with a few considerations, as listed in the table below.

No	Considerations	Descriptions
1	**Fluorochrome selection**	- Antibodies and cytokines with choice of available fluorochromes were matched by relative brightness (refer to **Table 2.3**). - Fluorochrome selection is instrument dependent, it varies with laser wavelength, laser power, filter and mirror sets available, optical alignment and pathway and PMT sensitivity.
2	**Relative antigen densities**	- The relative antigen densities could be estimated from the data sheet. The lowest antigen was paired with the brightest fluorochrome. - But this is limited by the availability of lasers, conjugate availability and potential spectral overlap considerations.
3	**Multiple laser lines optimization**	- PFC assay was optimized by using multiple laser lines, avoid “packing” a laser line, choosing optimal laser/fluorochrome combinations to minimize spillover background and to optimize signal to noise ratio.
4	**Antibody titration**	- Each antibody was titrated to optimize the amount of antibodies used. This will be elaborated in section 2.2.
5	**Background and negative control settings**	- Unstained control and single colour controls used were conjugated with the same fluorochromes used in the experiment. - They must be run at the same voltage as the fluorescence minus one FMO controls with the same cells of interest. - The purpose of running single colour controls was for spectral overlap compensation adjustment. - Meanwhile unstained control was meant to denote the background autofluoroscence of the cells.

6	**Fluorescence minus one (FMO) controls**	- They were included in the experimental design as bright single positives may change threshold levels between dim and background in other dimensions. - The use of autofluoroscence and isotypic controls is less accurate to determine threshold over background.
7	**Spectral overlap compensation**	- It was adjusted by bi-exponential compensation matrix. - Compensation percentages of each parameter were adjusted to achieve equal mean fluorescence value for the single colour positive population and the negative population measured.

Table 2.4 Considerations of Polychromatic Flow Cytometry Assay Experimental Design.

2.3 Antibody Optimization

1. Viable PBMCs isolated from normal volunteer donor's blood were counted.
2. Cells were resuspended with wash buffer (PBS+0.1% BSA) to obtain 0.5 million cells/ 50ul.
3. 0.5 million cells (=50ul) were transferred to each well of Plate 1a (Table 2.5) and Plate 1b, as shown in Table 2.6, (surface antibody titration), meanwhile 1 million cells were transferred to Plate 2, as shown in Table 2.7 (intracellular antibody titration) as below. Each well was topped up to 200ul with wash buffer after adding the cell suspension.
4. Plates 1a, 1b and 2 were spun down at 350g for 5 minutes at 4°C.
5. Supernatant was discarded and the plates were blotted dry.
6. Master mix antibodies were added in dark as shown in the table for Plate 1a and 1b below. Plates were incubated in dark for 45 minutes.
7. As for plate 2, cells were resuspended with 200ul Fix buffer (PBS + 1% PFA). Cells were incubated for 20 minutes at room temperature.
8. Plate 2 was later spun at 350g for 5 minutes at 4°C. Cells were washed twice with 2x Permeabilization buffer (0.1% Saponin in PBS + 0.5% BSA).
9. Cells in Plate 2 were incubated in 200ul Permeabilization buffer for 30 minutes at room temperature.
10. Plate 2 was spun down at 350g for 5 minutes at 4°C. Supernatant was discarded and the plates were blotted dry.
11. Master mix antibodies were added in dark as shown in the table for Plate 2 below. Plate 2 was incubated in dark for 45 minutes.

12. After incubation both plates were washed twice with buffer (wash buffer for Plate 1a and 1b while Permeabilization buffer for Plate 2), spun down at 350g for 5 minutes at 4°C.
13. For all plates, supernatant was discarded and cells were resuspended with 200ul Fix buffer.
14. All samples were transferred to FACS tube and analyzed with flow cytometer.

Per 1 million cells	**1ul**		**3ul**		**5ul**		**10ul**	
CD62L-FITC + CD8-PB								
CD16-PB + CD13-APC								
CD138-FITC + CD38-APC								
FITC iso + APC iso								
CD3-PC7								
CD45RA-PerCpCy5.5								
CD27-APC								

Table 2.5 Plate 1a. Table shows the surface monoclonal antibodies titration in duplicates.

Per 1 million cells	**1ul**		**3ul**		**5ul**		**10ul**	
IL7R-PE								
CD25-PE								
CXCR6-PE								
CD14-PE								
CD19-PE								
PE iso								

Table 2.6 Plate 1b. Table shows the surface monoclonal antibodies titration in duplicates.

Per 1 million cells	1ul		3ul		5ul		10ul	
CD62L-FITC + CD8-PB								
Foxp3-A647 + CD25-PE								
CD45RA-PerCpCy5.5								
CD4-FITC								
Bcl2-PE								
PE iso								

Table 2.7 Plate 2. Table shows the intracellular monoclonal antibodies titration in duplicates.

15. Antibody master mix for each surface antibody was prepared as shown in **Table 2.8** below. Total volume for each well is 100ul.

Total quantity of different antibody	Each antibody volume (ul)	Wash buffer volume (ul)	Total volume for duplicates (ul)	Total cell number for each well, 50ul	Final concentration/ well/ 1 million cells (ul/1 million cells)
ONE	1	99			1
	3	97			3
	5	95			5
	10	90		0.5 million cells	10
			100		
TWO	1	98			1
	3	94			3
	5	90			5
	10	80			10

Table 2.8 Master mix preparation for extracellular antibody titration for Plate 1a and 1b.

16. For Plate 2, the master mix was prepared as below as more cells were used than in the extracellular preparation.

Total quantity of different antibody	**Each antibody volume (ul)**	**Wash buffer volume (ul)**	**Total volume for duplicates (ul)**	**Total cell number for each well, 100ul**	**Final concentration/ well/ 1 million cells (ul/1 million cells)**
ONE	2	98	100	1.0 million cells	1
	6	94			3
	10	90			5
	20	80			10
TWO	2	96			1
	6	88			3
	10	80			5
	20	60			10

Table 2.9 Master mix preparation for intracellular antibody titration for Plate 2.

2.4 Procedures

2.4.1 Buffer Preparation:

Intracellular Fixation and Permeabilization Buffers:

1. Intracellular fixation and permeabilization buffers were prepared from BD Human FoxP3 Buffer Set (BD Pharmingen: 560098). These buffers need to be made fresh for each experimental set.
2. Foxp3 Buffer A (x10 concentrated) was diluted 1:10 with room temperature deionized water.
3. To make a working solution of Buffer C, FoxP3 Buffer B was diluted into 1x FoxP3 Buffer A at a ratio of 1:50 (Buffer A: Buffer A).
4. The buffers were brought to room temperature before use.

Red Blood Cell (RBC) Lysis Buffer:

1. FACS Lysing Solution x10 concentrated (BD: 349202) was diluted 1:10 with room temperature deionized water. The prepared solution is stable for a month if stored in glass container at room temperature.

Wash Buffer:

1. Wash buffer was prepared with 1x PBS + 0.1% BSA.

2.4.2 PBMC Preparation: PBMC (Peripheral blood mononuclear cells) separation on Ficoll Gradient

1. All tubes of whole blood collected from patients were pooled into a T75 flask. The remaining blood in the collection tubes was rinsed with 2ml 0.4% Sodium citrate and added into the T75 flask.
2. 1:1 dilution was prepared by adding 0.4% Sodium citrate into the T75 flask and it was mixed by shaking.
3. 15ml of Ficoll paque was aliquoted into 50ml of BD falcon tubes. 20ml of blood diluted with sodium citrate from step 2 was layered slowly and gently on top of the Ficoll paque.
4. The buckets of tubes were balanced before centrifugation.
5. Tubes were centrifuged at 1900 rpm for 20 minutes at room temperature with acceleration: 3 and brake off.
6. Transfer the buffy coat layer (white ring) into 50ml tubes and top up with Sodium citrate solution, mixed by inverting the tubes.
7. 1st PBMC washing: sample was spun at 2000rpm for 10 minutes at room temperature. It is important to remove as much ficoll as possible as it can interfere with the RNA extraction later.
8. Optionally, if pellet appears red, it was resuspended in 5ml of RBC lysis buffer and incubated for 2 minutes. The mixture was later topped up with wash buffer and centrifuged at 1600 rpm for 5 minutes at 4°C.
9. PBMCs were collected and the supernatant was discarded. The pellet was resuspended and tubes filled with Sodium citrate (or running buffer).
10. 2nd PBMC washing: sample was centrifuged at 1600rpm for 5 minutes at 4°C.

11. Supernatant was removed and PBMCs were pulled to one tube, topped up with wash buffer and cells were counted.
12. 3rd PBMC washing: step 10 was repeated.
13. Supernatant was removed and cells were suspended in wash buffer with a concentration of 10million cells/ ml.

2.4.3 Control Layout

Tube	**Control**	**Antibody (volume in µl)**
1	FITC single	CD62L (10)
2	PE single	IL7R (5)
3	Intracellular PE single	Bcl2 (10)
4	Intracellular A647 single	FoxP3 (10)
5	PC7 single	CD3 (5)
6	PerCpCy5.5 single	CD45RA (10)
7	APC single	CD13 (10)
8	PB single	CD8 (2.5)
9	Unstained	None

Table 2.10 Unstained and single colour controls. Each tube contains 10ul of FcR Blocking Reagent on top of the antibody cocktail.

2.4.4 Staining Layout: Extracellular Staining for T cells, Granulocytes and B cells

Tube	Cell	Label	FITC	PC7	PerCpCy5.5	PB	APC	PE
			(volume in µl)					
10	**T cells**	IL7R	CD62L (10)	CD3 (5)	CD45RA (10)	CD8 (2.5)	---	IL7R (5)
11		CD25	CD62L (10)	CD3 (5)	CD45RA (10)	CD8 (2.5)	---	CD25 (5)
12		CXCR6	CD62L (10)	CD3 (5)	CD45RA (10)	CD8 (2.5)	---	CXCR 6 (10)
13		PE FMO	CD62L (10)	CD3 (5)	CD45RA (10)	CD8 (2.5)	---	PE iso (10)
14	**Granulocytes**	Mo/ Neut	CD62L (10)	---	---	CD16 (2.5)	CD13 (10)	CD14 (10)
15		FITC/ APC FMT	FITC iso (10)	---	---	CD16 (2.5)	APC iso (10)	CD14 (10)
16	**B Cells**	B Plasma	CD138 (10)	---	---	---	CD38 (10)	CD19 (10)
17		B Memory	CD62L (10)	---	---	---	CD27 (10)	CD19 (10)
18		FITC/ APC FMT	FITC iso (10)	---	---	---	APC iso (10)	CD19 (10)

FMT: Fluorescence minus two

Table 2.11 Staining layout for T cells, Granulocytes and B cells. Each tube contains 10ul of FcR Blocking Reagent on top of the antibody cocktail.

2.4.5 Staining Layout: Intracellular Staining for T cells and T Regulatory cells.

Tube	Cell	Label	FITC	PC7	PerCpCy5.5	PB	AF647	PE
			(volume in µl)					
19	**T cells**	Bcl2	CD62L (10)	CD3 (5)	CD45RA (10)	CD8 (2.5)	---	Bcl2 (10)
20		Bcl2 FMO	CD62L (10)	CD3 (5)	CD45RA (10)	CD8 (2.5)	---	PE iso (10)
21	**T regulatory cells**	FoxP3	CD4 (10)	---	---	---	FoxP3 (10)	CD25 (5)
22		FoxP3 FMO	CD4 (10)	---	---	---	AF647 iso (10)	CD25 (5)
23		FoxP3 FMO	CD4 (10)	---	---	---	---	PE iso (10)

FMO: Fluorescence minus one

Table 2.12 Staining layout for intracellular T cells and T regulatory cells. Each tube contains 10ul of FcR Blocking Reagent on top of the antibody cocktail.

2.4.6 Staining Procedures

2.4.6.1 Procedure for Extracellular Staining

1. Surface marker antibodies were added to corresponding FACS tubes
2. 100ul of whole blood was aliquoted to each tube and mixed well.
3. Cells were incubated for 20 minutes in dark at room temperature.
4. Samples were vortexed before 2ml of RBC lysis buffer was added to each tube.
5. Samples were vortexed thoroughly.
6. Cells were incubated for 10 minutes in dark at room temperature.
7. Sample was then centrifuged at 350g for 8 minutes at room temperature.
8. Supernatant was decanted. Pellet was then resuspended with 2ml running buffer.
9. Step 7 was repeated to wash the cells.
10. Supernatant was discarded. Cells were resuspended in 250ul running buffer.
11. Samples were then stored at 4°C and analyzed within 4 hours. Cells were vortexed thoroughly at low speed to reduce aggregation before acquiring.

2.4.6.2 Procedure for Intracellular Staining

1. Surface marker antibodies were added to corresponding FACS tubes. Each tube was topped up to 50ul with 1x PBS.
2. 100ul of cells (=1x 10^6 cells) was aliquoted to each tube and mixed well, total volume of the tube was 150ul at this point.
3. Cells were incubated for 20 minutes in the dark at room temperature.
4. 2ml wash buffer was added to each tube.
5. Samples were centrifuged at 1000rpm for 10 minutes at 4°C.
6. Supernatant was discarded. Pellet was resuspended in remaining volume.

7. 2ml of Buffer A was added and vortexed to fix the cells.
8. Mixture was incubated in the dark for 10 minutes at room temperature.
9. Sample was centrifuged at 2000rpm for 5 minutes at room temperature.
10. Supernatant was discarded to remove fixative. This step needs extra care as the pellet is buoyant at this stage.
11. Pellet was washed with 2ml running buffer to wash the cells.
12. Samples were centrifuged at 2000rpm for 5 minutes at room temperature.
13. Supernatant was discarded to remove wash buffer and pellet was resuspended gently in 0.5ml 1x Buffer C, in order to permeabilize the cells.
14. Mixture was incubated for 30 minutes in the dark at room temperature.
15. Sample was then washed with 2ml running buffer, centrifuged at 2000rpm for 5 minutes at room temperature.
16. Supernatant was discarded. Pellet was washed again by repeating step 14.
17. Conjugated intracellular antibodies or isotype controls were added to corresponding tubes. Pellets were mixed well with the antibodies or isotype controls.
18. Sample was incubated in dark for 30 minutes in dark at room temperature.
19. 2ml running buffer was added, sample was then centrifuged at 2000rpm for 5 minutes.
20. Supernatant was discarded and pellet was resuspended in 200ul of running buffer.
21. Samples were then stored at 4°C and analyzed within 4 hours.

	Specificity	Fluorochrome	Clone	Source	Cat No.
T cell memory populations	CD8	Pacific Blue	RPA-T8	BD	558207
	SELL(CD62L)	FITC	DREG-56	BD	555543
	CD45RA	PerCP-Cy5.5	HI100	ebioscience	45-0458-73
	IL7R (CD127)	PE	HIL-7R-M21	BD	557938
	CD25	PE	143-13	Biosource	AHS2517
	CD25	PE	143-13	Abcam	ab16178
	CXCR6	PE	56811	R&D	FAB699P
	Isotype	PE	MOPC-21	BD	554680
	Bcl2	PE	MOPC-21	BD	556535
	CD3	PC7	UCHT1	BeckmanCoulter	6607100
Monocyte/Neutrophil populations	CD16	Pacific Blue	3G8	BD	558122
	CD13	APC	WM15	BD	557454
	CD14	PE	M5E2	BD	555398
	Isotype	FITC	M18-254	BD	553478
	Isotype	APC	X40	BD	340442
B cell	CD38	APC	HIT2	BD	555462
	CD138	FITC			
	CD19	PE	HIB19	BD	555413
	SELL (CD62L)	FITC	DREG-56	BD	555543
	CD27	APC	323	eBioscience	17-0279
	Isotype	FITC	M18-254	BD	553478
	Isotype	APC	X40	BD	340442

	Specificity	Fluorochrome	Clone	Source	Cat No.
Treg	FoxP3	AF647	259D/C7	BD	560045
	CD4	FITC	RPA-T4	BD	555346
	CD25	PE	143-13	Abcam	ab16178
	Isotype	PE	MOPC-21	BD	554680
	Isotype	AF647	MOPC-21	BD	557732
Blocking Regent	FcR block			Miltenyi	130-059-901

Table 2.13 List of antibodies used in the study.

2.5 RNA Isolation

This part of the work was kindly contributed by Dr Vojislav Jovanovic.

2.5.1. PBMC (Peripheral blood mononuclear cells) separation on Ficoll Gradient

1. All tubes of full blood were pooled into a T75 flask. The remaining blood in the collection tubes was rinsed with 2ml 0.4% Sodium citrate and added into the T75 flask.
2. 50ml of 0.4% Sodium citrate was added into the T75 flask and it was mixed by shaking.
3. 15ml of Ficoll paque was aliquoted into 50ml of BD falcon tubes. 20ml of blood diluted with sodium citrate from step 2 was layered slowly and gently on top of the Ficoll paque.
4. The buckets of tubes were balance before centrifugation.
5. Tubes were centrifuged at 1900 rpm for 20 minutes at room temperature with acceleration: 3 and brake off.
6. Transfer the buffy coat layer (white ring) into 50ml tubes and top up with Sodium citrate, solution was mixed by inverting the tubes.
7. 1st PBMC washing: sample was spun at 2000rpm for 10 minutes at room temperature. It is important to remove as much ficoll as possible as it can interfere with the RNA extraction later.
8. Optionally, if pellet appears red, 5ml of RBC lysis buffer was resuspended and incubated for 2 minutes. The mixture was later topped up with running buffer and centrifuged at 1600 rpm for 5 minutes at 4°C.
9. PBMCs were collected and supernatant was discarded. Pellet was resuspended and tubes were filled up with Sodium citrate (or running buffer).
10. 2nd PBMC washing: sample was centrifuged at 1600rpm for 5 minutes at 4°C.

11. Supernatant was removed and PBMCs were pulled to one tube, topped up with running buffer and cells were counted.
12. 3rd PBMC washing: step 10 was repeated.
13. Supernatant was removed and cells were suspended in running buffer with a concentration of 10million cells/ ml.
14. 500ul (= 5x 10^6) PBMCs were added respectively into a RNA and a genomic DNA labeled 15ml falcon tubes. Tubes were later kept on ice.
15. 50ul (=0.5 x 10^6) of PBMCs were aliquoted in each of 4 single-color controls (APC only, FITC only, PE only and unstained control).
16. Remaining PBMCs were divided into two 15ml falcon tubes for separation purposes (pre-CD14 and pre-CD19 falcon tubes).
17. Tubes were spun at 1600 rpm for 5 minutes at room temperature.
18. Supernatant was discarded and pellet was resuspended with the remaining volume.
19. Anti-CD14 or anti-CD19 microbeads together with FCR blocking reagent (TCRM3) were added into each PBMC tube respectively at the concentration of 2ul of microbeads/ 1 million of cells.
20. Mixture was incubated in fridge for 20 minutes on the AutoMACS.
21. 5ml running buffer was added into each tube.
22. Tubes were centrifuges at 1600rpm for 5 minutes at 4°C.
23. Pellet was resuspended with 500ul running buffer.
24. 20ul of pre-CD14or pre-CD19 was added to corresponding FACS tubes and samples were kept on ice.

2.5.2. Neutrophil Preparation (In Parallel with 2.5.1)

1. From step A3, after the buffy coat was harvested, Ficoll was removed from tubes and RBC lysis buffer added and pellet resuspended.
2. Mixture was incubated in fridge for 30 minutes.
3. Sample was centrifuged at 1600rpm for 5 minutes at 4°C.
4. Supernatant was removed, pellet was resuspended in 50ml of RBC lysis buffer.
5. Step B3 was repeated.
6. Supernatant was decanted and cells were pooled. Cells were resuspended in running buffer. Cells were counted.
7. Step B3 was repeated.
8. Supernatant was discarded and cells were resuspended in the remaining volume. Cells were kept on ice.
9. Anti-CD16 microbeads and FCR blocking agent (TCRM3) were added into the sample at the concentration of 1ul microbeads/ 1 million cells.
10. Repeat step A20-A23.
11. 20ul of pre-CD16 was added to corresponding FACS tubes and samples were kept on ice.

2.5.3. AutoMACS Cell Sorting

PBMCs were separated to CD4+, CD8+, CD14+, CD19+ and CD16+ cells by using automated magnetic cell sorter from Miltenyi, AutoMACS cell sorter. These cells were later lysed and digested with Qiagen QIAshredder to yield RNA, which was later delivered to Cambridge UK for microarray gene profiling.

AutoMACS Running Buffer: Optimized separation buffer from Miltenyi. It is sterile filtered buffer containing BSA, EDTA and 0.09% Azide.

AutoMACS Rinsing Buffer: For rinsing and cleaning cycles on AutoMACS's fluidics system. Ready purchased from Miltenyi. It contains BSA stock solution and it is preservative free.

1. Waste bottle level was checked before placing the instrument into the biosafety cabinet.
2. "Running" and "Rinsing" buffer were loaded in the hood and kept on ice.
3. A container was placed at the bottom of the nozzle and "Clean" program was run.
4. CD16, CD19 and CD14 fractions were separated on AutoMACS.
5. 15ml falcon tubes for positive and negative fractions were placed at the nozzle respectively. "Separation" button was pressed and "Possel" button was clicked too. "Possel" means positive selection, it allows the machine to select and keep the positively selected cells.
6. Volume of the negative fraction for CD14 and CD19 was recorded.

7. Tubes with the positive and negative fractions were kept on ice.
8. The following antibodies were added into the corresponding FACS tubes.

- CD14+ 50ul
- CD14- 50ul
- CD16+ 50ul
- CD16- 300ul
- CD19+ 50ul
- CD19- 50ul

9. "Q-rinse" was clicked. Tube was placed at the nozzle to collect liquid.
10. Program "Sleep" was used if there was no other samples for separation.
11. Cells were counted.
12. CD14- and CD19- fractions were centrifuged at 1600rpm for 5 minutes at 4°C. Pellet was resuspended in small volume.
13. CD14- sample was incubated with anti-CD4 microbeads. CD19- sample was incubated with anti-CD8 microbeads at the concentration of 2ul microbeads per 1 million cells.
14. Mixture was incubated in the fridge.
15. 5ml running buffer was added to the tube and sample was centrifuged at 1600 rpm for 5minutes at 4°C.
16. Pellet was resuspended in 500ul of running buffer.
17. 20ul of pre-4+ and CD8+ fractions were transferred into the FACS tubes.
18. CD14- and CD19- were separated.
19. Cells were counted.

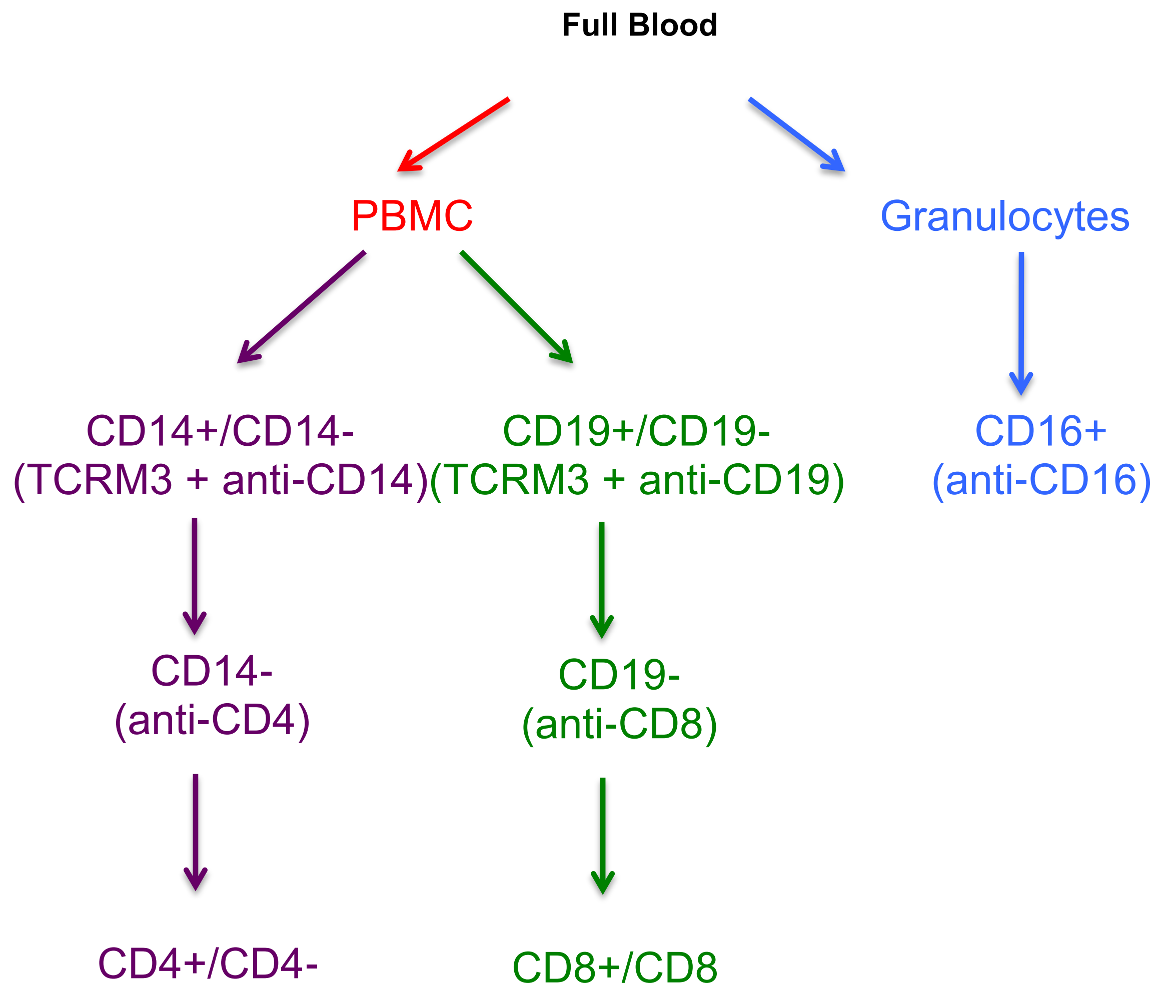

Figure 2.0 An overview of cell sorting using AutoMACS from PBMCs and Granulocytes.

2.5.4. Cell digestion with Qiagen QIAshredder columns (after cell sorting on AutoMACS)

1. Centrifuge all the 15ml falcon tubes:CD14+, CD19+, CD4+, CD8+, CD16+ and PBMCs for RNA, at 1600rpm for 5 minutes at 4°C.
2. 4ml of RLT and 40ul of β–mercaptoethanol were added into each the tube.
3. Supernatant was removed completely and pellet was resuspended in respective amount of RLT buffer mix.
4. Sample was mixed well by pipetting up and down until the mixture becomes gluish.
5. Digested cells were transferred into columns.
6. Sample was centrifuged for 2 minutes at 13,000rpm at room temperature. Column was discarded and tube was closed using rubber lid.
7. Sample was then stored at -80°C and ready to be delivered to Cambridge for microarray profiling.

CHAPTER 3

RESULTS PART 1

3.1 Antibody Optimization

Immunofluorescence reagents are titrated to ensure proper quality control, to minimize wastage of reagents and to reduce lot-to-lot variation. Comparisons should also be made between lots when new batch of antibodies are purchased. The same volume of reagent should be used at each dilution point.

Antibodies have a range in which they bind to antigens. If too little antibody is used in the labeling, there will be an inaccurate amount of light produced by fluorescence and depending on the magnitude, a particle positive for the antibody may not be detected. However using too much antibody will increase the background and may mask the true amount of the antigen in the sample. Therefore, it is important to find an optimal concentration of fluorescent antibody that approached the saturation level, but is slightly below it. This ensures a fluorescent signal emission that is linearly proportional to the antigen present in the sample.

0.5 million PBMCs of healthy donors were used to perform each extracellular monoclonal antibody titration, meanwhile 1 million PBMCs of healthy donor were aliquoted for each intracellular monoclonal antibody optimization. To titrate the antibodies, a starting volume is determined, which is typically 1ul, followed by 3ul, 5ul and 10ul. Cells were stained and analyzed on flow cytometer on the same day. Positive and negative populations were gated on the histograms. The concentration with the best separation between positive and negative populations, with the least background noise interference was chosen as the optimal concentration.

For T Lymphocytes specific antibodies optimization, as shown in **Figure 3.1.1**, separation of negative and positive populations was obvious in (a), (b), (c) and (e). Negative peaks only were observed in (d), (f) and (h). Meanwhile (g) displayed positive peaks of different titrations. Titrations reached a plateau at 3ul for (a), (b), (c) and (d). 1ul was too low a titration concentration for them. Meanwhile 5ul and 10ul gave the same optimal results as 3ul. Titration 10ul of chosen for (e), (f), (g) and (h) as they gave the optimal separation compared to others, with the least background interference.

Figure 3.1.2 shows the optimization of monocytes and granulocytes specific antibodies, CD16-PB (a) titration was optimal at 3ul and 5ul as 10ul gave a high background noise. 3ul titration was sufficient to give optimal results. CD14-PE(b) and CD13-APC (c) titration were optimal at 10ul. Its histograms shifted to the right whenever a higher concentration was used. APC isotype and FITC isotype gave optimal titration at both 5ul and 10ul. 5ul was chosen to reduce reagent cost.

For plasma B and memory B cells specific antibodies titration in **Figure 3.1.3**, 3ul was sufficient to generate optimal results for CD19-PE (a) and CD138-FITC (d). Titrations leveled off the rising curve at 5ul for CD38-APC (b) and at 10ul for CD27-APC (c).

As shown in **Figure 3.1.4** intracellular antibody titrations, a greater volume of antibodies was required to reach optimal optimizations for Bcl2-PE (a), FoxP3 (b) and A647 isotype (c). Titrations were at plateau for 10ul for all three antibodies.

All the above results of optimal antibody concentrations were summarized in **Table 3.1.1**.

	Fluorochrome conjugated Antibodies	**Concentration (ul/ 1 million cells)**
T Lymphocytes	CD3-PC7	6
	CD8- PB	6
	CD62L- FITC	6
	CD25-PE	6
	IL7R-PE	20
	CXCR5-PE	20
	CD45RA-PerCpCy5.5	20
	PE isotype	20
Monocytes/ Granulocytes	CD16-PB	6
	CD14-PE	20
	CD13- APC	20
	APC isotype	10
	FITC isotype	10
B plasma/ B memory	CD19-PE	6
	CD38-APC	10
	CD27-APC	20
	CD138-FITC	6
Intracellular T lymphocytes	Bcl2-PE	20
	Foxp3 A647	20
	A647 isotype	20

Table 3.1.1 Summary of the optimal fluorochrome-conjugated antibodies concentration.

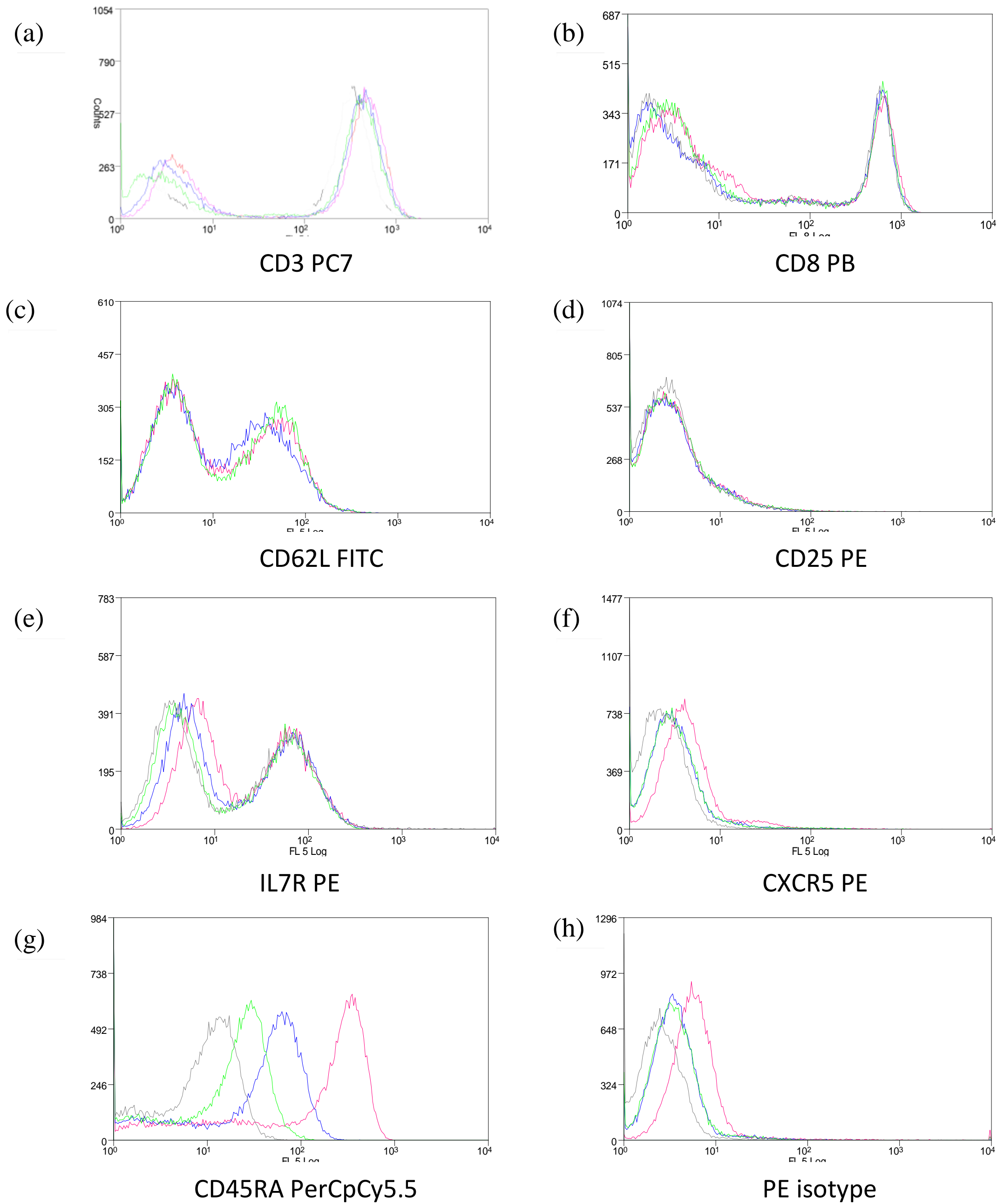

Figure 3.1.1 Antibody optimization for T lymphocyte specific antibodies. This was performed by using titration of 1ul (grey), 3ul (green), 5ul (blue) and 10ul (red) for each antibody in 0.5million PBMCs of healthy donor: CD3-PC7 (a), CD8-PB(b), CD62L-FITC (c), CD25-PE (d), IL7R-PE (e), CXCR5-PE (f), CD45RA PerCpCy5.5 (g) and PE isotype (h). Results are representative of three different healthy donors. CD45RA-PerCpCy5.5 was highly concentration dependent, as was PE isotype and CXCR5-PE. No significant difference of concentration was observed for CD3-PC7, CD8-PB, CD62L-FITC, CD25-PE and IL7R-PE.

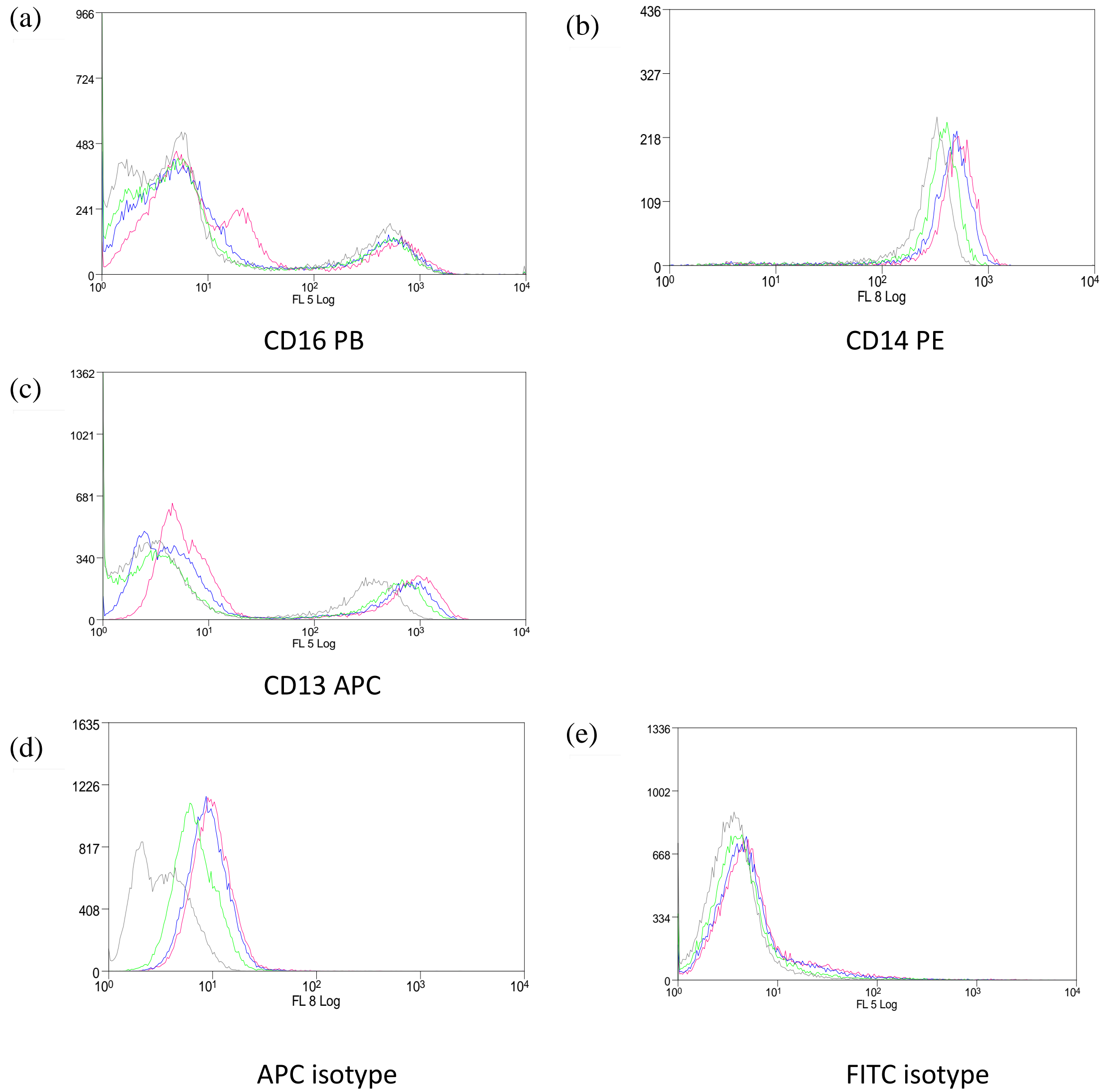

Figure 3.1.2 Monocytes and granulocytes specific antibodies optimization. Experiment was performed by using titration of 1ul (grey), 3ul (green), 5ul (blue) and 10ul (red) for each antibody in 0.5million PBMCs of healthy donor: CD16-PB (a), CD14-PE(b), CD13-APC(c), APC isotype (d) and FITC isotype (e). Experiment was repeated on three different healthy donors. APC isotpe, CD14-PE and CD13 APC were concentration dependent. No significant difference of concentration was observed for FITC isotype and CD16-PB.

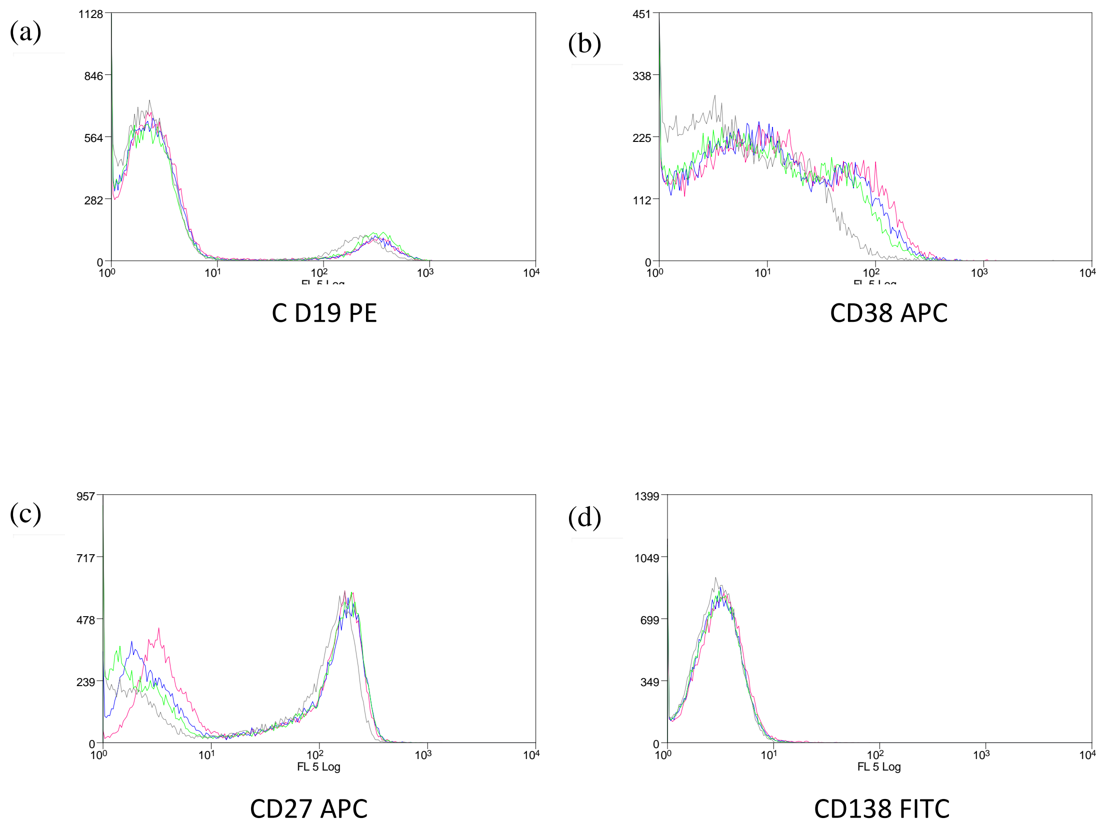

Figure 3.1.3 Antibody optimization for plasma B cells and memory B cells specific antibodies. Experiment was performed by using titration of 1ul (grey), 3ul (green), 5ul (blue) and 10ul (red) for each antibody in 0.5million PBMCs of healthy donor: CD19-PE (a), CD38-APC (b), CD27-APC(c) and CD138-FITC (d). No significant difference of concentration was observed in this panel.

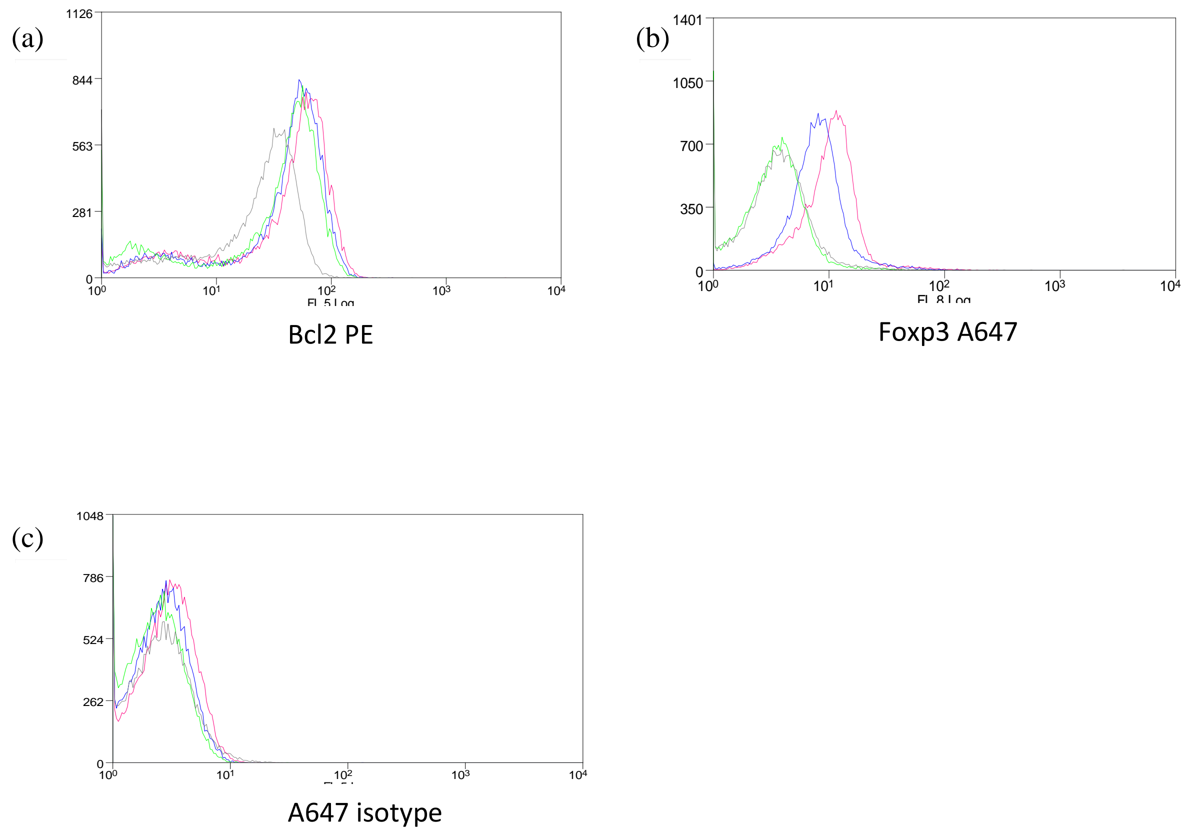

Figure 3.1.4 Intracellular antibodies titration specific to Bcl2 and T regulatory cells. Titration was performed by using titration of 1ul (grey), 3ul (green), 5ul (blue) and 10ul (red) for each antibody in 1 million PBMCs of healthy donor: Bcl2 PE (a), Foxp3-A647 (b) and A647 isotype (c). Bcl2-PE and Foxp3-A647 are concentration dependent but no significant difference of concentration was observed for A647 isotype.

3.2 SGH Patients' Clinical Information

A total of 25 subjects with confirmed transcriptional profiling (performed in Cambridge) were analyzed. However the UK and Singapore cohorts were not studied at the same time and were not designed for direct comparison. Nonetheless there were interesting differences in the two cohorts.

Of the 25 subjects, 19 of them were SLE patients and 6 were healthy donors. 16 of these patients were from SGH while 3 were from NUH. Results of patients' clinical data were all from SGH. With informed consent, 50ml of blood were collected from the patients by the research nurse. However, some patients were too weak to give sufficient blood for flow analysis.

75% of these SLE patients from SGH were Chinese, 19% were Malay and only 6% were Indian. As expected, 87.5% of the SLE patients from SGH who are involved in this study are female and overall 86% of these female patients are Chinese. Most of the patients from SGH have disease duration of more than five years and they are in remission. Some were on immunosuppressive drugs, particularly Prednisolone on the date of blood collection.

These patients from SGH are 100% ANA positive and 75% showed symptoms of arthritis and hematologic disorder respectively.

3.2.1 Prognostic Subgroup Classification

Referring to the microarray results (Figure 1.5), 12 of the subjects (9 SLE patients and 3 healthy controls) are categorized as prognostic subgroup v8.1. Meanwhile 13 (10 SLE patients from SGH and 3 healthy controls) were in prognostic subgroup v8.2 (**Table 3.2.1**). This shows that unlike the UK cohort who are mostly CD8.2, the Asian cohort in Singapore displays an equal distribution of these two categories.

16 of patients' blood were collected from SGH meanwhile 3 were from NUH. Most of the patients from SGH were in remission and their conditions stabilized with drug therapy. NUH SLE patients were usually at flare and were recently diagnosed SLE on date the blood was taken. Unfortunately collection of NUH subjects' clinical information was not as complete as SGH. The patients' clinical characteristics shown below are those from SGH (Table 3.2.1, Table 3.2.2, Table 3.2.3, Table 3.2.4, Figure 3.2.1, Table 3.2.5, Table 3.2.6, Table 3.2.7, Table 3.2.8 and Table 3.2.9) .

Profile	Subjects	Details
v8.1	Patients = 9	6 from SGH, 3 from NUH
	Controls = 3	
	Total v8.1 = 12	
v8.2	Patients = 10	10 from SGH
	Controls = 3	
	Total v8.2 = 13	

Table 3.2.1 Confirmed transcriptional profiling of subjects involved in the study. A total of 25 subjects were involved in this study. Table shows clinical information and confirmed transcriptional profiling for healthy control subjects and patients with Systemic Lupus Erythematosus (SLE). Patients display a homogeneous distribution of v8.1 and v8.2 categories. 16 patients were from Singapore General Hospital (SGH) except three of them who were from National University Hospital (NUH).

3.2.2 Characteristics of Patients

75% of these patients from SGH are Chinese, followed by Malay (19%) and Indian (6%), as shown in **Table 3.2.2**. 87.5% of them are female; this shows that SLE clearly has a female preponderance. As most of the SLE patients from SGH were diagnosed with the disease years ago and mostly are in remission, they tend to be older than the expected child-bearing years (**Table 3.2.3**).

	Female (n=14)	**Male (n=2)**
Chinese (n=12) (75%)	**10**	**2**
<= 20	0	0
21-40	5	1
41-60	5	1
>=60	0	0
Malay (n=3) (19%)	**3**	**0**
<= 20	1	0
21-40	1	0
41-60	1	0
>=60	0	0
Indian (n=1) (6%)	**1**	**0**
<= 20	0	0
21-40	0	0
41-60	1	0
>=60	0	0

Table 3.2.2 Clinical characteristics of patients from SGH. Ethnic group, gender and age group (on date of blood taken) distribution profile of the SLE patients from SGH. Chinese females in age group 21-60 are the majority (75%) of the patients involved in this study, followed by Malay (19%) and Indian (6%).

	v8.1 (n=6)	**v8.2 (n=10)**
Female:Male	5:1	9:1
Malay:Chinese:Indian	1:4:1	2:8:0
20s:30s:40s:50s	0:3:1:2	1:2:7:0
Disease Duration **<5: 5-10: 10-20: 20-30**	0:1:4:1	2:4:2:2

Table 3.2.3 Clinical information of patients from SGH involved in this study. Gender, race, age group and disease duration of SLE patients from SGH. In both prognostic subgroups, it shows that SLE in Singapore has a female preponderance.

3.2.3 BILAG Scores of Patients

The patients generally had mild lupus and low lupus activity and this explains why 56% of them were assessed with a BILAG Score C, 19% scored D, 12.5% scored B and E respectively (**Table 3.2.4**). Patients who scored BILAG Score C had insignificant changes in their lupus activity over time, no indication of new symptoms or a change in immuno-suppression was detected. Their conditions were generally stable for which symptomatic therapy would be sufficient. Comparison between prognostic subgroup v8.1 and v8.2 shows that patients who fall in subgroup v8.1 display a relatively higher lupus activity with higher BILAG Scores (**Figure 3.2.1**).

BILAG Scores	No (n=16)	%
A	0	0.0
B	2	12.5
C	9	56.3
D	3	18.8
E	2	12.5

Table 3.2.4 BILAG scores of SLE patients from SGH involved in this study. The BILAG is a validated tool for assessing lupus activity and comprises clinical as well as laboratory parameters. It is thought to be more sensitive change compared to other tools such as SLAM (Systemic Lupus Activity Measurement) and SLEDAI (SLE Disease Activity Index). 56% of these SLE patients display a BILAG Score C, followed by 19% of D and equal distribution (12.5%) of B and E.

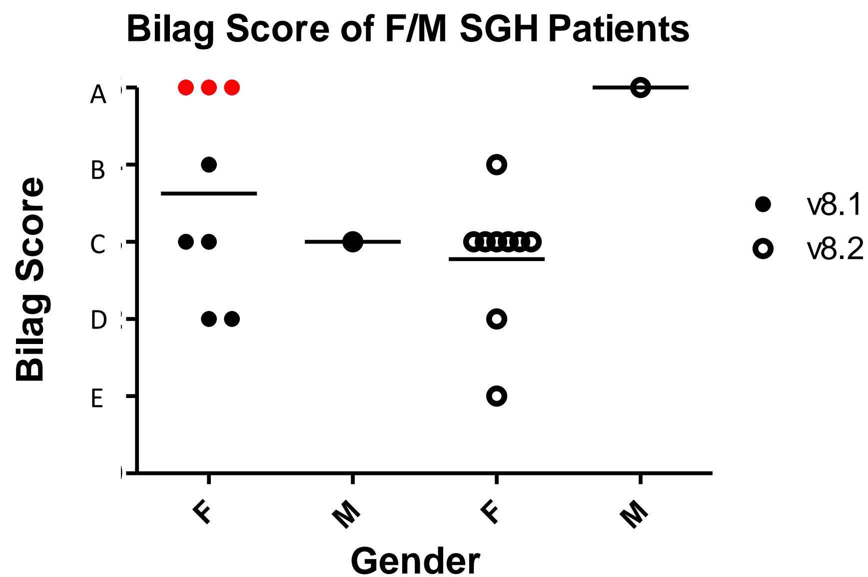

Figure3.2.1 BILAG score of SLE patients from SGH, with respective prognostic group and gender distribution. Three red filled dots represent active SLE patients from NUH who were on flare on date of blood collection. Prognostic group v8.1 generally has a higher BILAG Score (higher lupus activity) compared to prognostic group v8.2. Both subgroups are mostly female SLE patients, with only one male respectively in each subgroup.

3.2.4 ACR Criteria of Patients

75% of SGH SLE patients had arthritis and hematologic disorder respectively. Half of them developed malar rash and 44% of them developed renal disorder. Patients from prognostic subgroup v8.1 seemed to display more prominent symptoms of malar rash (83%), hematologic disorder (83%), arthritis (67%) and renal disorder (67%). All the three patients who had serositis were also from subgroup v8.1 (**Table 3.2.5**). All the SLE patients involved in this study were tested Antinuclear Antibody (ANA) positive and had elevated levels of ANA (>1:20 titration). Almost all of them, 94% were Anti-dsDNA positive and all had higher than normal range of Anti-dsDNA readings (> 1:10 titration). The same patients from subgroup v8.2 were both ACA (anti-cardiolipin) IgM and ACA IgG positive (**Table 3.2.6**).

ACR Criteria	v8.1 (n=6)	v8.2 (n=10)	Total (n=16)	%
Malar Rash	5	3	8	50.0
Discoid Rash	0	3	3	18.8
Photosensitivity	2	3	5	31.3
Oral Ulcers	3	2	5	31.3
Arthritis	4	8	12	75.0
Serositis	3	0	3	18.8
Renal Disorder	4	3	7	43.8
Neurological Disorder	0	1	1	6.3
Hematologic Disorder	5	7	12	75.0
Antinuclear Antibody	6	10	16	100.0

Table 3.2.5 ACR criteria of SLE patients from SGH involved in this study. All the SLE patients were tested antinuclear antibody positive. 75% of them show symptoms of hematologic disorder and arthritis. Half of them developed malar rash, 7 of them developed renal disorder, 31% of them are photosensitive and have oral ulcers respectively. Discoid rash, serositis and neurological disorder are less common in these patients.

3.2.5 Autoantibodies Found in Patients

Autoantibodies	v8.1(n=6)	v8.2(n=10)	Total (n=16)	%
*ANA	6	10	16	100.0
*Anti-dsDNA	5	10	15	93.8
Anti-Ro	1	1	2	12.5
Anti-La	0	1	1	6.3
Anti-Sm	0	0	0	0.0
Anti-RNP	1	2	3	18.8
Anti-Scl-70	0	1	1	6.3
Anti-Jo 1	0	0	0	0.0
ACA IgM	0	2	2	12.5
ACA IgG	0	2	2	12.5
Lupus Anticoagulant	1	1	2	12.5

Table 3.2.6 shows autoantibodies found in SLE patients from SGH. All the patients are ANA positive, followed by 93.8% Anti-dsDNA positive, Anti-RNP positive, 12.5% for Anti-Ro, ACA IgM, ACA IgG and Lupus Anticoagulant positive respectively. 6.3% of them are Anti-La and Anti-Scl-70 positive respectively. None of them is Anti-Sm and Anti-Jo 1 positive.

*ANA levels higher than 1:20 titration
*Anti-dsDNA levels higher than 1:10 titration

3.2.6 Classification of Renal Biopsy in Patients

69% of the patients were not renal biopsy-classified (**Table 3.2.7**). Of those who were classified, one fell under Class III, 2 were categorized Class IV, one had global sclerosis and minor abnormalities respectively. Those classified were all from subgroup v8.2 except one who was from subgroup v8.1 and was classified Class IV.

Renal Biopsy Class	No (n=16)	%	Subgroup
no classification	11	68.8	NA
Class II & III	1	6.3	v8.2
Class IV	2	12.5	both v8.1
Focal global sclerosis	1	6.3	v8.2
Minor abnormalities	1	6.3	v8.2

Table 3.2.7 Renal Biopsy classification of SLE patients from SGH involved in this study. 68.8% of the patients were unclassified, 2 (12.5%) of them were Class IV, one (6.3%) of them was reported Class II & III, developed focal global sclerosis and minor abnormalities respectively.

3.2.7 Medications Taken on Date of Blood Collection

Some of these patients were under treatment on date the blood was taken (**Table 3.2.8**). 56% of them were on Prednisolone, 50% were on Hydroxycholoquine, 19% on Azathiopine and MMF respectively. Patients who were on Hydroxycholoquine were mostly from subgroup v8.2 (88%).

	v8.1 (n=6)	v8.2 (n=10)	Total (n=16)	%
Prednisolone	4	5	9	56.3
Azathiopine	2	1	3	18.8
Cyclophosphamide	0	0	0	0.0
Hydroxycholoquine	1	7	8	50.0
Methotrexate	0	0	0	0.0
MMF	2	1	3	18.8
Rituximab	0	0	0	0.0

Table 3.2.8 Medications taken of SGH SLE patients on date of blood taken. 56% of them were on Prednisolone, 50% on Hydroxycholoquine, 19% on Azathiopine and MMF respectively.

3.2.8 Blood Test Results of Patients

High level of mean reading of Creatinine (Cr) was detected in patients in subgroup v8.1, as shown in **Table3.2.9**. Among these five patients tested, three were in the normal range (48, 52 and 82 μmol/ litre) with only one outlier scoring an extraordinary high level of Cr, 245 μmol/ litre, which increases the mean reading enormously. This suggests that the high mean reading of the Cr of prognostic subgroup v8.1 is not a genuine figure. Erythrocyte Sedimentation Rate (ESR) readings were generally high for both subgroups. White Blood Cells (WBC), Neutrophils, Lymphocytes, Complement Component 3 (C3) and Complement Component 4 (C4) of these patients were in normal range.

Mean	v8.1 (total n=6)	v8.2 (total n=10)	Normal Range
Cr (μmol/ l)	107.6 (n=5)	70.8 (n=8)	53-106 μmol/ l
CRP (mg/l)	NA (n=0)	6.9 (n=1)	0-1.0 mg/dl
ESR (mm/h)	71 (n=2)	23 (n=7)	0-15 mm/ hr
WBC ($x10^9$/l)	5.48 (n=6)	5.93 (n=10)	3.4- 10 x 10^9/ l
Neutrophils ($x10^9$/l)	3.36 (n=6)	3.44 (n=10)	1.8-6.8 x 10^9/ l
Lymphocytes ($x10^9$/l)	1.6 (n=6)	1.53 (n=10)	0.9-2.9 x 10^9/ l
C3 (mg/l)	870 (n=1)	1007 (n=3)	640-1660 mg/ l
C4 (mg/l)	320 (n=1)	180 (n=3)	150-450 mg/ l

Table 3.2.9 Blood tests done around date of blood collection of SLE patients from SGH. Results in red indicate the abnormal range of readings. All the WBC, Neutrophils and Lymphocytes counts were safe in normal range.

3.2.9 Discussion

The age of these female patients tended to be older than the expected child-bearing years as they were diagnosed with SLE earlier and had disease duration of more than 5 years. In light of this, most patients had mild lupus and this explains the low BILAG Score of mostly C, D and E. Immunosuppressive drug therapy also plays a role in the low lupus activity which causes them to have very minimal or undetected changes in their lupus activity. Nevertheless, patients in subgroup v8.1 displayed a relatively higher BILAG Scores suggesting a higher lupus activity than prognostic subgroup v8.2.

Comparing to SGH SLE patients in subgroup v8.2, prognostic subgroup v8.1 seemed to develop obvious symptoms like malar rash (83%), hematologic disorder (83%), arthritis (67%) and renal disorder (67%). All the three patients who had serositis were also from subgroup v8.1. This supports the view that Singapore SLE patients of prognostic subgroup v8.1 have more serious manifestations.

All the patients from SGH involved in this study were found to have elevated ANA and Anti-dsDNA readings. However, a negative ANA test alone does not rule out SLE, but alternative diagnoses should be considered. Patterns of staining of ANA may give some clues to diagnoses but these patterns may change with serum dilution. Only the rim (peripheral) pattern is highly specific for SLE. ANA test should be used only when there is clinical evidence of a connective tissue disease. In this case, Anti-dsDNA is a more specific (95% specificity) test than ANA as it is more specifically targeted by IgG and IgM antibodies directed against host double-stranded DNA, rather than measuring heterogeneous antinuclear

antibodies in patient's serum in ANA diagnosis. Titers of Anti-dsDNA correlate well with disease activity and with occurrence of glomerulonephritis and Anti-dsDNA is not found in drug-induced SLE.

88% of SLE patients from SGH who were on Hydroxycholoquine on day of blood collection were from subgroup v8.2. Does this suggest that Hydroxycholoquine reduces the manifestations of the disease? This needs to be confirmed with a large patient population. The possibility of subgroup switching also needs to be monitored. Anegret and colleagues described that corticosteroids and cyclophosphamide could significantly restore the decreased number of T reg cells (Kuhn*, et al* 2009). But none of the patients involved in this study was on these drugs at the time of blood collection. Two other groups found a significant increase of CD4+CD25+ Treg in cell proportion following B cell depletion with Rituximab in lupus patients (Sfikakis*, et al* 2007, Vallerskog*, et al* 2007). But unfortunately there was no SGH patient involved who was on Rituximab for us to verify this observation in our study.

ESR readings were generally high for both subgroups, suggesting the possibility of infections and inflammatory disease in these SLE patients which causes the aggregations of erythrocytes in plasma settle rapidly. However, even though there is a good correlation between ESR and Cr, Cr levels in these patients were mostly in the normal range. This is due to the incomplete data collection and the mean levels analyzed were performed on different patients. WBC and Lymphocytes counts of these SLE patients were safely in the normal range, suggesting that current infection and inflammation was involved. The neutrophil count was surprisingly in the normal range of 1.8-6.8 x 10^9 cells/l, despite the fact the high level of ANA found in the serum. ANA are produced in SLE or other autoimmune disease and

destroy neutrophils and subsequently reduce the number of neutrophils in blood. The concentration of C3 and C4 of these patients was also in the normal range and the expected increased level of C3 and decreased level of C4 were not seen in these patients.

CHAPTER 4

RESULTS PART 2

4.1 T Lymphocyte Analysis

4.1.1.1 Extracellular Staining Analysis for IL7R, CD25 and CXCR6

100ul of whole blood was lysed and stained with anti-CD3, CD8, CD45RA and CD62L to give four T cell populations: T naïve (Tn), T central memory (Tcm), T effector memory (Tem) and T revertant memory (Temra), as shown in **Figure 4.1.1**.

Proportions of all four CD8 and CD4 T memory subsets, particularly the T memory population (Tcm+ Tem) of the Asian cohort were compared with the UK cohort in both prognostic subgroups v8.1 and v8.2, with and without the inclusion of healthy controls.

Samples were also stained to quantify expression of IL7R, CD25 and CXCR6 respectively. The objectives of quantifying these markers are elaborated in later sub-chapters. But generally the data analysis for T lymphocytes is depicted in **Figure 4.1.1,** based on a single patient's data. Whole blood cells were lysed and stained with multicolour antibodies. CD3+CD4+ and CD3+CD8+ populations were later gated to further analyze memory T subsets using CD62L versus CD45RA.

In multicolor flow experiments, it is not possible to set gates based on an entirely unstained or fully isotype stained control. A control is defined as changing one variable condition at a time. Fluorescence Minus One (FMO) controls leave out one reagent at a time, it acts like the opposite of single colour controls. Thus FMO method was used as gating strategy in the analysis of expression quantification, as shown in **Figure 4.1.2**. Mean Fluorescence Intensity (MFI) for each histogram was collected in geometric mean.

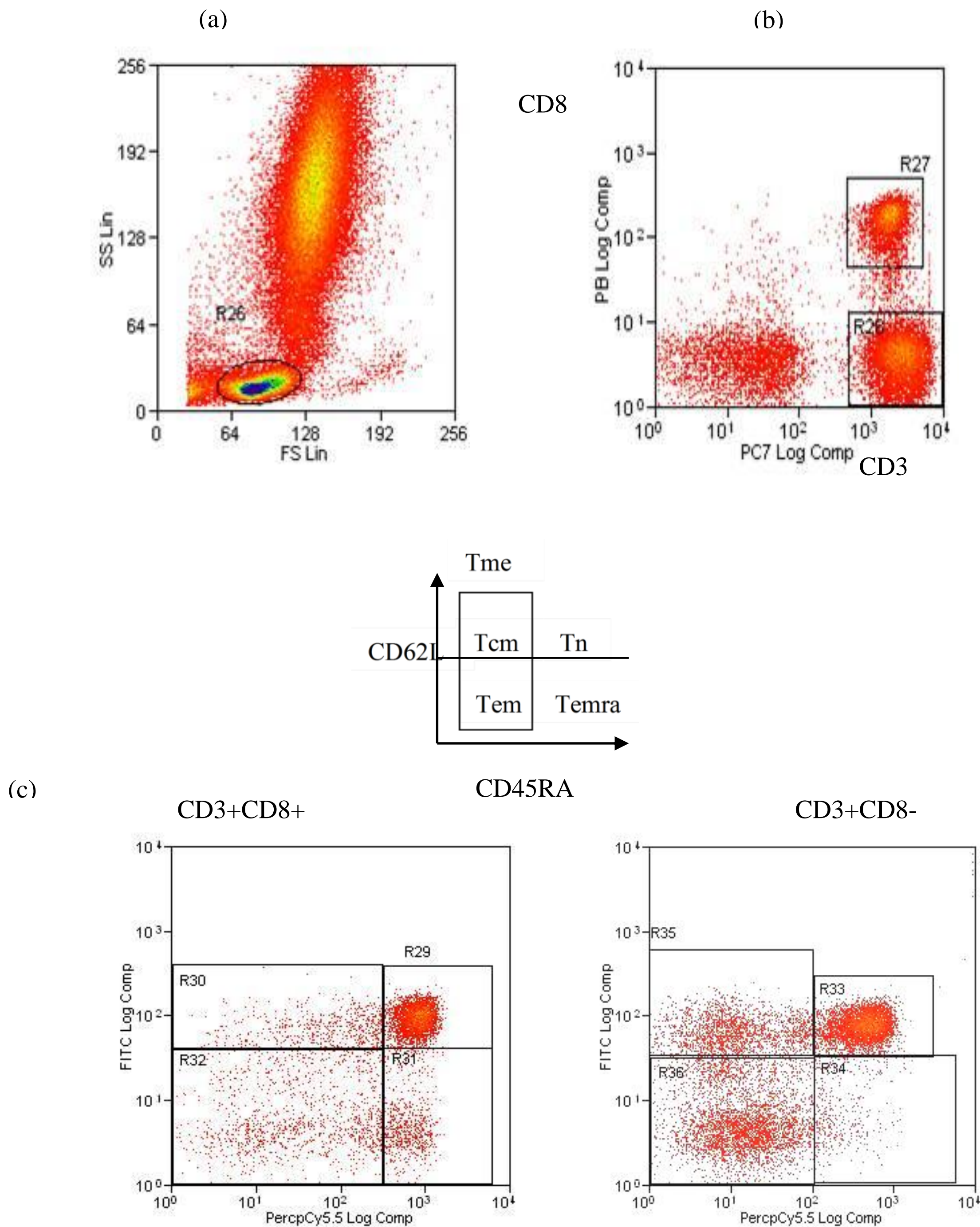

Figure 4.1.1 FACS data analysis for T lymphocytes. Whole blood cells from a patient were lysed and stained with monoclonal antibodies specific for CD3-PC7, CD8-PB, CD45RA-PerCpCy5.5 and CD62L-FITC, samples were acquired using a Beckman Coulter CyAn. (a) Lymphocyte population gated in scatter plot was then applied to (b). CD3+CD8+ and CD3+CD8- populations were then applied in (c) to characterize four subsets of T Memory populations. CD62L^{hi}CD45RA^{hi} cells were termed T naïve, CD62L^{hi}CD45RA^{lo} cells were termed T central memory cells, CD62L^{lo}CD45RA^{lo} cells were termed T effector memory cells and CD62L^{lo}CD45RA^{hi} cells were termed T revertant cells. T memory cells are a combination of T central memory and T effector cells.

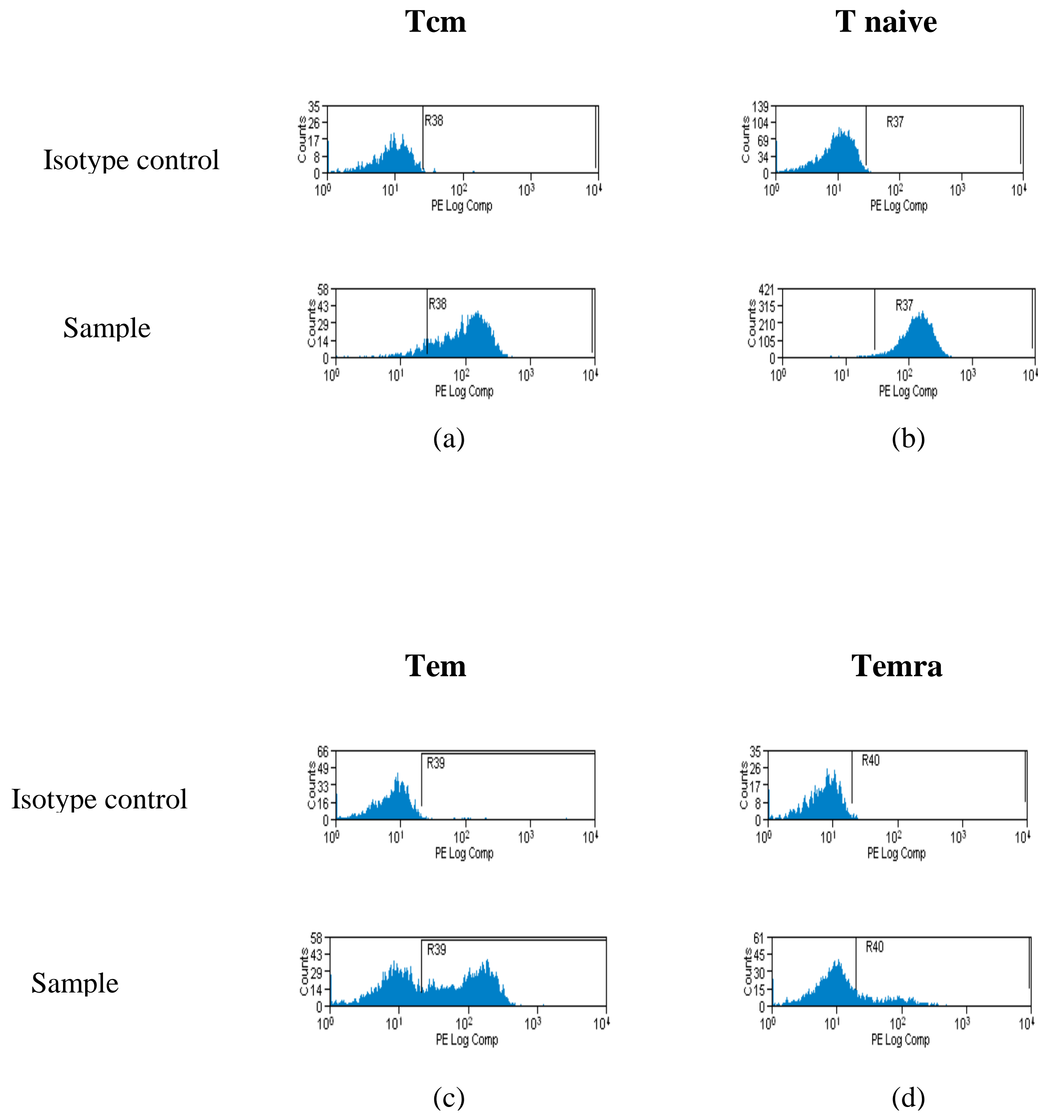

Figure 4.1.2 Gating Strategy for T Lymphocytes Analysis. Lysed whole blood cells were stained with monoclonal antibodies specific for CD3-PC7, CD8-PB, CD45RA-PerCpCy5.5, CD62L-FITC and PE-conjugates (IL7R or CD25 or CXCR6). Positive gatings were established using fluorescence minus one (FMO) isotype controls as shown in the histograms for T central memory (a), T naïve (b), T effector memory(c) and T revertant cells (d). Mean Fluorescence Intensity (MFI) for each histogram is the geometric mean of the gated histogram.

4.1.1.2 CD8 and CD4 T Memory Subsets

Comparisons between the UK and Asian SLE cohorts (with and without healthy controls) for CD8 and CD4 T memory subsets proportions in prognostic subgroups v8.1/4.1 or v8.2/ v4.2 were laid out from **Figure 4.1.3 to 4.1.6**. Red dots represents the active SLE patients from NUH.

Compared to the UK SLE cohort, the Asian cohort displays a different trend of CD8 T memory cells. The Singapore SLE cohort had almost double the proportion of naïve CD8 T cells in both prognostic subgroups, 40% in Asian 20% in UK cohort (**Figure 4.1.3 (a) & (c)**). Patients in subgroup v8.1 had one third less CD8 T memory cells (a combination of CD8 T central memory, CD45RA-CD62L+ and CD8 T effective memory CD45RA- CD62L- cells). However, both UK and Singapore SLE cohort had a similar range of CD8 Temra (CD45RA+CD62L-) cells. Inclusion of healthy controls did not change the profile of Singapore SLE cohort significantly **(Figure 4.1.3 (b)**).

Comparisons between v8.1 and v8.2 in T memory population (T central memory and T effector memory cells) are highlighted in **Figure 4.1.4**. It is reported that subgroup v8.1 has a higher CD8 T memory population compared to subgroup v8.2 in the UK cohort (**Figure 4.1.4 (c)**). However this does not occur in Singapore cohort with (**Figure 4.1.4 (b)**) and without (**Figure 4.1.4 (a)**) the inclusion of healthy donors. The Singapore cohort had a homogenous distribution of CD8 T memory cells in both prognostic subgroups.

Referring to **Figure 4.1.5**, the proportion of CD4 T memory subsets was very much similar to the UK CD8 T memory subsets profile, regardless the inclusion of healthy controls’ data, as

shown in **Figure 4.1.5**. There were generally more CD4 T memory cells and a smaller CD4 T naïve cell proportion in subgroup v4.1 than v4.2. Similar to the UK cohort, a higher CD4 T memory population in subgroup v4.1 is clearly seen in **Figure 4.1.6 (a) & (b)** and this is statistically significant. This shows the possibility of CD4 being a better gene signature for Asian cohort than CD8.

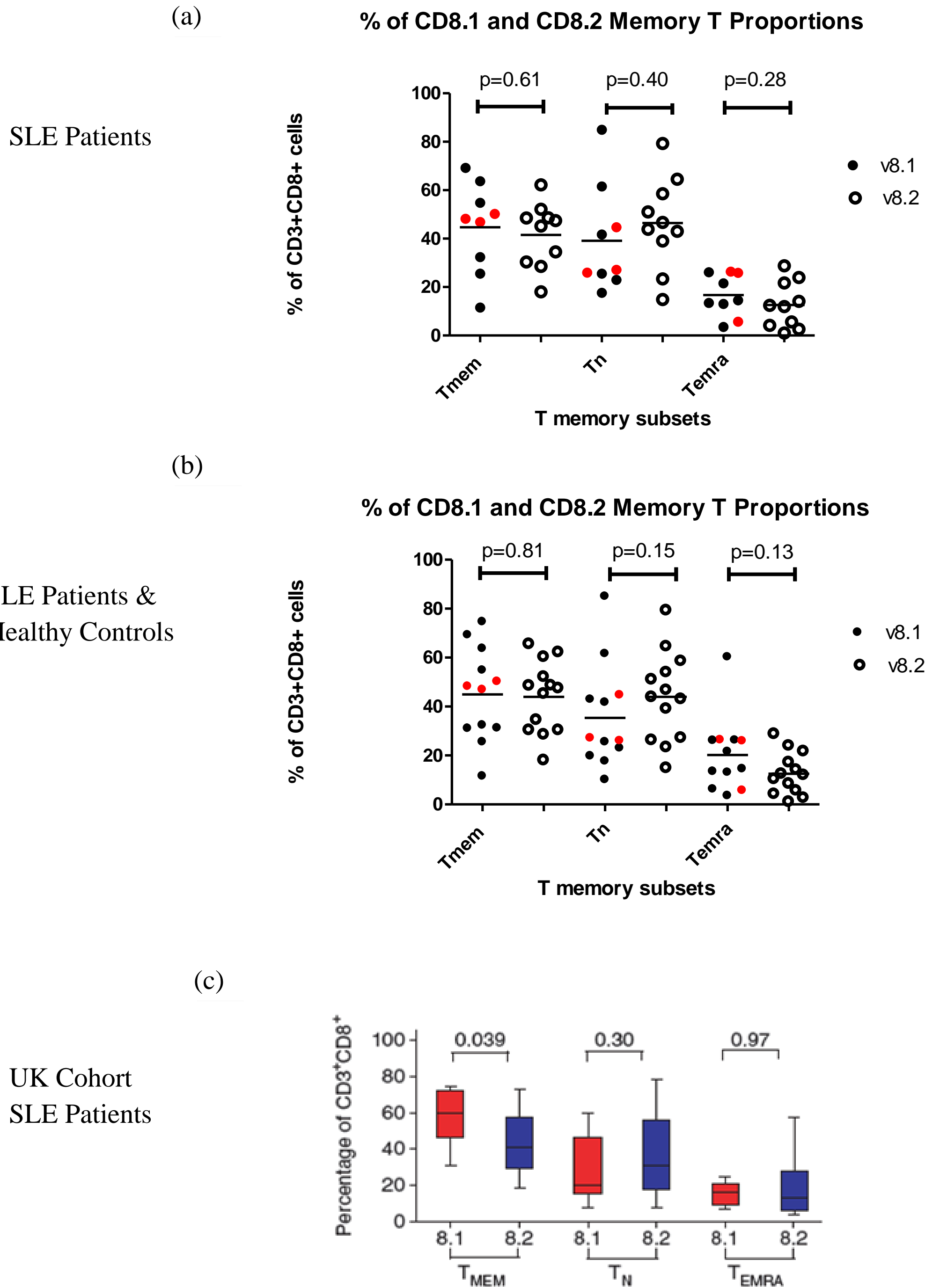

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Figure 4.1.3 Comparison of CD8 T memory subsets in Asian and UK cohort. Proportions of CD8 T memory subsets of Asian cohort in prognostic group v8.1 and v8.2, without (a) and with (b) the inclusion of data of healthy controls, as compared to UK cohort (c). The Asian cohort displays a different trend of CD8 T memory cell subset proportions compared to the UK cohort. The Asian cohort has double the proportion of T naive cells: 40% in Asian (a): 20% in UK cohort(c). The Asian also displayed a lower T memory populations (T central memory + T effector memory cells) in prognostic group v8.1. Both UK and Asian cohorts display a similar trend of CD8 T memory subsets proportions in prognostic group v8.2.

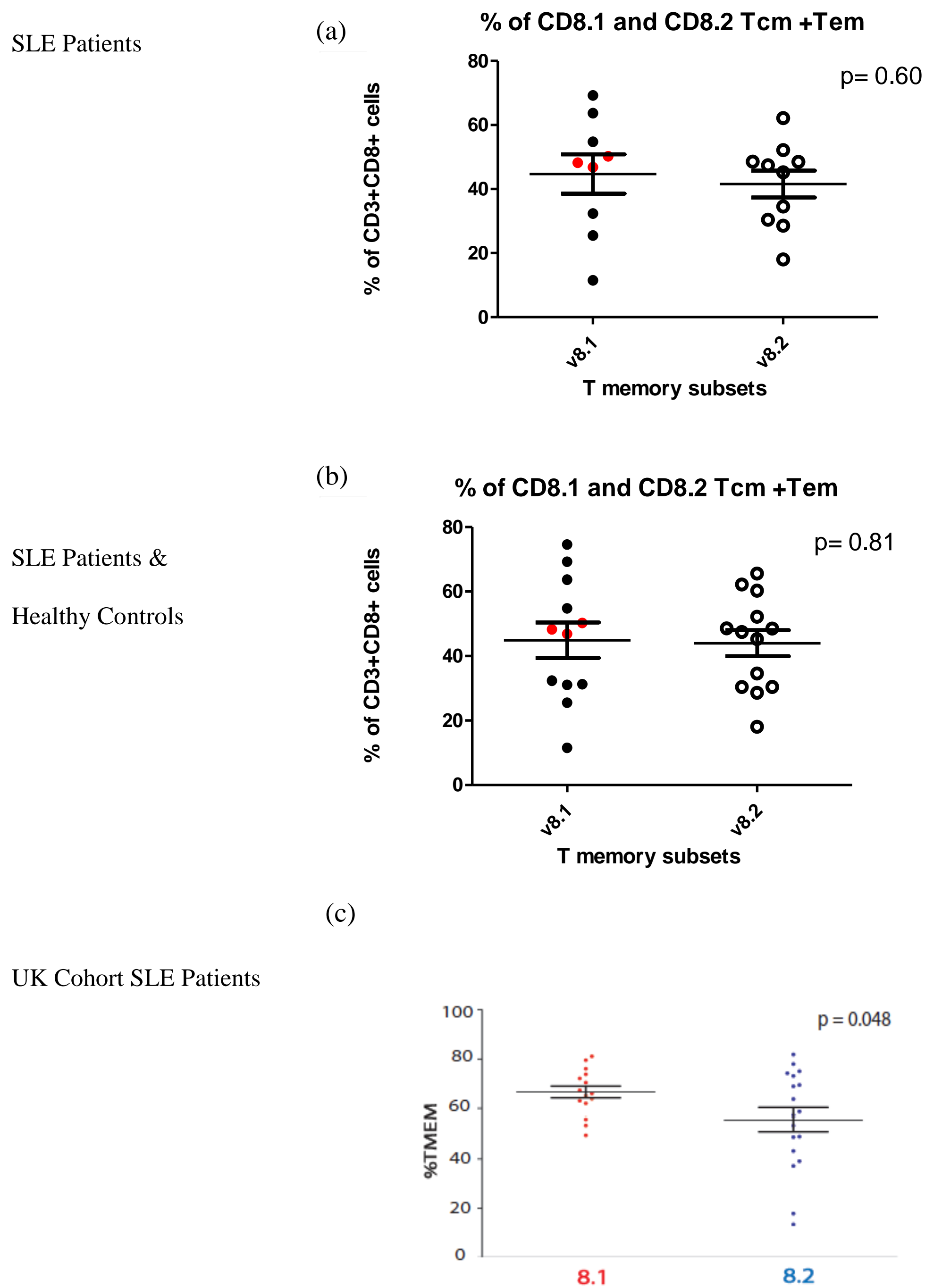

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Figure 4.1.4 CD8 T memory (Tcm +Tem) comparison between Asian and UK cohort. Asian cohort v8.1 has a slightly higher CD8 T memory cells (Tcm +Tem) percentage compared to v8.2 (a)(b), similar to the trend shown in the published data by Cambridge (c). But they are not statistically significant.

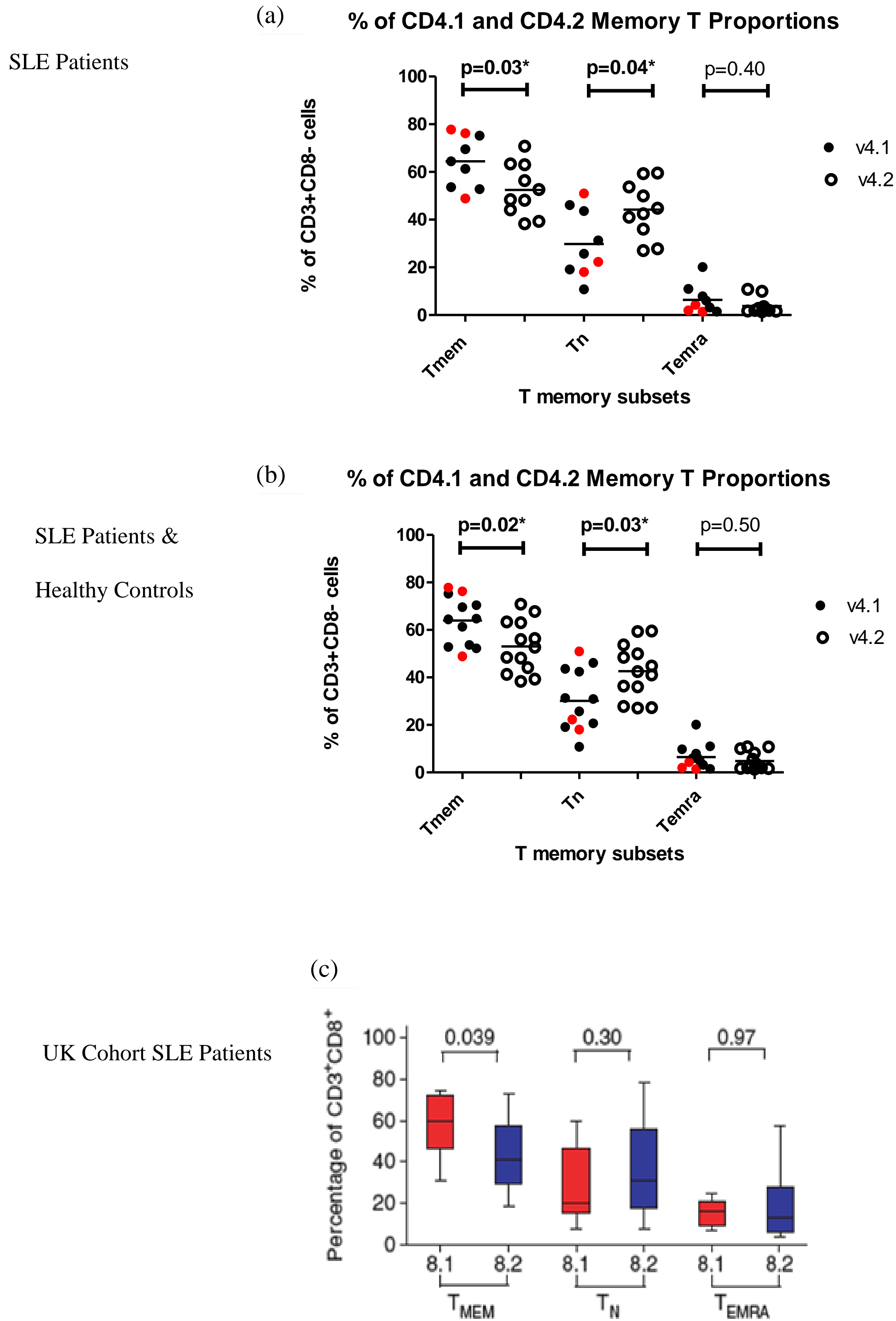

Figure 4.1.5 Comparison of CD4 T memory subsets in Asian and UK cohort. Proportions of CD4 T memory subsets of Asian cohort in prognostic group v4.1 and v4.2, without (a) and with (b) the inclusion of data of healthy controls, as compared to CD8 T memory subsets of UK cohort (c). Asian cohort displays a similar trend of CD4 T memory cell subset proportions compared to the UK cohort.

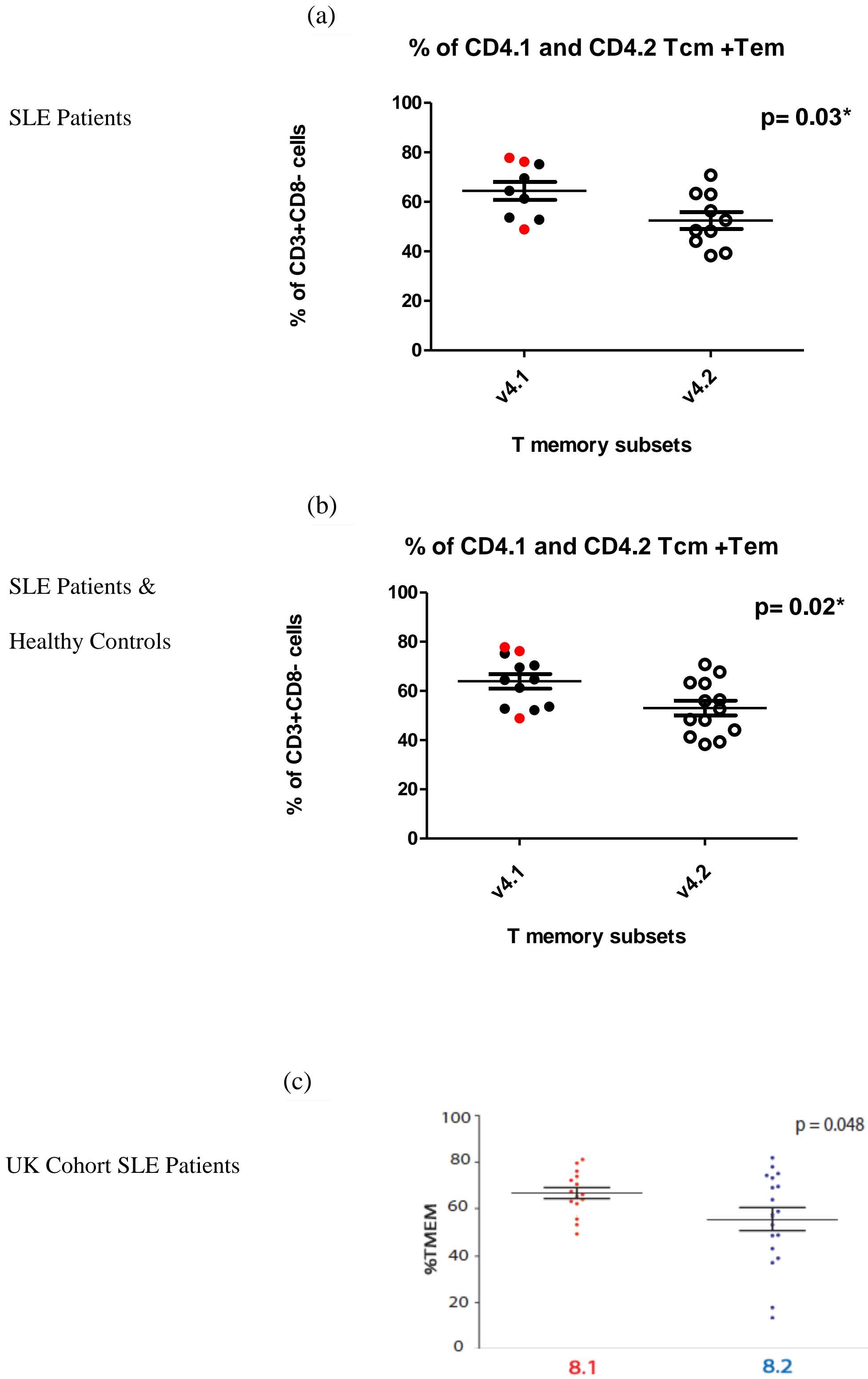

Figure 4.1.6 CD4 T memory (Tcm +Tem) comparison between Asian and UK cohort. Like the UK cohort, the Asian cohort v4.1 has a slightly higher CD4 T memory cells (Tcm +Tem) percentage compared to v4.2 (a)(b), similar to the trend shown in the published data by Cambridge (c). They are statistically significant.

4.1.1.3 Quantification of IL7R Expression

Memory T cell precursors are present at the peak of the immune response. But memory T cells do not display their function, such as survival, which are progressively acquired with transcriptional signatures as the antigen is cleared (Kaech*, et al* 2002). Effector T cells which do not persist long-term in the absence of antigen, are unable to undergo homeostatic proliferation as they fail to acquire key properties of memory cells like IL7R at early time points.

The survival of memory T cells is dependent on the expression of anti-apoptotic molecules like Bcl2 (Rathmell and Thompson 2002) and the cells' capacity to respond to homeostatic cytokines like IL-7 which enhances survival (Li*, et al* 2003). IL7R expression identified the memory precursors at early time points. IL7R hi cells were found to contain high amounts of anti-apoptotic molecules and conferred protective immunity (Huster*, et al* 2004, Kaech*, et al* 2003).

As displayed in **Figure 4.1.7**, no significant difference between the expression levels (measured by Mean Fluorescence Intensity, MFI) was detected between subgroups v8.1/ 4.1 and v8.2/ 4.2 in both CD8 and CD4 T subsets. However, CD4 T memory and CD4 T naïve subsets shows a higher cell proportions expressing IL7R+ in v4.1 than in v4.2 (**Figure 4.1.8 (b) & (d)**).

CD8 T Memory **CD4 T Memory**

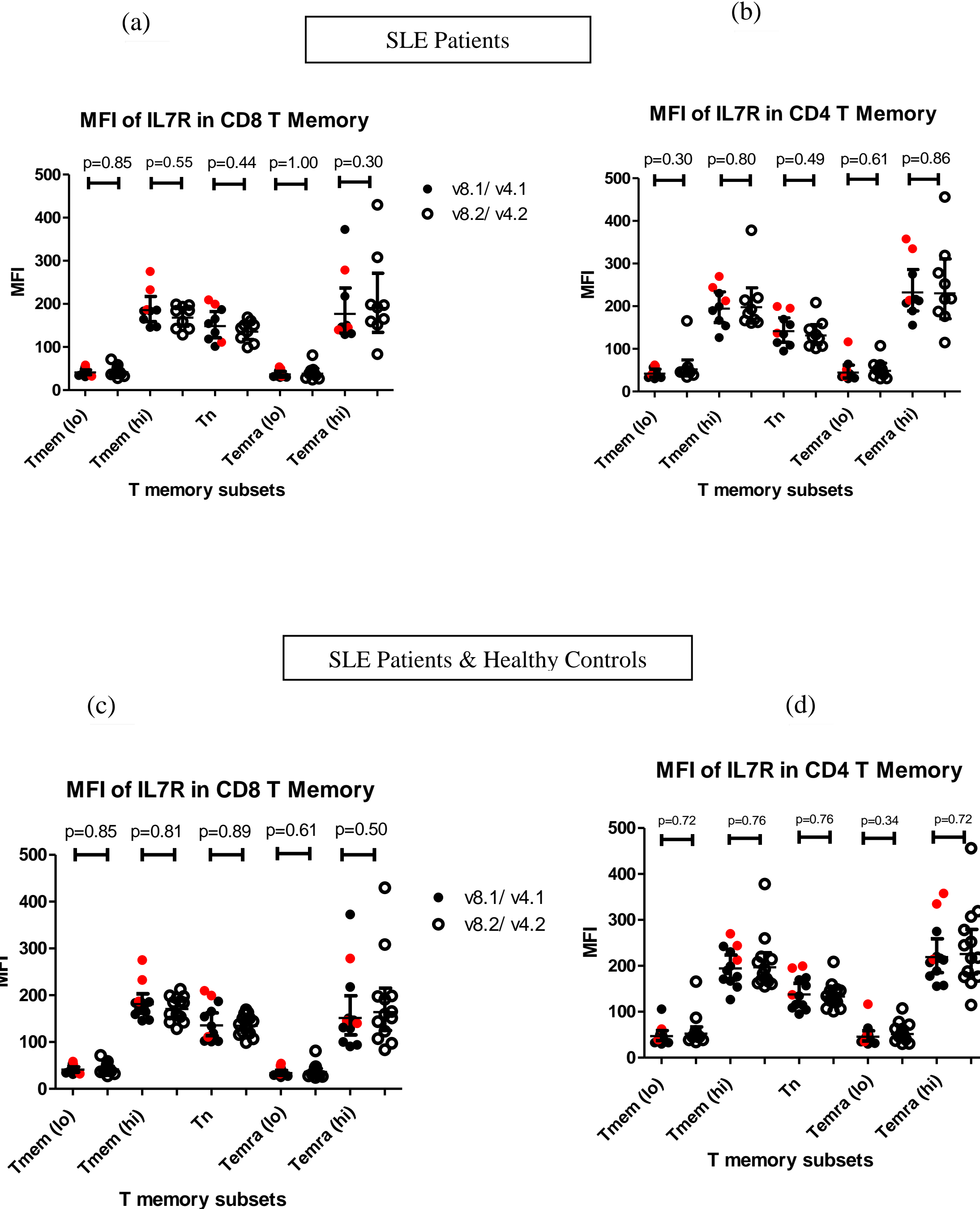

Figure 4.1.7 Expression of IL7R in CD4 and CD8 T Memory subsets, between v8.1 and v8.2. Geometric Mean fluorescence intensity (MFI) of cells expressing IL7R+ in CD8 (a, c) and CD4 T (b, d) memory subsets, with (a, b) and without (c, d) the inclusion of healthy controls.

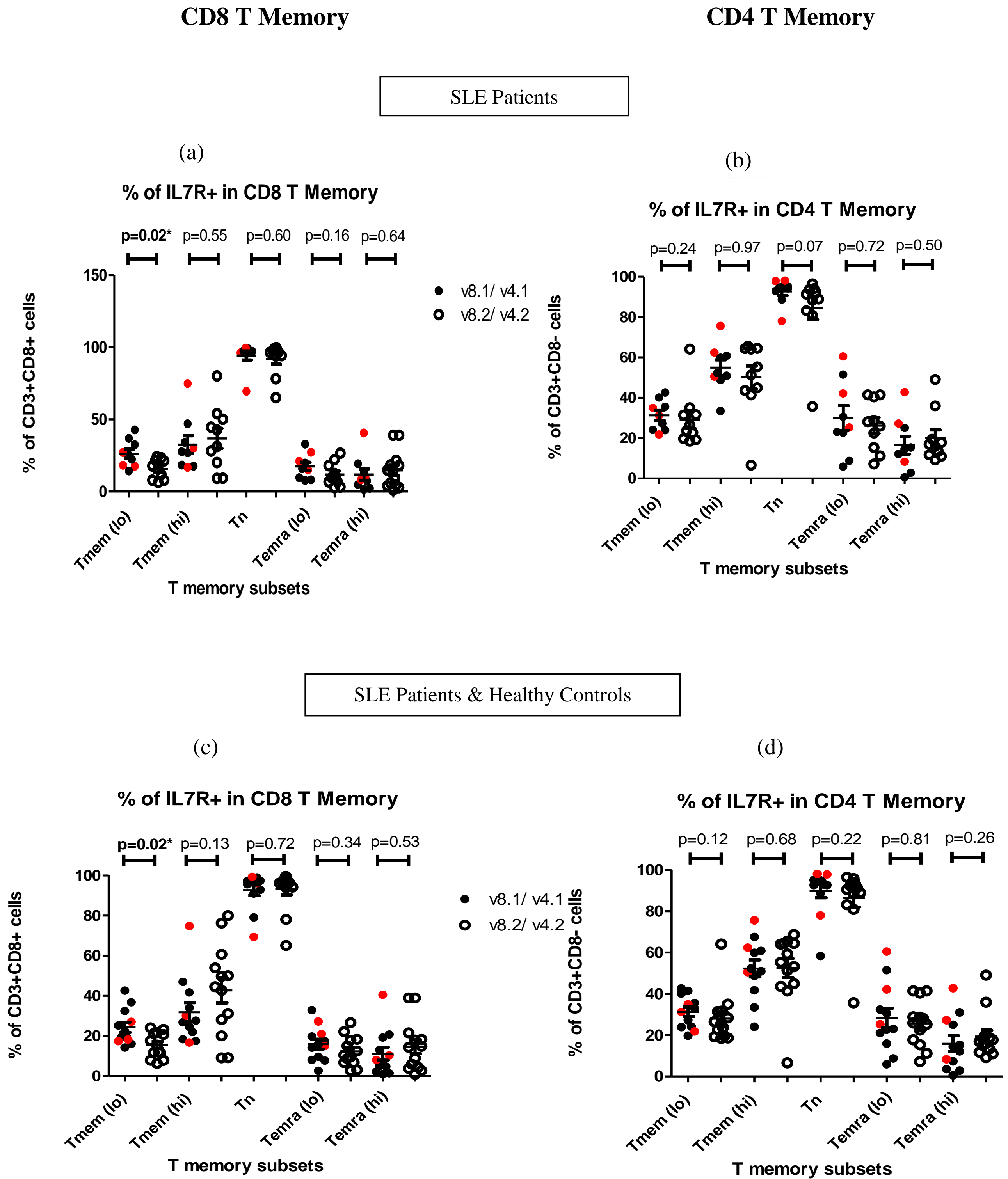

Figure 4.1.8 Cell proportions of IL7R+ CD4 and CD8 T Memory subsets, between v8.1 and v8.2. Proportions of cells expressing IL7R+ in CD8 (a, c) and CD4 T (b, d) memory subsets, with (a, b) and without (c, d) the inclusion of healthy controls.

4.1.1.4 Quantification of CD25 Expression

Quantification of CD25 (also termed as IL2R), is an indicator of T lymphocyte activation that can be used to track disease activity and progression (Smith 1988, Smith 1990). CD25 is the alpha chain of the IL-2 receptor. It is a type I transmembrane protein present on activated T cells and B cells, on macrophages, and on a subset of non-activated $CD4^+$ regulatory T cells. These membrane-bound molecules CD25 (55-65kDa in size) are expressed and released in soluble form, sIL2R (a truncated form of its receptor, 45-55kDa in size) by activation of T lymphocytes (Nelson*, et al* 1986, Rubin*, et al* 1986). The rate of release of soluble CD25 correlates to its cell surface expression and thus to the level of activation of the T lymphocytes (Rubin*, et al* 1986, Symons*, et al* 1988). Serum levels of soluble CD25 are claimed to be proportional to the disease activity in patients with SLE (Spronk*, et al* 1994, ter Borg*, et al* 1990).

Both CD8 and CD4 T subsets (except CD8 Temra) of subgroup v8.2/ 4.2 exhibited elevated expression of CD25 (**Figure 4.1.9**). But this data was not statistically significant. On the contrary, prognostic subgroup v8.1/ 4.1 generally gives a slightly higher cell percentage in both CD8 and CD4 T subsets, as shown in **Figure 4.1.10**.

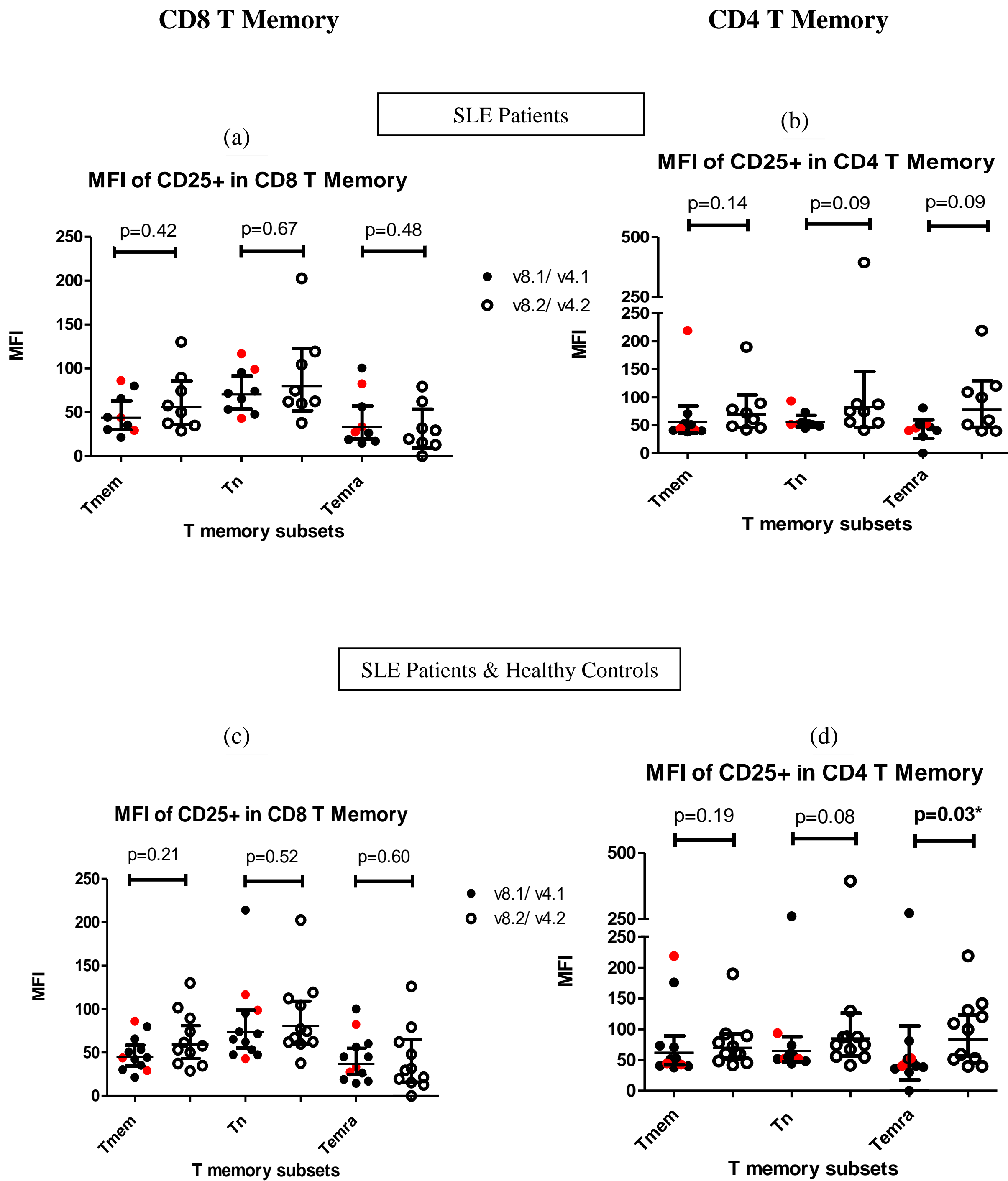

Figure 4.1.9 Expression of CD25 in CD4 and CD8 T Memory subsets, between v8.1 and v8.2. Mean fluorescence intensity (MFI) of cells expressing CD25+ in CD8 (a, c) and CD4 T (b, d) memory subsets, with (a, b) and without (c, d) the inclusion of healthy controls.

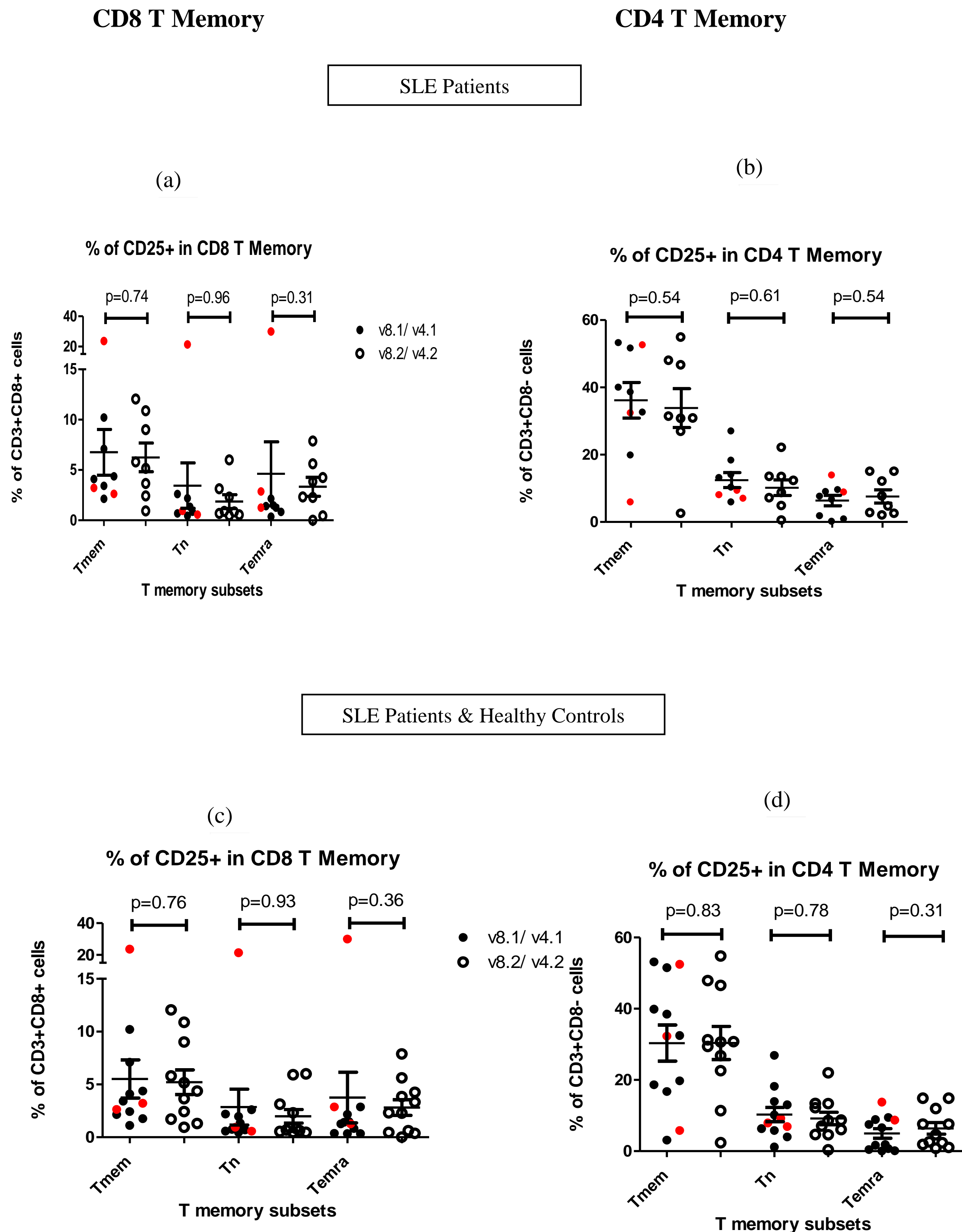

Figure 4.1.10 Cell proportions of CD25+ CD4 and CD8 T Memory subsets, between v8.1 and v8.2. Proportions of cells expressing CD25+ in CD8 (a, c) and CD4 T (b, d) memory subsets, with (a, b) and without (c, d) the inclusion of healthy controls.

4.1.1.5 Quantification of CXCR6 Expression

CXCR6 is a unique receptor for CXCL16, a chemokine expressed on the cell surface as membrane-bound molecules (Shimaoka*, et al* 2004). Based on conserved cysteine motifs, chemokines which induce leukocyte migration are generally classified as C, CC, CXC, CX3C chemokines (Yoshie*, et al* 2001). It has been shown that CXCR6 is expressed on the peripheral blood T cells of Th1 phenotype, NK cells and B cells (Kim*, et al* 2001). Toshihiro and colleagues described that CXCL16 play an important role in T cell migration and that it was stimulated in the synovium of patients with RA (Nanki*, et al* 2005). They also found that CXCR6 was expressed more frequently on synovial T cells of RA patients rather than in peripheral blood and stimulation of these cells with IL-15 will increase expression of CXCR6. Sato and colleagues observed that CXCR6 was required for lymphocyte proliferation or amplification of the inflammatory reaction. CXCR6 also facilitates effector CD8 T cell localization from blood into sites of pathological inflammation and thus contributes to the recruitment of activated lymphocytes into an inflamed liver (Sato*, et al* 2005).

There was generally no significant difference shown in CXCR6 expressions in both subgroups in both the CD8 and CD4 T subsets. However, expression of CXCR6 appeared higher in CD4 T naïve of subgroup v4.2; regardless the inclusion of healthy controls (**Figure 4.1.11**). But the data was not statistically significant. The CD4 T naïve cell proportion expressing CXCR6+ also appeared to be higher in subgroup v4.2 than subgroup v4.1. Generally across the cell proportion expressing CXCR6+ data, subgroup v8.2/4.2 appeared to be a higher proportion of T subsets (**Figure 4.1.12**).

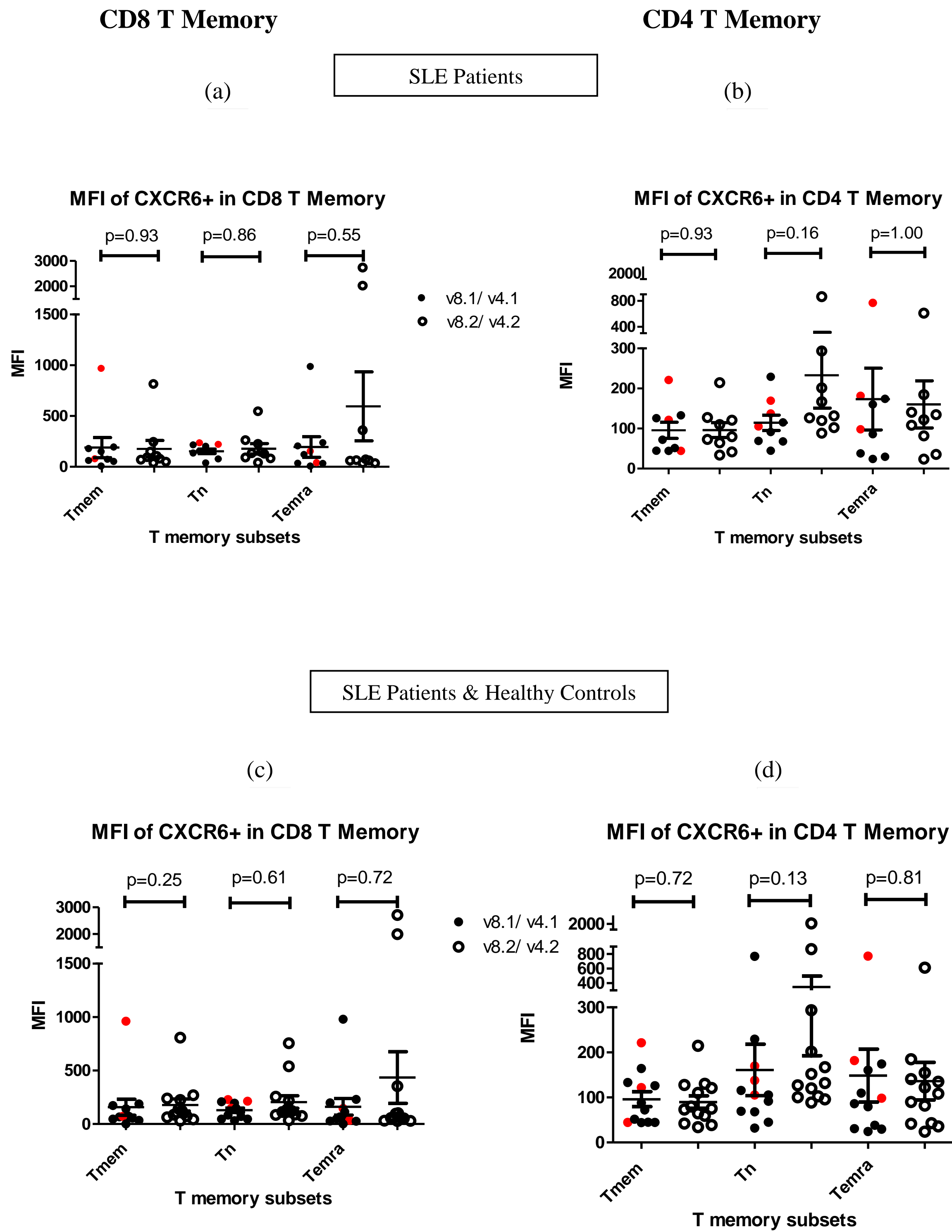

Figure 4.1.11 Expression of CXCR6 in CD4 and CD8 T Memory subsets, between v8.1 and v8.2. Mean fluorescence intensity (MFI) of cells expressing CXCR6+ in CD8 (a, c) and CD4 T (b, d) memory subsets, with (a, b) and without (c, d) the inclusion of healthy controls.

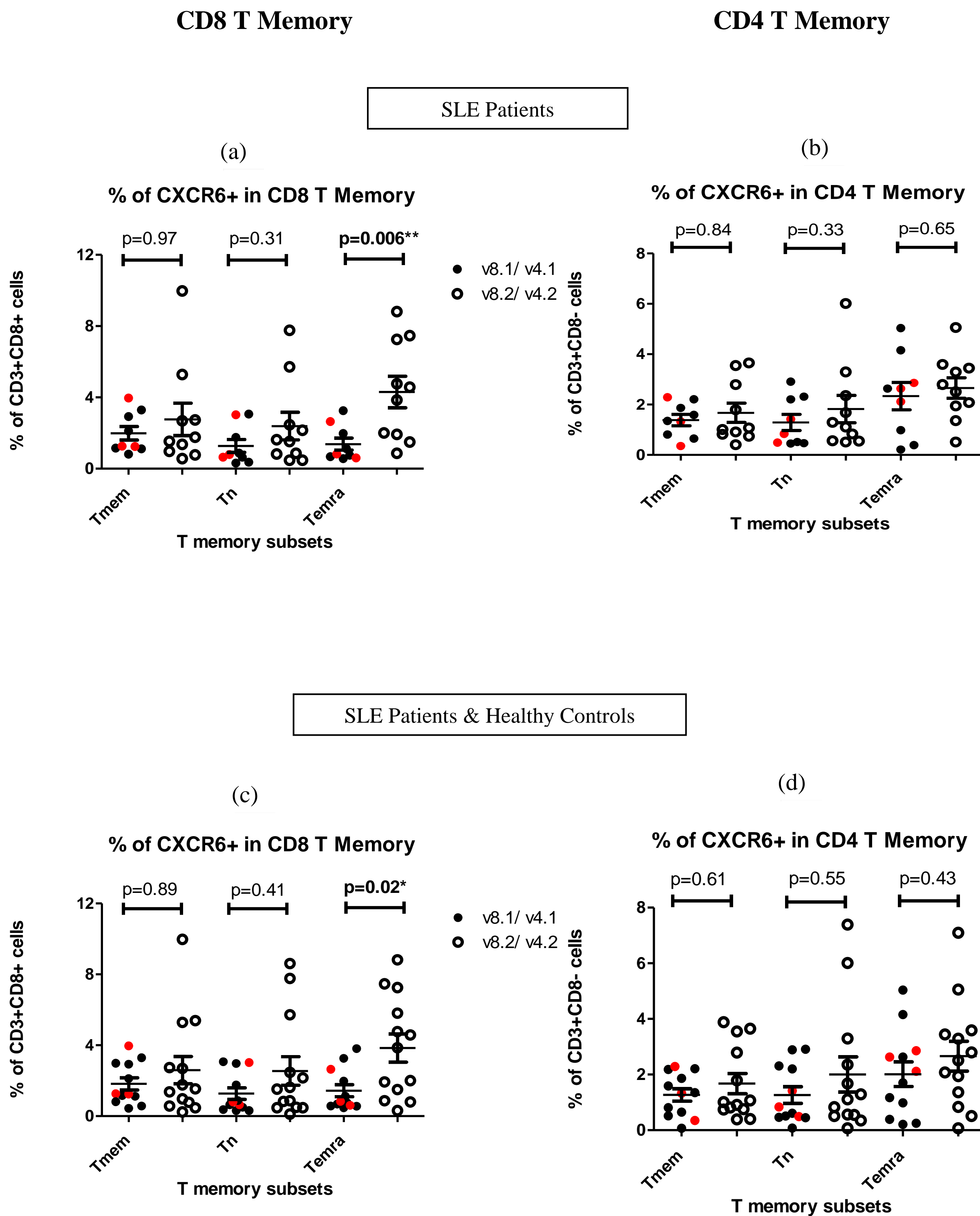

Figure 4.1.12 Cell proportions of CXCR6+ CD4 and CD8 T Memory subsets, between v8.1 and v8.2. Proportions of cells expressing CXCR6+ in CD8 (a, c) and CD4 T (b, d) memory subsets, with (a, b) and without (c, d) the inclusion of healthy controls.

4.1.2 Intracellular Bcl2 Analysis

Bcl2 is expressed in a variety of hematopoietic lineages including T cells, it is a proto oncogene which was identified due to its involvement in non-Hodgkin B cell lymphoma. A t(14:18) interchromosomal translocation juxtaposes the Bcl2 gene which consequently leads to transcription of high levels of Bcl2 (Graninger*, et al* 1987) and eventually enhances cell survival. The intracellular protein is found on the mitochondrial membrane (Hockenbery*, et al* 1990), perinuclear membrane and endoplasmic reticulum (Alnemri*, et al* 1992, Jacobson*, et al* 1993). Bcl2 appears to enhance lymphoid cell survival by interferring with apoptosis rather than promoting cell propagation (Rose*, et al* 1994).

Ohsako described that expression of Bcl2 in T lymphocytes from SLE patients was significantly higher, compared to inactive SLE patients and healthy individuals (Ohsako*, et al* 1994). In autoimmune mice, the abnormal expression of a number of genes, including Bcl-2 gene influence apoptosis have been identified (Veis*, et al* 1993). The aberrant expression of these genes (also called autogenes) is believed to result in defective apoptosis (Talal 1994) (Mountz*, et al* 1994) and development of maglinancy. Studies have shown that SLE might be due to the failure of immune system to eliminate autoreactive immune cells, which leads to the abnormal longetivity of these cells and elevated autoantibody production (Ohsako*, et al* 1994, Rose*, et al* 1994).

The profile pattern of Bcl2 expression in the Singapore cohort CD8 T memory subsets (**Figure 4.1.14(a) & (d)**) is very much similar to the UK cohort (**Figure 4.1.14(c)**), but with significant higher fluorescence intensity of Bcl2 expression. Two possibilities to explain the phenomenon are:

- The Asian cohort seems to have one-fold more intense or defective anti-apoptotic Bcl2 expression in T lymphocytes compared to the UK cohort, judging that the MFI readings of Bcl2 expression is twice as high as the UK cohort.

- This does not represent increased Bcl2 expression and could it be simply due to the different signal-to-noise ratio of instruments used in Singapore Immunology Programme and Cambridge Institute of Medical research. This needs to be further clarified by repeating the same sample in both institutes with the same batch of antibodies used, same brand and model of instrument used.

All CD4 and CD8 T memory subsets display higher cell proportions expressing Bcl2+ in subgroup v8.1/4.1 and they are statistically significant, as shown in **Figure 4.1.15**. This indicates that cells in subgroup v8.1/4.1 are more anti-apoptotic than v8.2/4.2.

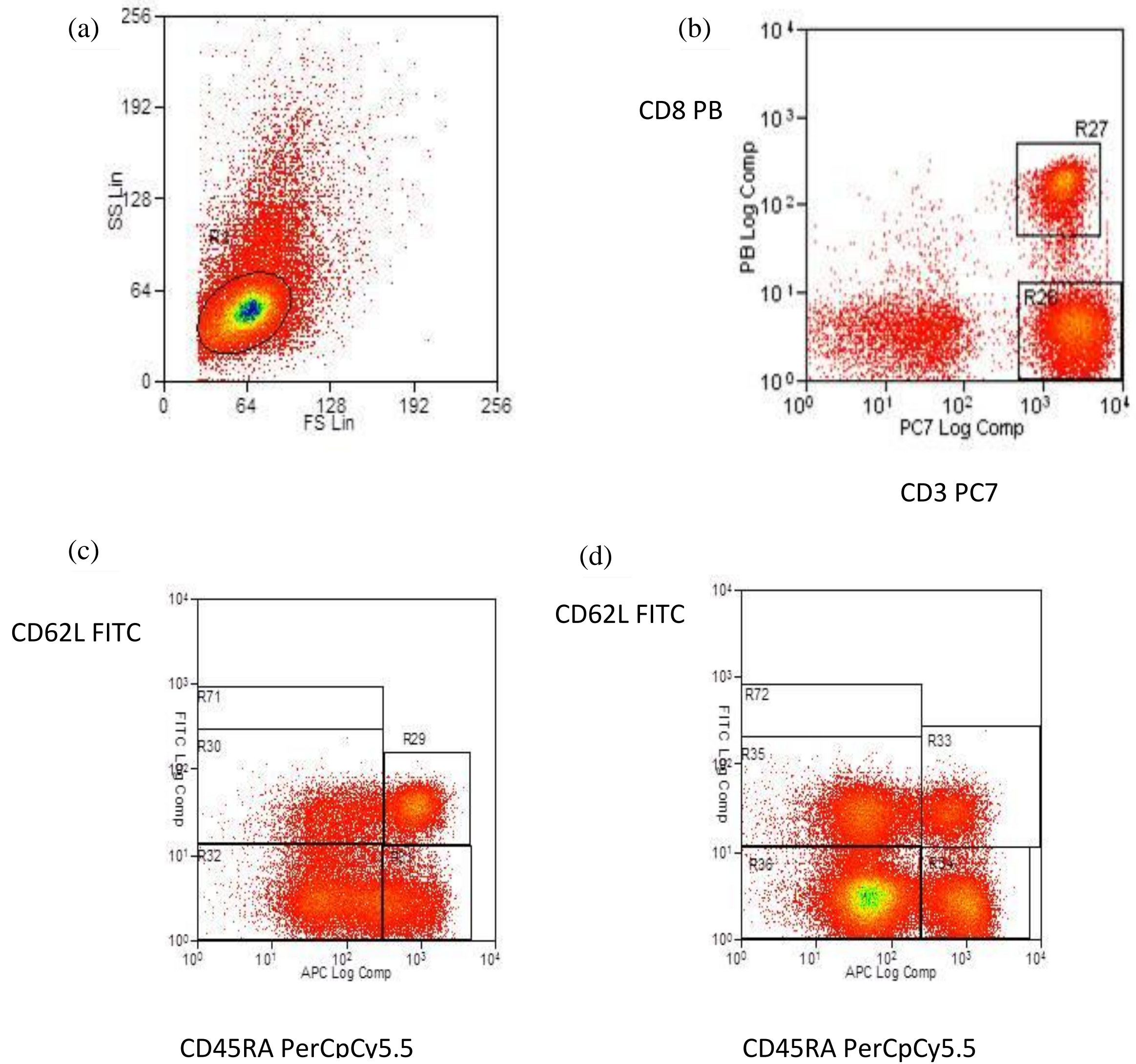

Figure 4.1.13 Example of flow data analysis of ficolled PBMC from a SLE patient for Bcl2 intracellular staining. Lymphocytes were gated in the scatter plot (a) and then applied to CD3 PC7- CD8 PB dot plot (b). CD3+CD8+ population was gated and applied to CD45RA PerCpCy5.5- CD62L FITC dot plot on (c). CD3+CD8- region was applied to CD45RA PerCpCy5.5- CD62L FITC dotplot on (d). Four distinguished T cell memory subsets can be found both dot plots (c) and (d).

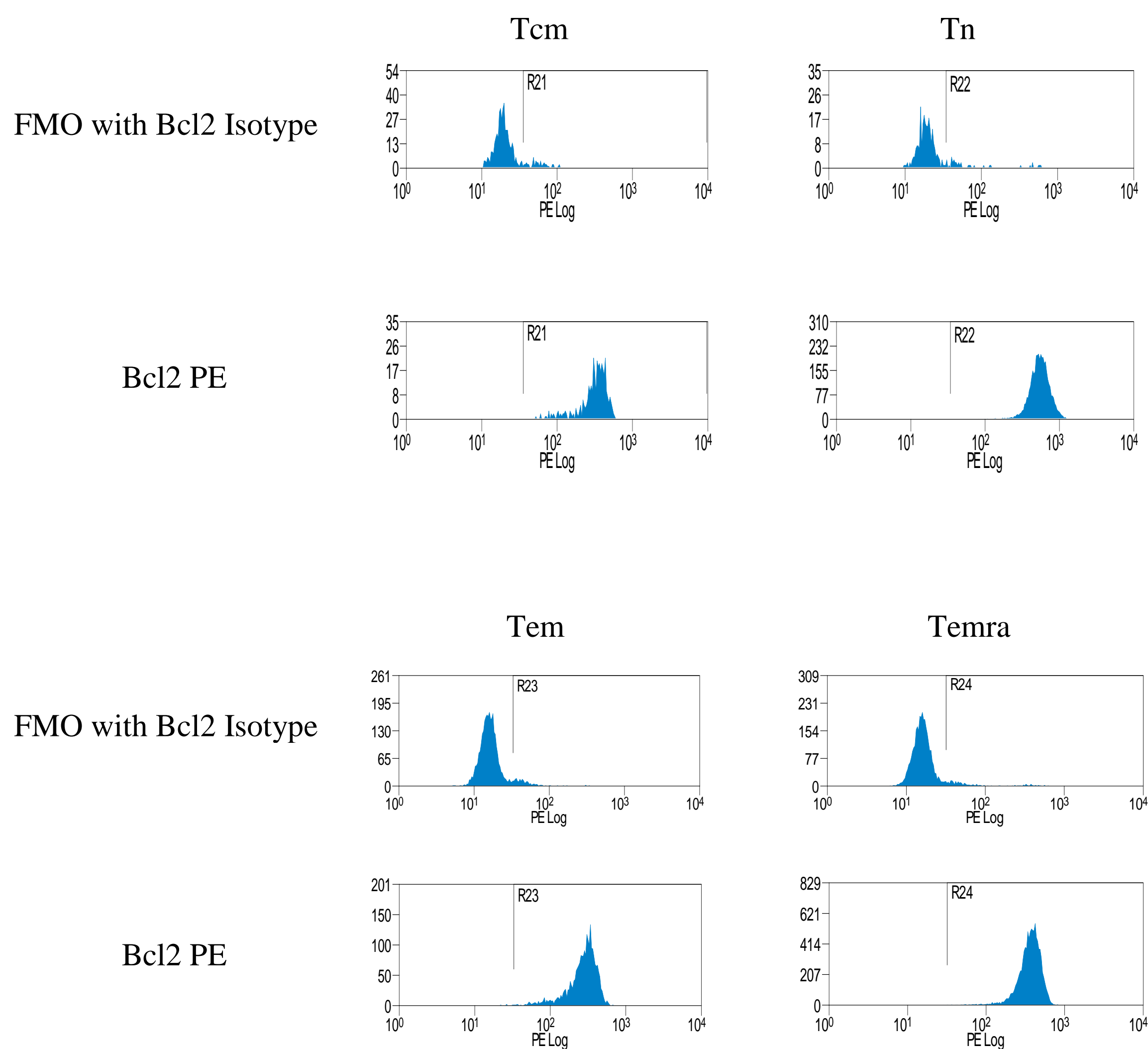

Figure 4.1.14 Example of Bcl2-PE expression analysis in T memory subsets of CD8 Tcells. Fluorescence Minus One (FMO) with Bcl2 isotype served as a baseline to measure Bcl expression in T naive cells, T central memory cells, T effector memory cells and T revertant cells of a healthy donor.

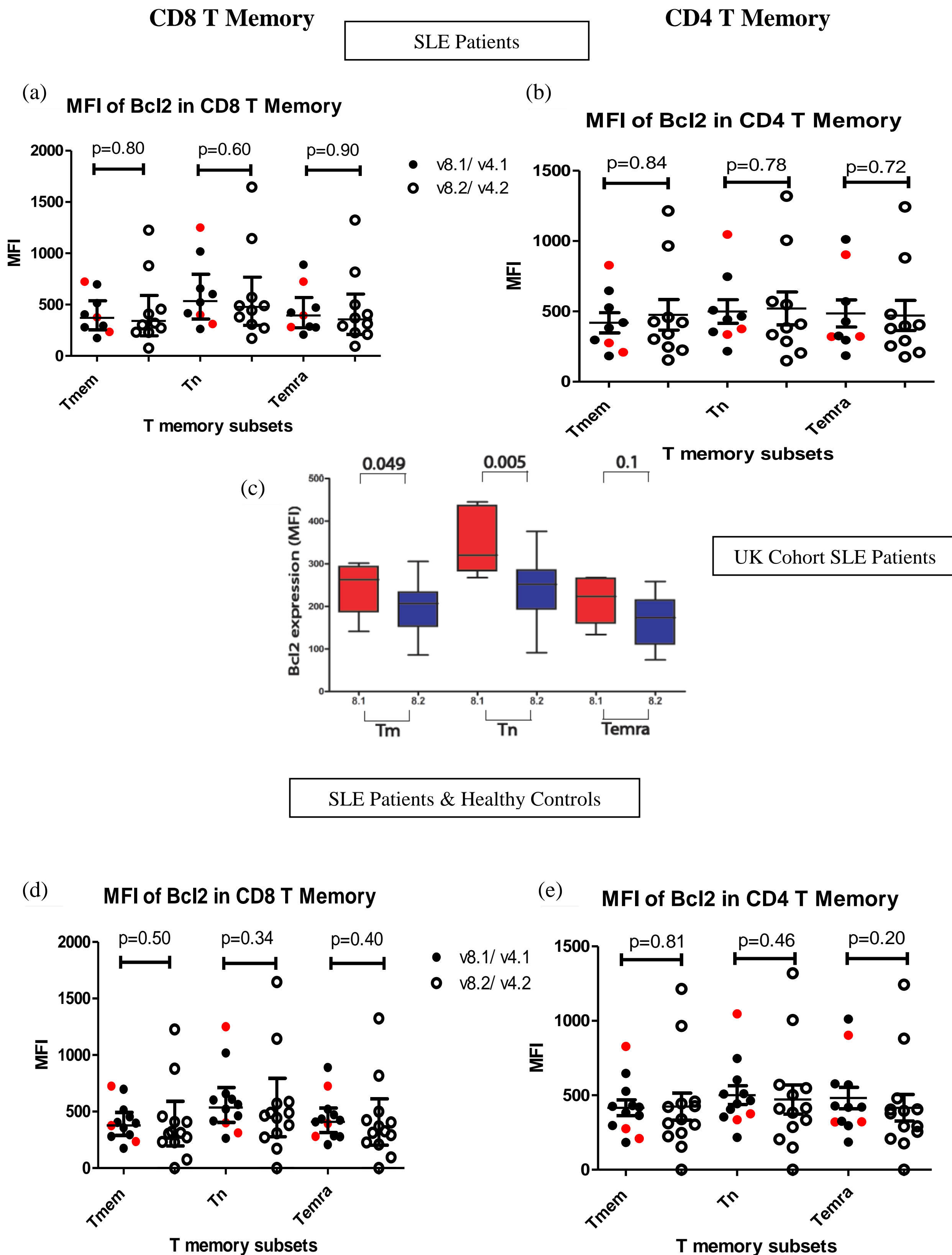

Figure 4.1.15 Expression of Bcl2 in CD4 and CD8 T Memory subsets, between v8.1 and v8.2. Geometric Mean fluorescence intensity (MFI, Geometric Mean) of cells expressing Bcl2+ in CD8 (a, d) and CD4 T (b, e) memory subsets, with (d, e) and without (a, b) the inclusion of healthy controls, as compared to UK cohort (c), courtesy by *E.F McKinney, P A Lyons et al Nature Medicine*

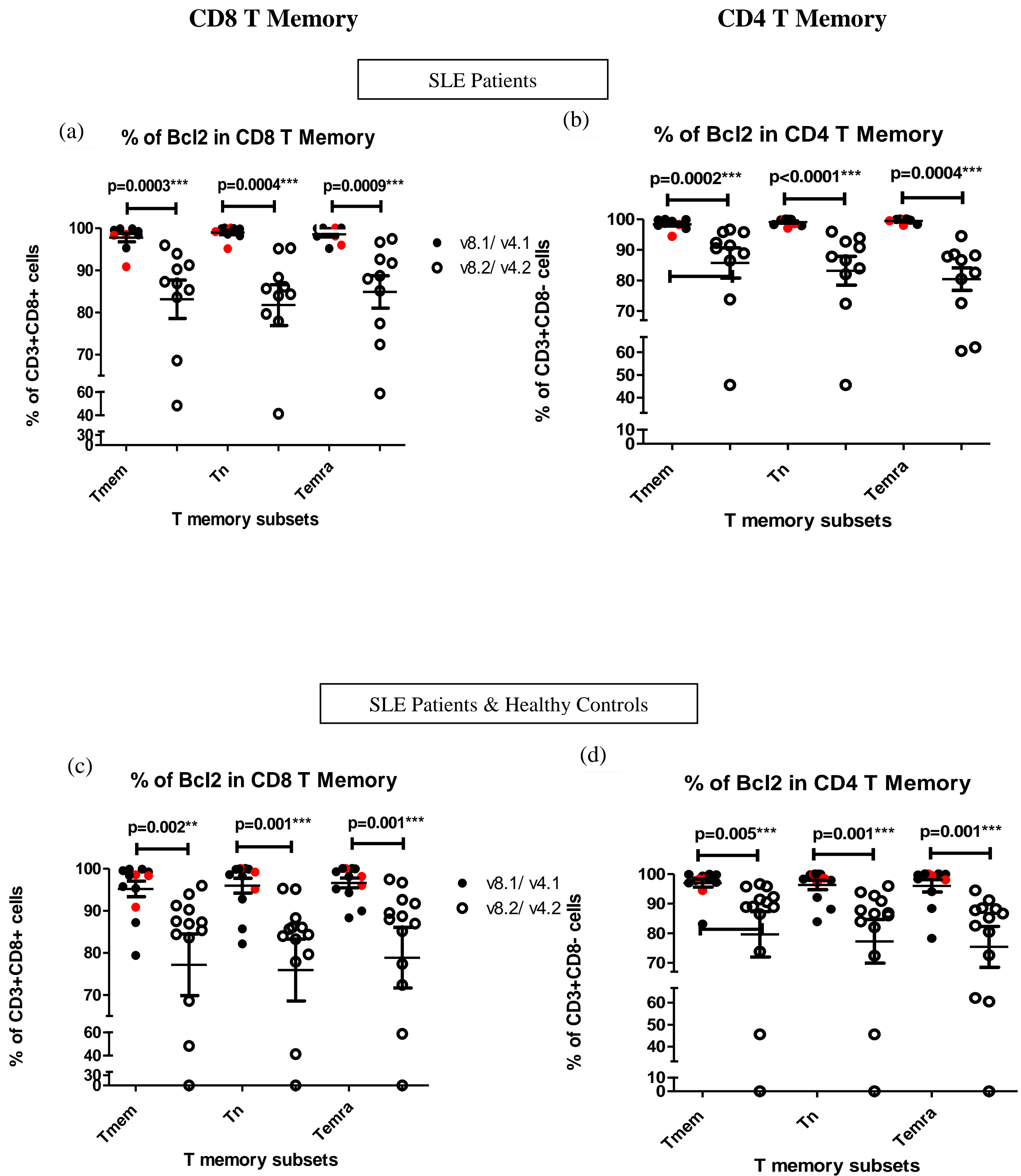

Figure 4.1.16 Cell proportions of Bcl2+ CD4 and CD8 T Memory subsets, between v8.1 and v8.2. Proportions of cells expressing Bcl2+ in CD8 (a, c) and CD4 T (b, d) memory subsets, with (c, d) and without (a, b) the inclusion of healthy controls.

4.2 Monocyte and Granulocyte Analysis

Differential expression of CD14 and CD16 (also known as FcγRIII) allow human blood monocytes to be divided into two major subpopulations: CD14++CD16- and CD14+CD16+ monocytes (Gordon and Taylor 2005). CD14++CD16- are often called classic monocytes as the phenotype resembles the original description of monocytes. It was shown that CD14+CD16+ monocytes expressed higher amounts of MHC class II molecules and CD32 (also known as FcγRII) (Ziegler-Heitbrock 1996). It was suggested that these cells resemble mature tissue macrophages (Ziegler-Heitbrock*, et al* 1993)(Ancuta*, et al* 2000).

CD14+CD16+ monocytes are smaller (an average size of 13.8µm) and less granular than regular monocytes (18.4µm in size). They account for 2.2% of the mononuclear cells (Passlick*, et al* 1989) These non-classical monocytes are potent producers of TNFα (Ancuta*, et al* 2000, Belge*, et al* 2002) but produce very low IL-10, suggesting that they are proinflammatory cells. They also show a higher antigen-presenting capacity. It appears that CD14+CD16+ monocytes play a critical role in infection and inflammation (Ziegler-Heitbrock 2007), they were found to be significantly increased in Rheumatoid Arthritis patients (Kawanaka*, et al* 2002).

A higher CD14+CD16lo monocyte percentage was found in v8.1 but the difference is not significant, as shown in **Figure 4.2.3(a)**.There is no significant difference of CD14+CD16+, CD14-CD16++, CD14-CD16+ and Eosinophils cell proportions between subgroups v8.1 and v8.2, as shown in **Figure 4.2.3(b)**. Unlike active lupus patients, most of the Singapore

patients involved in this study are quiescent and in remission, thus less inflammation and infection as exhibited by these patients. TNFα should be included in future research experiments to serve as a potential biomarker for prognostic subgroups v8.1 and v8.2.

Figure 4.2.4(a) shows that CD16loCD14+ of subgroup v8.1 displays a higher cell proportion expressing CD13+. Eosinophils in subgroup v8.2 show a slightly higher cell percentage expressing CD13+ than subgroup v8.1. All the subsets of v8.1 display higher cell proportions expressing CD62L+, as shown in **Figure 4.2.4(b)**. But these results were not statistically significant.

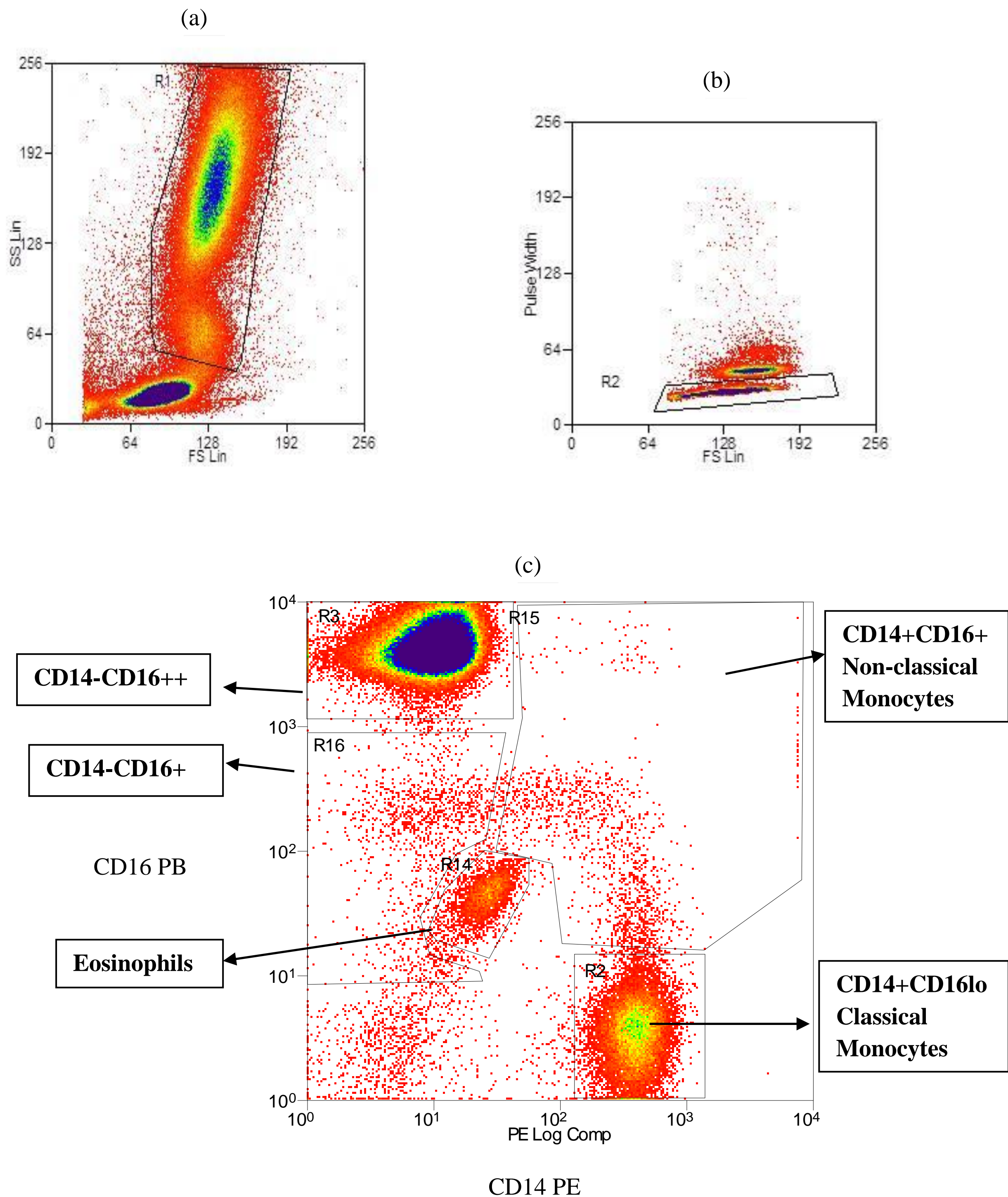

Figure 4.2.1 Flow data analysis of lysed RBC from a SLE patient for monocytes and granulocytes analysis. Granulocytes and monocytes were gated in the scatter plot (a) and single cells were gated at (b). Five populations were gated on CD14PE- CD16PB dotplot: CD14+CD16+, CD14+CD16++, CD14-CD16+, Eosinophils and CD14+CD16lo.

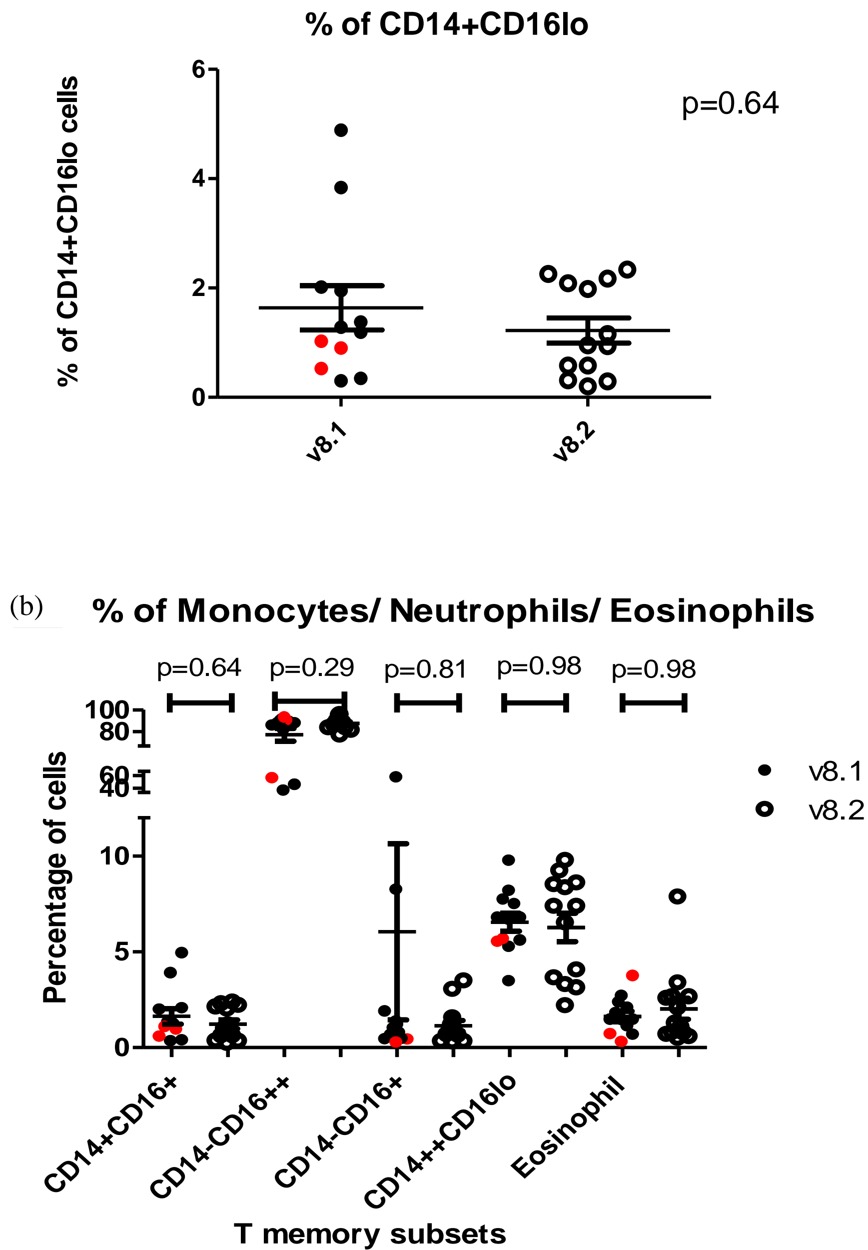

Figure 4.2.3 CD14+CD16lo cell proportions in v8.1 and v8.2 and cell proportions of different subgroups of monocytes and neutrophils. Comparison of CD14+ CD16lo cell proportions between prognostic group v8.1 and v8.2 (a). An overview of cell proportions of different subpopulations between prognostic groups v8.1 and v8.2: CD14+CD16+, CD14-CD16++, CD14-CD16+, CD14++CD16lo and Eosinophils.

(a)

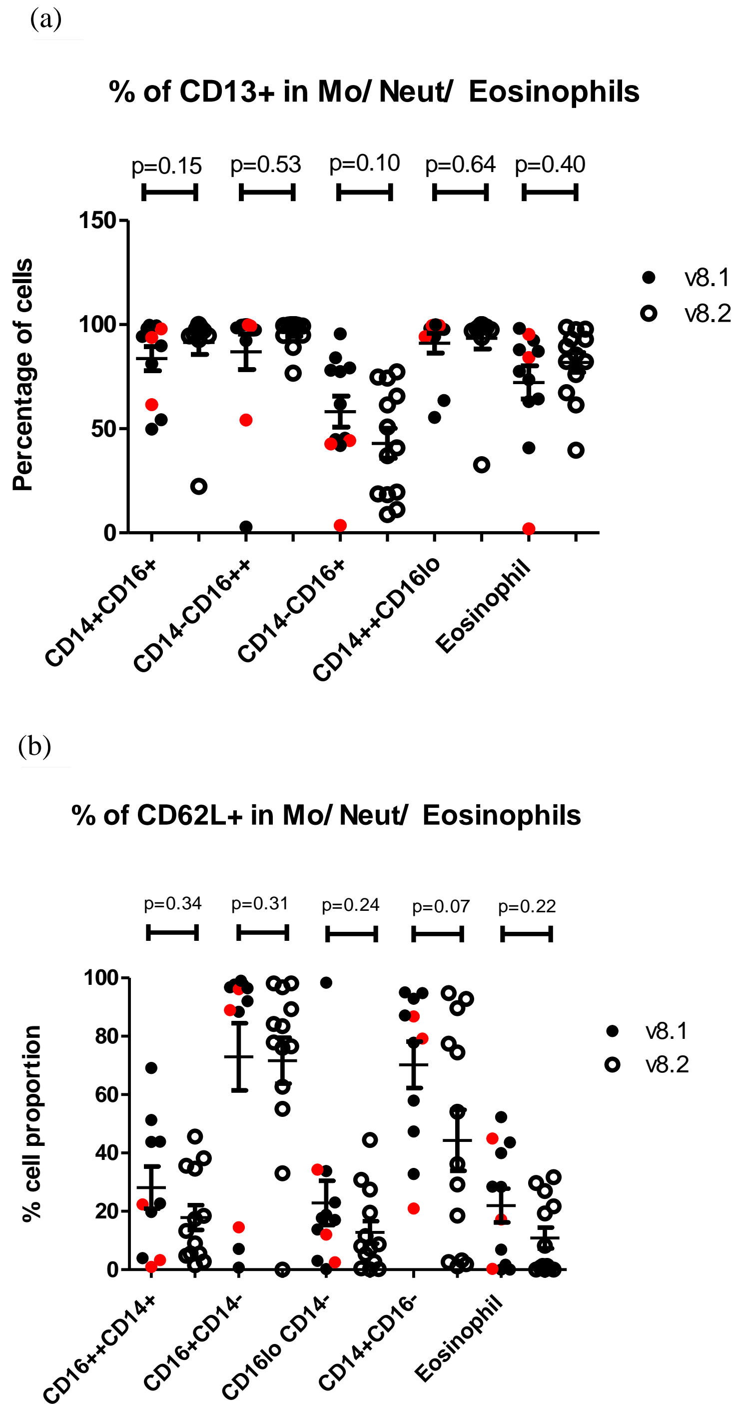

Figure 4.2.4 Monocytes and neutrophils cell subsets proportions expressing CD13+ and CD62L+. Cell proportions of different populations between prognostic groups v8.1 and v8.2: (CD14+CD16+, CD14-CD16++, CD14-CD16+, CD14++CD16lo and Eosinophils) expressing CD13+ (a) and CD62L (b). Red filled dots represent NUH patients who were on flare on date of blood collection.

4.3 Plasma B and Memory B Cell Analysis

SLE is an autoimmune disease where B-cell functions are closely correlated with disease activity. Abnormal B cell and T cell recognition of self-antigens leads to the production of autoantibody. Autoantibodies produced by B-cell-derived plasma cells also contribute significantly to the disease pathogenesis such as immune complex formation, complement activation and tissue destruction (Yanaba*, et al* 2008). The immune complexes formed have a central role in the effector phase of the disease (Kotzin 1996).

In vivo, antigen-specific activation and differentiation of B cells occur in germinal centers. Within germinal centers, CD19+CD27- naïve B cells undergo activation, proliferation, somatic hypermutation, Ig isotype switching and positive/ negative selection by antigen (MacLennan*, et al* 1992, Zhang*, et al* 2008, Zheng*, et al* 1997) . Activated B cells mature into antibody producing plasma cells (CD19+CD38+) or become memory B cells (CD19+CD27+) (Liu and Banchereau 1997). An enlarged pool of memory B cells poses a risk for autoimmunity since these cells have lower activation thresholds (Dorner*, et al* 2009, Sato*, et al* 2004).

Active SLE patients are reported to have a slightly higher proportion of CD19+ B cells and increased frequency of CD19+CD27+ peripheral B cells, due to a reduction of CD19+CD27- naïve peripheral B cell pool and a lesser decline in CD19+CD27+ memory B cells (Odendahl*, et al* 2000). CD19+CD38+CD138+ mature plasma B cells were found to have higher frequencies in active but not inactive SLE patients (Odendahl*, et al* 2000).

My investigation results show that there is no significant difference in CD19+ B cells and cell frequencies in Memory B and Plasma B cells between the two prognostic groups, as depicted in **Figure 4.3.1**.

Figure 4.3.2(a) shows no significant difference of cell proportion expressing CD19+ B cells in both subgroup v8.1 and v8.2. Proportion of CD27+CD62L+ B cells is higher in subgroup v8.1 than in v8.2. Subgroup v8.2 displays a higher cell proportions in both CD27-CD62L+ and CD38+CD138- B cell subsets (**Figure 4.3.2(b)**).

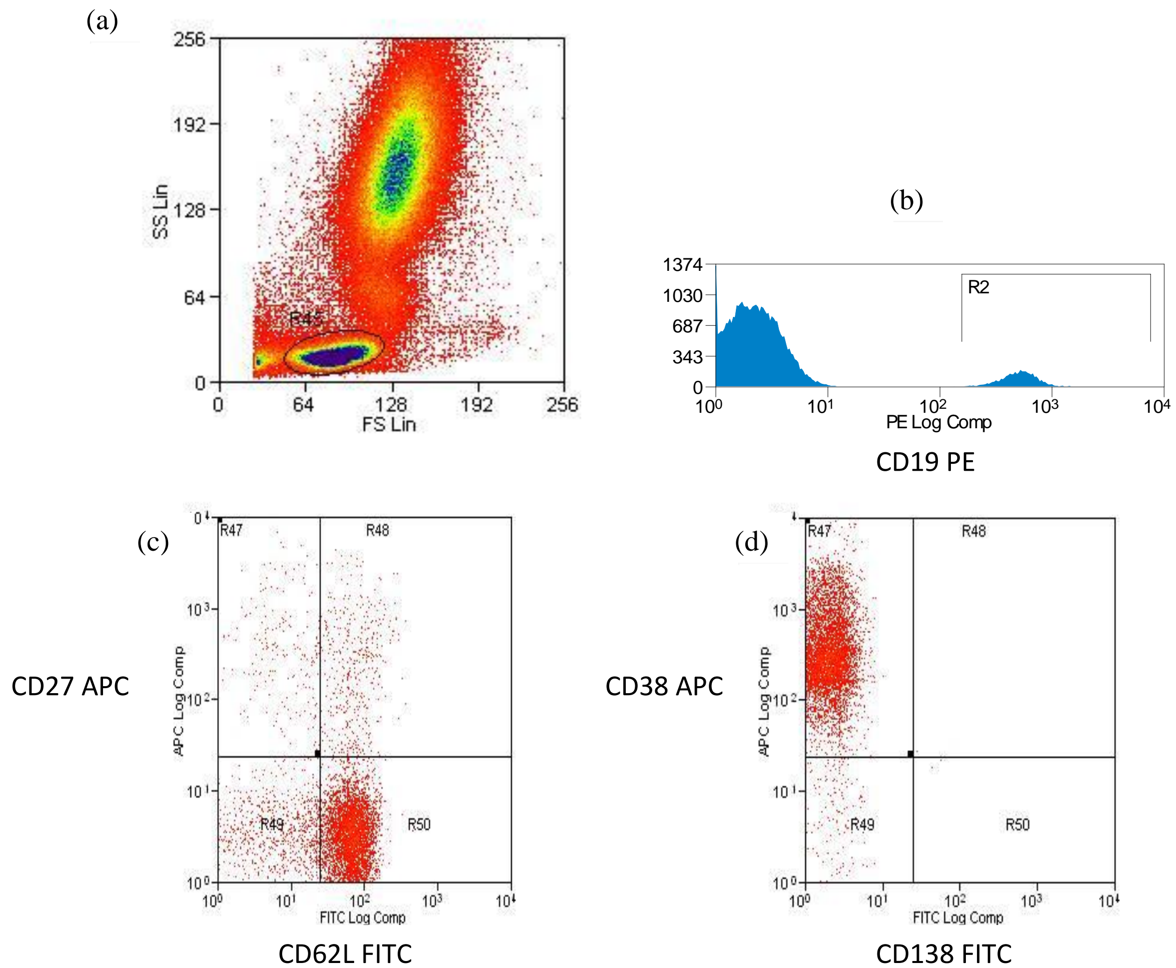

Figure 4.3.1 An example of flow data analysis of whole blood after RBC lysis for B plasma and B memory cells. Lymphocytes were gated in (a) and CD19+ B cells were gated in (b), these two gates were later applied in CD62LFITC-CD27APC(c) and CD138FITC-CD38APC(d) dotplots.

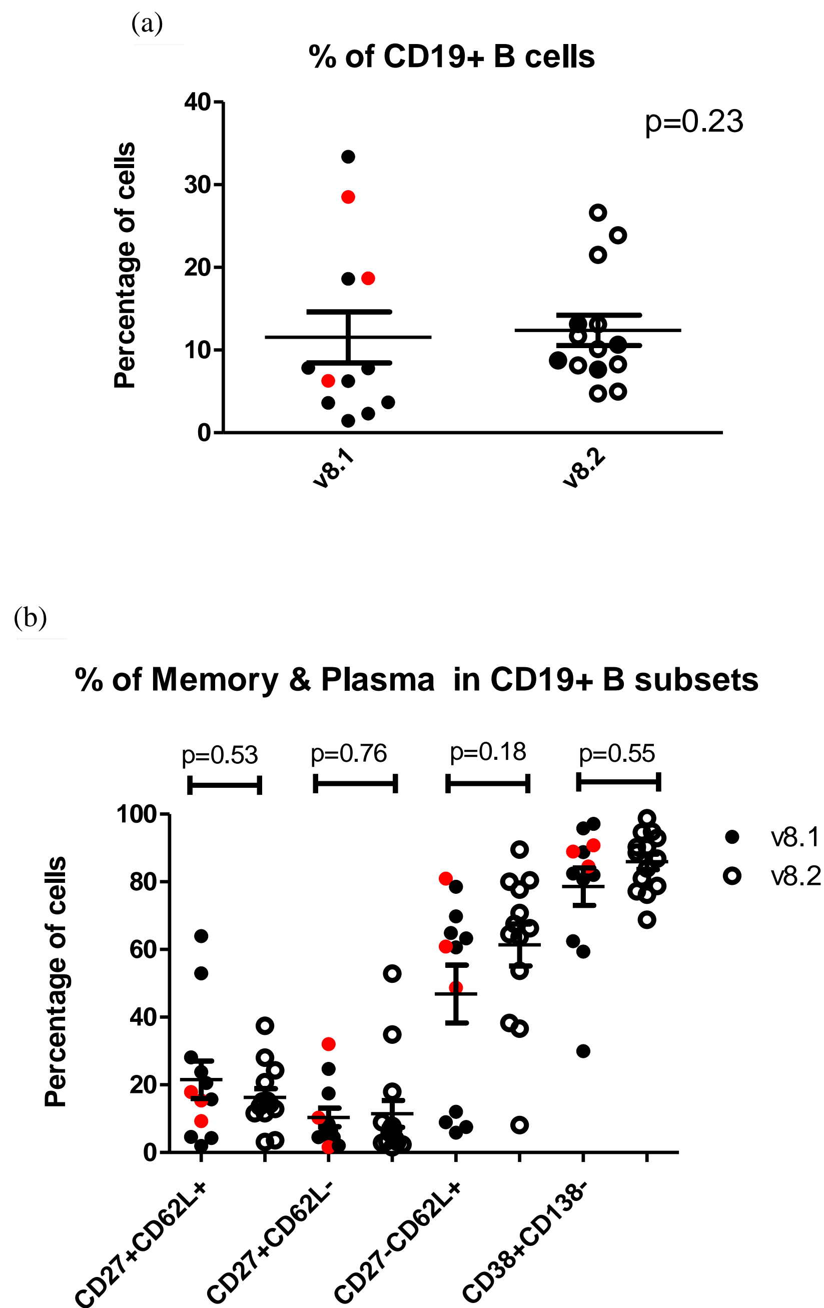

Figure 4.3.2 CD19+ cell proportions in v8.1 and v8.2 and cell proportions of different subgroups of B memory and B plasma cells (CD38+CD138-). Proportions of CD19+ B cells in prognostic group v8.1 and v8.2 (a). Cell proportions of different populations between prognostic groups v8.1 and v8.2 : CD27+CD62L+, CD27+CD62L-, CD27-CD62L+, CD38+CD138- (b).

4.4 Regulatory T Cell Analysis

Natural T reg cells are characterized by a constitutive expression of CD25 on the surface of CD4 T lymphocytes. They show a potent immunosuppressive function and contribute to immunologic self-tolerance by suppressing potentially auto-reactive T cells. Deficiency in T regulatory function may result in increased helper T cell activity or directly enhanced B cell activity. Several studies have been published regarding the phenotype and function of CD4+CD25+ T reg in patients with SLE. However, the literatures were conflicting. As compared to healthy controls, most labs found significantly decreased frequencies of CD4+CD25++ T reg in SLE patients (Barath*, et al* 2007a, Barath*, et al* 2007b, Bonelli*, et al* 2008, Crispin*, et al* 2003, Fathy*, et al* 2005, Lee*, et al* 2006, Lyssuk*, et al* 2007, Mellor-Pita*, et al* 2006, Miyara*, et al* 2005, Valencia*, et al* 2007, Vargas-Rojas*, et al* 2008, Zhang*, et al* 2008). In contrast, Sfikakis and colleagues showed that the T regulatory functional marker Foxp3 was increased significantly in all patients (n=7) examined, following B cell depletion with Rituximab in patients with lupus nephritis (Sfikakis*, et al* 2007). Vallerskog and colleagues also reported that rituximab-induced B cell depletion in SLE patients increased the proportion of T reg cells (Vallerskog*, et al* 2007). However, further studies should be performed to determine whether modulations of T reg by therapeutic B cell depletion contributes and predicts lupus activity.

The population of CD4+CD25+ in subgroup v8.2 showed a slightly higher expression of Foxp3+ than in v8.1 (**Figure 4.4.2(a)**). However, subgroup v8.1 had a higher cell proportion expressing Foxp3+ than subgroup v8.2 (**Figure 4.4.2(b)**). No significant difference of T reg functional Foxp3 expression in the two prognostic subgroups of v8.1 and v8.2 was observed. The cell proportion of T reg in v8.1 (~50%) was slightly higher than v8.2 (~40%).

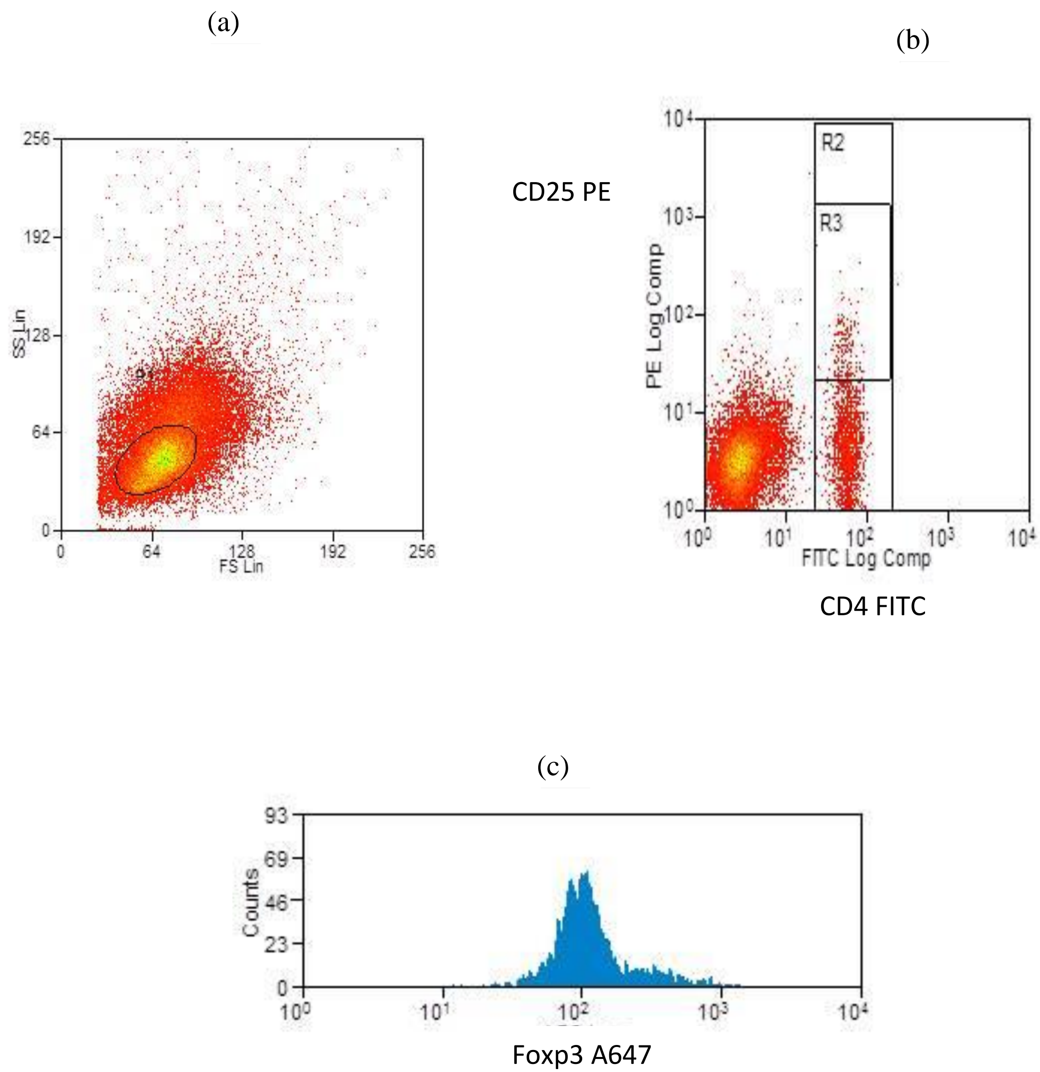

Figure 4.4.1 An example of flow data analysis of ficolled PBMCs for regulatory T cells. Lymphocytes were gated in (a) and CD4+CD25+ T cells were gated in (b), these two gates were later applied in Foxp3 A647 histogram (c).

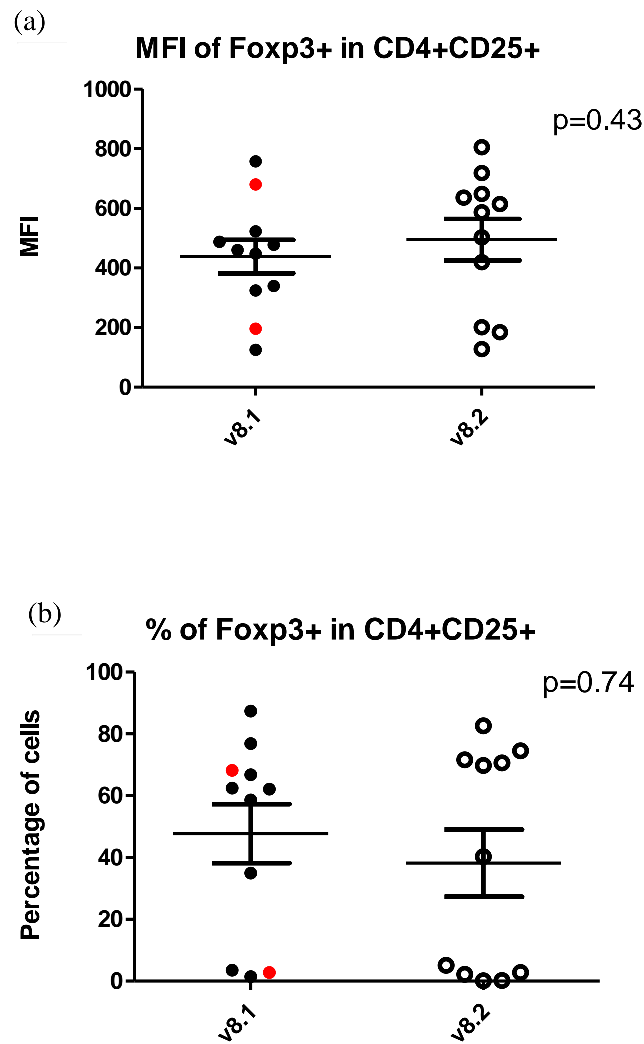

Figure 4.4.2 Quantification of CD4+CD25+ cells expressing Foxp3 in MFI and cell percentage. MFI (Geometric Mean) of Foxp3 A647 in CD4+CD25+ T Lymphocytes between prognostic group v8.1 and v8.2 (a). (b) shows the CD4+CD25+ T cell proportions expressing Foxp3 in prognostic groups v8.1 and v8.2.

CHAPTER 5

DISCUSSION

5.1 Aims of Study

SLE is an autoimmune disease with manifestations derived from the involvement of multiple organs. Recent findings from various studies have improved our understanding of SLE and reshaped current models. Even though the survival rate of the SLE patients has increased significantly over the years, complications arising from the use of toxic immunosuppressive drugs still cause considerable health difficulties.

Here, we review recent findings by our collaborators to identify prognostic subgroups based on the mRNA transcriptional profiling in an Asian cohort. As mentioned earlier in Chapter 1: Introduction, phenotypic analysis of the CD8 T cells revealed increases in the proportion of memory cells, as well as higher expression of BCL-2 and IL7R in the v8.1 group compared to v8.2. This study was performed in SLE patients from UK cohort. It was also reported that lupus patients in the 8.1 group had a poorer prognosis with a shorter time to first flare and an increased flare rate.

We aimed to determine if similar differences to the European population exist in these prognostic subgroups (v8.1 and v8.2). In addition, we aimed to study the phenotypes of CD4 and CD8 T cells in normal and lupus diagnosed in Asian populations. On top of all these, we aimed to see if flow cytometry would be a quicker, less painful for patients (for lesser blood volume) more efficient and economic tool to substitute microarray to classify SLE patients to the different prognostic subgroups.

5.2 Discussion: Main Observations and Findings

The Singapore cohort was observed to have relatively homogeneous proportions of memory cells (CD8 Tcm + Tem). The CD8 T naïve populations (~49% respectively) in both subgroups v8.1 and v8.2 are relatively similar in proportions, this suggests that, compared to the UK cohort, CD8 T memory subsets of the Singapore cohort were less activated with a lower cell percentage of CD8 T memory and higher CD8 T naïve. In the UK cohort, the proportion of CD8 T memory cells (CD8 Tcm +Tem) in subgroup v8.1 was higher than in v8.2. But this is not the case in Singapore. The local cohort has a similar cell percentage (40%) in both subgroups. Therefore the pattern of CD8 T memory subsets dot plot profile in CD45RA versus CD62L (McKinney*, et al* 2010) does not serve as a sensible and valid biomarker to classify the subgroup in the Singapore population. In comparison to subgroup v8.2, patients that fall in prognostic subgroup v8.1 are reported to have more severe SLE disease manifestations, shorter time to first flare, more frequent disease flare per month of follow-up.

Nonetheless, the trend of CD4 T memory subsets observed was very much similar to the UK cohort CD8 T memory subsets profile, and they are statistically significant. CD4 T memory (Tcm +Tem) displayed the highest proportions, followed by Naïve T cells and T revertant cells showed to be the least subgroup. CD4 T memory cells (CD4 Tcm+Tem) display a higher cell proportion in v4.1 T memory as featured in the UK cohort CD8 T memory cells. Naïve T cells population has a lower cell proportion in subgroup v4.1 compared to v4.2. This suggests that more naïve T cells had been converted to become CD4 T memory cells (Tcm+Tem), indicating that these SLE patients in subgroup 4.1 have a more active immune

response. This also suggests the possibility that the CD4 T memory proportion serves as a better diagnostic biomarker for the Asian cohort.

The proportion of cells expressing IL7R+ in CD8 T memory subsets are higher in v8.1 than v8.2. IL7R is a cardinal feature acquired by memory T cells at early time points to enhance their survival. This suggests that CD8 T memory subsets in prognostic group v8.1 are more rapid and advanced in acquiring IL7R feature than group v8.2. As identified by Huster, this IL7R+ CD8 T memory cells contain higher number of anti-apoptotic molecules and thus enhance their survival (Huster, et al 2004).

Contrary to the belief that subgroup v8.1 of CD8 T memory subsets is more aggressive and intense than v8.2, CD25 expression appeared to be higher in CD8 and CD4 T memory subsets of subgroup v8.2 (data was not statistically significant). However, subgroup v8.1 has a higher cell proportion expressing CD25+ in both profiles of CD8 and CD4 T memory subsets. This could indicate the possibility of subgroup v8.2 having a higher lupus activity but lower cell count.

Generally group v8.2/4.2 appeared to display a higher proportion of cells expressing CXCR6+ in all T memory subsets for both CD8 and CD4 T subsets (data not statistically significant). Sato and his group observed that CXCR6 was required for lymphocyte proliferation or amplification of the inflammatory reaction. It was also reported that CXCR6 facilitates effector CD8 T cell migration from the blood into sites of pathological inflammation and thus contributes to the recruitment of activated lymphocytes into inflamed

liver (Sato*, et al* 2005). My results indicate that group v8.2/4.2 could have more cells proliferating and migrating from the blood stream into sites of pathological inflammation.

The Asian CD8 T subsets display a similar trend of Bcl2 expression in MFI (Geometric Mean) compared to UK cohort but with a significantly higher fluorescence intensity. Both CD4 and CD8 T memory subsets showed a higher proportion of cells expressing Bcl2+. My findings were in line with the observations of Ohsako who believed that higher Bcl2+ expression in SLE patients might be due to a defective of the immune system to eradicate autoreactive immune cells. This consequently caused the abnormal survival of these immune cells which constantly expressed the anti-apoptotic marker Bcl2+.

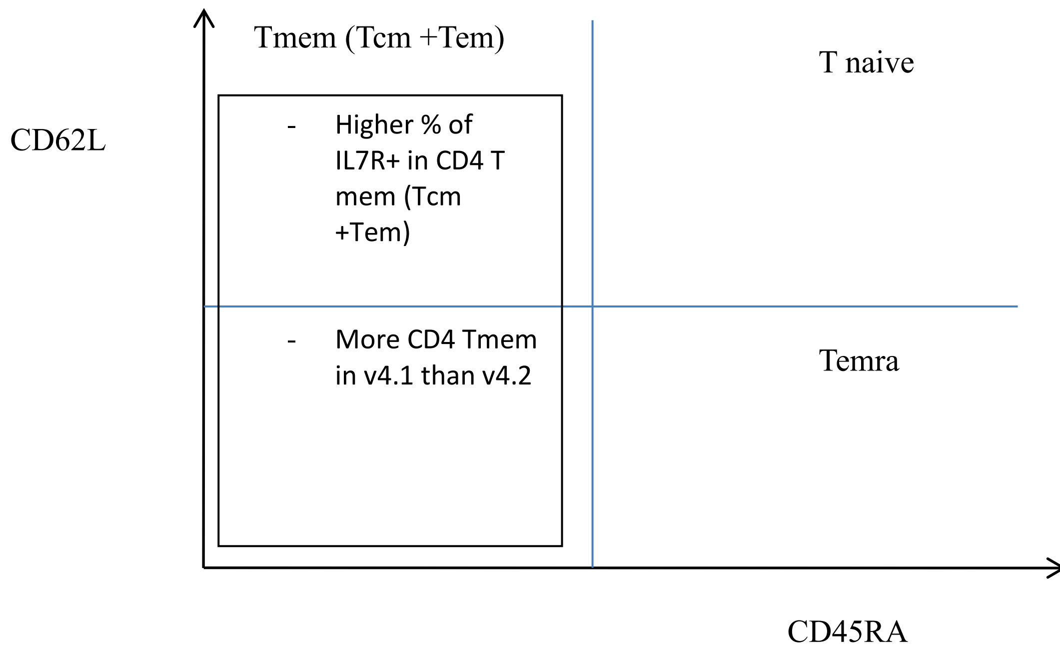

Figure 5.1 Summarizes the possible biomarkers to classify patients under prognostic subgroup of v8.1 in T memory cell analysis compared to subgroup v8.2. Prognostic subgroup v8.1/ 4.1 has a higher cell proportion of CD4 Tmemory (Tcm +Tem) in v4.1 and higher frequencies of cells expressing IL7R in CD4 T memory (Tcm + Tem). In general, in all CD4 and CD8 T memory subsets, subgroup v8.1 presents a higher cell proportions expressing Bcl2+, lower cell frequencies expressing CXCR6+ and lower Mean Fluorescence Intensity of CD25 expression.

5.3 Limitations of the Study

Of the total 25 subjects participated in the study, 19 were SLE patients. Among them, only 3 of the SLE patients were from NUH and were at disease flare and with active inflammation on the date of blood collection. The rest of the SLE patients involved were from SGH and were in remission with a stable condition and an average of more than 10 years of disease. Access to active SLE patients is limited as this is usually dependent on the conditions of the patients (as assessed by doctors) and most were considered too weak to donate blood for the study. Some were not thought fit to donate the volume of blood needed for the study. This explains why we do not have sufficient patients to phenotype by flow cytometry. Repeated blood donation at different times after first flare seemed almost impossible. These patients usually do not return for the blood donation after they recover from the first flare. This complicates our intention to monitor the changes in disease development and phenotypic expression in patients over time.

To boost the number of active SLE patients, this study should be extended to other neighboring countries like Indonesia, Thailand and Vietnam which do have a high prevalence rate of SLE. Furthermore such an SLE study has not been conducted in these countries.

One of the issues we encountered was low RNA yield which was not sufficient for microarray profiling in Cambridge. Unlike the UK cohort who donated 100ml of blood, the Singapore cohort was only allowed to give 50ml, as limited by the Singapore IRB. Storage duration and conditions, delivery conditions from Singapore to Cambridge UK could also

affected the stability of RNA. The possibility of sending the RNA to a reliable local microarray facility lab would prevent the loss of RNA, save time and reduce cost of producing the array.

Instrument background noise (electronic noise) should be verified by running instrumental set up calibration beads, such as 8 Peak beads to ensure the separation of signal to background noise is significant. Tracking beads are useful in this context to track the performance and target brightness of each Photomultiplier tubes, filters and mirrors of each detector. These beads should be used concurrently in both labs (Singapore and Cambridge) in order to ensure a fair comparison of results generated.

More functional intracellular markers like IFN-γ and TNF-α should be included in this study to check the function and status of the memory cells in different prognostic subgroups. Current panel is not expansive enough to answer the functional aspects of T memory subsets. More markers should be screened based on the gene profiling of microarray to create a panel of biomarkers to segregate patients of different levels of disease manifestations.

Communications among doctors, researchers and research nurses could be more effective by constantly having meetings and discussions about the findings of the study. In depth trainings need to be more organized and accurate in patient management, blood collection and screening as well as in data collection. More awareness and involvement from all parties are needed to benefit the patients in long run.

In conclusion, the study was underpowered. It is limited by the number of SLE patients, especially those with active SLE. Nevertheless, from the results obtained, CD4 seems to be a better gene signature for Asians than CD8. This synchronizes with the recent gene profiling of an Asian cohort from Cambridge (data yet to be released), where they did find that CD4 is a better gene signature to classify the patients into the different prognostic subgroups. This shows that flow cytometry can draw similar conclusions to that obtained by microarray and suggests that flow cytometry can be used to recognize different prognostic subgroups of patients and allow individualized therapy to reduce SLE severe manifestations. However, more biomarkers should be identified and included in the panel to empower the credibility of the study.

5.4 Future Work

Telomeres are the repeating hexameric sequence of nucleotides (TTAGGG) found at the ends of linear chromosomes (Blackburn 2001, Hodes*, et al* 2002). Each division of a cell leads to the loss of 50-100 base pairs of telomeric DNA due to the inability of DNA polymerase to fully replicate the ends of chromosomes (Hodes*, et al* 2002, Wright and Shay 2000). The induction of telomerase activity can compensate for telomere loss (Hooijberg*, et al* 2000, Plunkett*, et al* 2005, Rufer*, et al* 2001). Upon repeated stimulation, cells lose the capacity to upregulate telomerase. Consequently, the progressive erosion of telomeres to a critical length leads to chromosomal instability and end-to-end chromosome fusion which has consequences that include cell-cycle arrest, apoptosis (McEachern*, et al* 2000) and replicative senescence (Akbar*, et al* 2004, Hodes*, et al* 2002). During ageing, the number of highly differentiated memory T cells that reaches replicative senescence increases (Pawelec*, et al* 2004). This

population of cells would have much reduced telomere length (Akbar*, et al* 2000) and remain functional but with a limited capacity for further clonal expansion following subsequent antigenic challenge (Khan*, et al* 2002, Ouyang*, et al* 2004). Such an expanded population might compete for survival factors with smaller less-frequently or less efficiently stimulated memory T cell populations. The accumulation of non-functional antigenic specific cells may reduce the available immunological space or other survival factors for T cells of other specificities, which are then lost through competition (Khan*, et al* 2004, Messaoudi*, et al* 2004).

The measurement of telomere length has been useful to assess the status and extent of cell differentiation of a population of lymphocytes, especially for memory T cells. After an immune response resolves, to establish cellular homeostasis, most of the clonally expanded T cells are cleared by apoptosis (Akbar*, et al* 1993a, Akbar*, et al* 1993b). Nevertheless, some antigen-specific T cells are retained and constitute the memory pool. Thus, by measuring the T memory subsets telomere length, one is able to predict the SLE disease progression in patients. Comparison of telomere lengths of prognostic subgroups v8.1 and v8.2, together with the quantifications of markers IL7R and Bcl-2 will be a good indicator of lupus activity and intensity and could form the basis of future study.

References

Ahmed, R. & Gray, D. (1996) Immunological memory and protective immunity: understanding their relation. *Science,* 272, 54-60.

Akbar, A.N. & Fletcher, J.M. (2005) Memory T cell homeostasis and senescence during aging. *Current opinion in immunology,* 17, 480-485.

Akbar, A.N., Beverley, P.C. & Salmon, M. (2004) Will telomere erosion lead to a loss of T-cell memory? *Nature reviews. Immunology,* 4, 737-743.

Akbar, A.N., Borthwick, N., Salmon, M., Gombert, W., Bofill, M., Shamsadeen, N., Pilling, D., Pett, S., Grundy, J.E. & Janossy, G. (1993a) The significance of low bcl-2 expression by CD45RO T cells in normal individuals and patients with acute viral infections. The role of apoptosis in T cell memory. *The Journal of experimental medicine,* 178, 427-438.

Akbar, A.N., Salmon, M., Savill, J. & Janossy, G. (1993b) A possible role for bcl-2 in regulating T-cell memory--a 'balancing act' between cell death and survival. *Immunology today,* 14, 526-532.

Akbar, A.N., Soares, M.V., Plunkett, F.J. & Salmon, M. (2000) Differential regulation of CD8+ T cell senescence in mice and men. *Mechanisms of ageing and development,* 121, 69-76.

Almanzar, G., Schwaiger, S., Jenewein, B., Keller, M., Herndler-Brandstetter, D., Wurzner, R., Schonitzer, D. & Grubeck-Loebenstein, B. (2005) Long-term cytomegalovirus infection leads to significant changes in the composition of the CD8+ T-cell repertoire, which may be the basis for an imbalance in the cytokine production profile in elderly persons. *Journal of virology,* 79, 3675-3683.

Alnemri, E.S., Robertson, N.M., Fernandes, T.F., Croce, C.M. & Litwack, G. (1992) Overexpressed full-length human BCL2 extends the survival of baculovirus-infected Sf9 insect cells. *Proceedings of the National Academy of Sciences of the United States of America,* 89, 7295-7299.

Ancuta, P., Weiss, L. & Haeffner-Cavaillon, N. (2000) CD14+CD16++ cells derived in vitro from peripheral blood monocytes exhibit phenotypic and functional dendritic cell-like characteristics. *European journal of immunology,* 30, 1872-1883.

Avihingsanon, Y. & Hirankarn, N. (2010) Major lupus organ involvement: severe lupus nephritis. *Lupus,* 19, 1391-1398.

Bachmann, M.F., Barner, M., Viola, A. & Kopf, M. (1999) Distinct kinetics of cytokine production and cytolysis in effector and memory T cells after viral infection. *European journal of immunology,* 29, 291-299.

Barath, S., Aleksza, M., Tarr, T., Sipka, S., Szegedi, G. & Kiss, E. (2007a) Measurement of natural (CD4+CD25high) and inducible (CD4+IL-10+) regulatory T cells in patients with systemic lupus erythematosus. *Lupus,* 16, 489-496.

Barath, S., Soltesz, P., Kiss, E., Aleksza, M., Zeher, M., Szegedi, G. & Sipka, S. (2007b) The severity of systemic lupus erythematosus negatively correlates with the increasing number of CD4+CD25(high)FoxP3+ regulatory T cells during repeated plasmapheresis treatments of patients. *Autoimmunity,* 40, 521-528.

Belge, K.U., Dayyani, F., Horelt, A., Siedlar, M., Frankenberger, M., Frankenberger, B., Espevik, T. & Ziegler-Heitbrock, L. (2002) The proinflammatory CD14+CD16+DR++ monocytes are a major source of TNF. *Journal of immunology,* 168, 3536-3542.

Blackburn, E.H. (2001) Switching and signaling at the telomere. *Cell,* 106, 661-673.

Bonelli, M., Savitskaya, A., von Dalwigk, K., Steiner, C.W., Aletaha, D., Smolen, J.S. & Scheinecker, C. (2008) Quantitative and qualitative deficiencies of regulatory T cells in patients with systemic lupus erythematosus (SLE). *International immunology,* 20, 861-868.

Boey, M.L., Peebles, C.L., Tsay, G., Feng, P.H. & Tan, E.M. (1988) Clinical and autoantibody correlations in Orientals with systemic lupus erythematosus. *Annals of the rheumatic diseases,* 47, 918-923.

Butcher, E.C. & Picker, L.J. (1996) Lymphocyte homing and homeostasis. *Science,* 272, 60-66.

Cervera, R., Font, J. & Shoenfeld, Y. (2006) European Working Party on Systemic Lupus Erythematosus: a 15-year report. *Autoimmunity reviews,* 5, 549-553.

Chalasani, G., Dai, Z., Konieczny, B.T., Baddoura, F.K. & Lakkis, F.G. (2002) Recall and propagation of allospecific memory T cells independent of secondary lymphoid organs. *Proceedings of the National Academy of Sciences of the United States of America,* 99, 6175-6180.

Crispin, J.C., Martinez, A. & Alcocer-Varela, J. (2003) Quantification of regulatory T cells in patients with systemic lupus erythematosus. *Journal of autoimmunity,* 21, 273-276.

Di Rosa, F. & Santoni, A. (2003) Memory T-cell competition for bone marrow seeding. *Immunology,* 108, 296-304.

Dorner, T., Jacobi, A.M. & Lipsky, P.E. (2009) B cells in autoimmunity. *Arthritis research & therapy,* 11, 247.

Dunne, P.J., Belaramani, L., Fletcher, J.M., Fernandez de Mattos, S., Lawrenz, M., Soares, M.V., Rustin, M.H., Lam, E.W., Salmon, M. & Akbar, A.N. (2005) Quiescence and functional reprogramming of Epstein-Barr virus (EBV)-specific CD8+ T cells during persistent infection. *Blood,* 106, 558-565.

Dunne, P.J., Faint, J.M., Gudgeon, N.H., Fletcher, J.M., Plunkett, F.J., Soares, M.V., Hislop, A.D., Annels, N.E., Rickinson, A.B., Salmon, M. & Akbar, A.N. (2002) Epstein-Barr virus-specific CD8(+) T cells that re-express CD45RA are apoptosis-resistant memory cells that retain replicative potential. *Blood,* 100, 933-940.

Dutton, R.W., Bradley, L.M. & Swain, S.L. (1998) T cell memory. *Annual review of immunology,* 16, 201-223.

Fathy, A., Mohamed, R.W., Tawfik, G.A. & Omar, A.S. (2005) Diminished CD4+CD25+ T-lymphocytes in peripheral blood of patients with systemic lupus erythematosus. *The Egyptian journal of immunology / Egyptian Association of Immunologists,* 12, 25-31.

Freitas, A.A. & Rocha, B. (1999) Peripheral T cell survival. *Current opinion in immunology,* 11, 152-156.

Garcia, S., DiSanto, J. & Stockinger, B. (1999) Following the development of a CD4 T cell response in vivo: from activation to memory formation. *Immunity,* 11, 163-171.

Geginat, J., Lanzavecchia, A. & Sallusto, F. (2003) Proliferation and differentiation potential of human CD8+ memory T-cell subsets in response to antigen or homeostatic cytokines. *Blood,* 101, 4260-4266.

Gett, A.V., Sallusto, F., Lanzavecchia, A. & Geginat, J. (2003) T cell fitness determined by signal strength. *Nature immunology,* 4, 355-360.

Goldrath, A.W. & Bevan, M.J. (1999) Selecting and maintaining a diverse T-cell repertoire. *Nature,* 402, 255-262.

Gordon, S. & Taylor, P.R. (2005) Monocyte and macrophage heterogeneity. *Nature reviews. Immunology,* 5, 953-964.

Graninger, W.B., Seto, M., Boutain, B., Goldman, P. & Korsmeyer, S.J. (1987) Expression of Bcl-2 and Bcl-2-Ig fusion transcripts in normal and neoplastic cells. *The Journal of clinical investigation,* 80, 1512-1515.

Hammarlund, E., Lewis, M.W., Hansen, S.G., Strelow, L.I., Nelson, J.A., Sexton, G.J., Hanifin, J.M. & Slifka, M.K. (2003) Duration of antiviral immunity after smallpox vaccination. *Nature medicine,* 9, 1131-1137.

Hockenbery, D., Nunez, G., Milliman, C., Schreiber, R.D. & Korsmeyer, S.J. (1990) Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. *Nature,* 348, 334-336.

Hodes, R.J., Hathcock, K.S. & Weng, N.P. (2002) Telomeres in T and B cells. *Nature reviews. Immunology,* 2, 699-706.

Hooijberg, E., Ruizendaal, J.J., Snijders, P.J., Kueter, E.W., Walboomers, J.M. & Spits, H. (2000) Immortalization of human CD8+ T cell clones by ectopic expression of telomerase reverse transcriptase. *Journal of immunology,* 165, 4239-4245.

Huster, K.M., Busch, V., Schiemann, M., Linkemann, K., Kerksiek, K.M., Wagner, H. & Busch, D.H. (2004) Selective expression of IL-7 receptor on memory T cells identifies early CD40L-dependent generation of distinct CD8+ memory T cell subsets. *Proceedings of the National Academy of Sciences of the United States of America,* 101, 5610-5615.

Jacobson, M.D., Burne, J.F., King, M.P., Miyashita, T., Reed, J.C. & Raff, M.C. (1993) Bcl-2 blocks apoptosis in cells lacking mitochondrial DNA. *Nature,* 361, 365-369.

Kaech, S.M., Hemby, S., Kersh, E. & Ahmed, R. (2002) Molecular and functional profiling of memory CD8 T cell differentiation. *Cell,* 111, 837-851.

Kaech, S.M., Tan, J.T., Wherry, E.J., Konieczny, B.T., Surh, C.D. & Ahmed, R. (2003) Selective expression of the interleukin 7 receptor identifies effector CD8 T cells that give rise to long-lived memory cells. *Nature immunology,* 4, 1191-1198.

Kawanaka, N., Yamamura, M., Aita, T., Morita, Y., Okamoto, A., Kawashima, M., Iwahashi, M., Ueno, A., Ohmoto, Y. & Makino, H. (2002) CD14+,CD16+ blood monocytes and joint inflammation in rheumatoid arthritis. *Arthritis and rheumatism,* 46, 2578-2586.

Khan, N., Hislop, A., Gudgeon, N., Cobbold, M., Khanna, R., Nayak, L., Rickinson, A.B. & Moss, P.A. (2004) Herpesvirus-specific CD8 T cell immunity in old age: cytomegalovirus impairs the response to a coresident EBV infection. *Journal of immunology,* 173, 7481-7489.

Khan, N., Shariff, N., Cobbold, M., Bruton, R., Ainsworth, J.A., Sinclair, A.J., Nayak, L. & Moss, P.A. (2002) Cytomegalovirus seropositivity drives the CD8 T cell repertoire toward greater clonality in healthy elderly individuals. *Journal of immunology,* 169, 1984-1992.

Kim, C.H., Kunkel, E.J., Boisvert, J., Johnston, B., Campbell, J.J., Genovese, M.C., Greenberg, H.B. & Butcher, E.C. (2001) Bonzo/CXCR6 expression defines type 1-polarized T-cell subsets with extralymphoid tissue homing potential. *The Journal of clinical investigation,* 107, 595-601.

Kotzin, B.L. (1996) Systemic lupus erythematosus. *Cell,* 85, 303-306.

Kuhn, A., Beissert, S. & Krammer, P.H. (2009) CD4(+)CD25 (+) regulatory T cells in human lupus erythematosus. *Archives of dermatological research,* 301, 71-81.

Lakkis, F.G. & Sayegh, M.H. (2003) Memory T cells: a hurdle to immunologic tolerance. *Journal of the American Society of Nephrology : JASN,* 14, 2402-2410.

Lanzavecchia, A. & Sallusto, F. (2002) Progressive differentiation and selection of the fittest in the immune response. *Nature reviews. Immunology,* 2, 982-987.

Lanzavecchia, A. & Sallusto, F. (2005) Understanding the generation and function of memory T cell subsets. *Current opinion in immunology,* 17, 326-332.

Lee, J.H., Wang, L.C., Lin, Y.T., Yang, Y.H., Lin, D.T. & Chiang, B.L. (2006) Inverse correlation between CD4+ regulatory T-cell population and autoantibody levels in paediatric patients with systemic lupus erythematosus. *Immunology,* 117, 280-286.

Lee, S.S., Li, C.S. & Li, P.C. (1993) Clinical profile of Chinese patients with systemic lupus erythematosus. *Lupus,* 2, 105-109.

Li, J., Huston, G. & Swain, S.L. (2003) IL-7 promotes the transition of CD4 effectors to persistent memory cells. *The Journal of experimental medicine,* 198, 1807-1815.

Liu, Y.J. & Banchereau, J. (1997) Regulation of B-cell commitment to plasma cells or to memory B cells. *Seminars in immunology,* 9, 235-240.

Lyons, P.A., McKinney, E.F., Rayner, T.F., Hatton, A., Woffendin, H.B., Koukoulaki, M., Freeman, T.C., Jayne, D.R., Chaudhry, A.N. & Smith, K.G. (2010) Novel expression signatures identified by transcriptional analysis of separated leucocyte subsets in systemic lupus erythematosus and vasculitis. *Annals of the rheumatic diseases,* 69, 1208-1213.

Lyssuk, E.Y., Torgashina, A.V., Soloviev, S.K., Nassonov, E.L. & Bykovskaia, S.N. (2007) Reduced number and function of CD4+CD25highFoxP3+ regulatory T cells in

patients with systemic lupus erythematosus. *Advances in experimental medicine and biology,* 601, 113-119.

Macallan, D.C., Wallace, D., Zhang, Y., De Lara, C., Worth, A.T., Ghattas, H., Griffin, G.E., Beverley, P.C. & Tough, D.F. (2004) Rapid turnover of effector-memory CD4(+) T cells in healthy humans. *The Journal of experimental medicine,* 200, 255-260.

Mackay, C.R. (1993) Homing of naive, memory and effector lymphocytes. *Current opinion in immunology,* 5, 423-427.

MacLennan, I.C., Liu, Y.J. & Johnson, G.D. (1992) Maturation and dispersal of B-cell clones during T cell-dependent antibody responses. *Immunological reviews,* 126, 143-161.

Masopust, D., Vezys, V., Marzo, A.L. & Lefrancois, L. (2001) Preferential localization of effector memory cells in nonlymphoid tissue. *Science,* 291, 2413-2417.

McEachern, M.J., Krauskopf, A. & Blackburn, E.H. (2000) Telomeres and their control. *Annual review of genetics,* 34, 331-358.

McKinney, E.F., Lyons, P.A., Carr, E.J., Hollis, J.L., Jayne, D.R., Willcocks, L.C., Koukoulaki, M., Brazma, A., Jovanovic, V., Kemeny, D.M., Pollard, A.J., Macary, P.A., Chaudhry, A.N. & Smith, K.G. (2010) A CD8+ T cell transcription signature predicts prognosis in autoimmune disease. *Nature medicine,* 16, 586-591, 581p following 591.

McLeod, I.X., Zhou, X., Li, Q.J., Wang, F. & He, Y.W. (2011) The class III kinase Vps34 promotes T lymphocyte survival through regulating IL-7Ralpha surface expression. *Journal of immunology,* 187, 5051-5061.

Mellor-Pita, S., Citores, M.J., Castejon, R., Tutor-Ureta, P., Yebra-Bango, M., Andreu, J.L. & Vargas, J.A. (2006) Decrease of regulatory T cells in patients with systemic lupus erythematosus. *Annals of the rheumatic diseases,* 65, 553-554.

Messaoudi, I., Lemaoult, J., Guevara-Patino, J.A., Metzner, B.M. & Nikolich-Zugich, J. (2004) Age-related CD8 T cell clonal expansions constrict CD8 T cell repertoire and have the potential to impair immune defense. *The Journal of experimental medicine,* 200, 1347-1358.

Miyara, M., Amoura, Z., Parizot, C., Badoual, C., Dorgham, K., Trad, S., Nochy, D., Debre, P., Piette, J.C. & Gorochov, G. (2005) Global natural regulatory T cell depletion in active systemic lupus erythematosus. *Journal of immunology,* 175, 8392-8400.

Mountz, J.D., Wu, J., Cheng, J. & Zhou, T. (1994) Autoimmune disease. A problem of defective apoptosis. *Arthritis and rheumatism,* 37, 1415-1420.

Mullbacher, A. (1994) The long-term maintenance of cytotoxic T cell memory does not require persistence of antigen. *The Journal of experimental medicine,* 179, 317-321.

Murali-Krishna, K., Lau, L.L., Sambhara, S., Lemonnier, F., Altman, J. & Ahmed, R. (1999) Persistence of memory CD8 T cells in MHC class I-deficient mice. *Science,* 286, 1377-1381.

Nanki, T., Shimaoka, T., Hayashida, K., Taniguchi, K., Yonehara, S. & Miyasaka, N. (2005) Pathogenic role of the CXCL16-CXCR6 pathway in rheumatoid arthritis. *Arthritis and rheumatism,* 52, 3004-3014.

Nelson, D.L., Kurman, C.C., Fritz, M.E., Boutin, B. & Rubin, L.A. (1986) The production of soluble and cellular interleukin-2 receptors by cord blood mononuclear cells following in vitro activation. *Pediatric research,* 20, 136-139.

Odendahl, M., Jacobi, A., Hansen, A., Feist, E., Hiepe, F., Burmester, G.R., Lipsky, P.E., Radbruch, A. & Dorner, T. (2000) Disturbed peripheral B lymphocyte homeostasis in systemic lupus erythematosus. *Journal of immunology,* 165, 5970-5979.

Ohsako, S., Hara, M., Harigai, M., Fukasawa, C. & Kashiwazaki, S. (1994) Expression and function of Fas antigen and bcl-2 in human systemic lupus erythematosus lymphocytes. *Clinical immunology and immunopathology,* 73, 109-114.

Opferman, J.T., Ober, B.T. & Ashton-Rickardt, P.G. (1999) Linear differentiation of cytotoxic effectors into memory T lymphocytes. *Science,* 283, 1745-1748.

Ouyang, Q., Wagner, W.M., Zheng, W., Wikby, A., Remarque, E.J. & Pawelec, G. (2004) Dysfunctional CMV-specific CD8(+) T cells accumulate in the elderly. *Experimental gerontology,* 39, 607-613.

Passas, C.M., Wong, R.L., Peterson, M., Testa, M.A. & Rothfield, N.F. (1985) A comparison of the specificity of the 1971 and 1982 American Rheumatism Association criteria for the classification of systemic lupus erythematosus. *Arthritis and rheumatism,* 28, 620-623.

Passlick, B., Flieger, D. & Ziegler-Heitbrock, H.W. (1989) Identification and characterization of a novel monocyte subpopulation in human peripheral blood. *Blood,* 74, 2527-2534.

Pawelec, G., Akbar, A., Caruso, C., Effros, R., Grubeck-Loebenstein, B. & Wikby, A. (2004) Is immunosenescence infectious? *Trends in immunology,* 25, 406-410.

Plunkett, F.J., Franzese, O., Belaramani, L.L., Fletcher, J.M., Gilmour, K.C., Sharifi, R., Khan, N., Hislop, A.D., Cara, A., Salmon, M., Gaspar, H.B., Rustin, M.H., Webster, D. & Akbar, A.N. (2005) The impact of telomere erosion on memory CD8+ T cells in patients with X-linked lymphoproliferative syndrome. *Mechanisms of ageing and development,* 126, 855-865.

Rathmell, J.C. & Thompson, C.B. (2002) Pathways of apoptosis in lymphocyte development, homeostasis, and disease. *Cell,* 109 Suppl, S97-107.

Reinhardt, R.L., Khoruts, A., Merica, R., Zell, T. & Jenkins, M.K. (2001) Visualizing the generation of memory CD4 T cells in the whole body. *Nature,* 410, 101-105.

Rogers, P.R., Dubey, C. & Swain, S.L. (2000) Qualitative changes accompany memory T cell generation: faster, more effective responses at lower doses of antigen. *Journal of immunology,* 164, 2338-2346.

Rose, L.M., Latchman, D.S. & Isenberg, D.A. (1994) Bcl-2 and Fas, molecules which influence apoptosis. A possible role in systemic lupus erythematosus? *Autoimmunity,* 17, 271-278.

Rubin, L.A., Jay, G. & Nelson, D.L. (1986) The released interleukin 2 receptor binds interleukin 2 efficiently. *Journal of immunology,* 137, 3841-3844.

Rufer, N., Migliaccio, M., Antonchuk, J., Humphries, R.K., Roosnek, E. & Lansdorp, P.M. (2001) Transfer of the human telomerase reverse transcriptase (TERT) gene into T lymphocytes results in extension of replicative potential. *Blood,* 98, 597-603.

Sato, S., Fujimoto, M., Hasegawa, M. & Takehara, K. (2004) Altered blood B lymphocyte homeostasis in systemic sclerosis: expanded naive B cells and diminished but activated memory B cells. *Arthritis and rheumatism,* 50, 1918-1927.

Sato, T., Thorlacius, H., Johnston, B., Staton, T.L., Xiang, W., Littman, D.R. & Butcher, E.C. (2005) Role for CXCR6 in recruitment of activated CD8+ lymphocytes to inflamed liver. *Journal of immunology,* 174, 277-283.

Sfikakis, P.P., Souliotis, V.L., Fragiadaki, K.G., Moutsopoulos, H.M., Boletis, J.N. & Theofilopoulos, A.N. (2007) Increased expression of the FoxP3 functional marker of regulatory T cells following B cell depletion with rituximab in patients with lupus nephritis. *Clinical immunology,* 123, 66-73.

Shayakul, C., Ong-aj-yooth, L., Chirawong, P., Nimmannit, S., Parichatikanond, P., Laohapand, T., Vasuvattakul, S., Vareesangthip, K., Vanichakarn, S., Malasit, P. & et al. (1995) Lupus nephritis in Thailand: clinicopathologic findings and outcome in 569 patients. *American journal of kidney diseases : the official journal of the National Kidney Foundation,* 26, 300-307.

Shimaoka, T., Nakayama, T., Fukumoto, N., Kume, N., Takahashi, S., Yamaguchi, J., Minami, M., Hayashida, K., Kita, T., Ohsumi, J., Yoshie, O. & Yonehara, S. (2004) Cell surface-anchored SR-PSOX/CXC chemokine ligand 16 mediates firm adhesion of CXC chemokine receptor 6-expressing cells. *Journal of leukocyte biology,* 75, 267-274.

Smith, K.A. (1988) Interleukin-2: inception, impact, and implications. *Science,* 240, 1169-1176.

Smith, K.A. (1990) Interleukin-2. *Scientific American,* 262, 50-57.

Sprent, J. & Surh, C.D. (2002) T cell memory. *Annual review of immunology,* 20, 551-579.

Spronk, P.E., ter Borg, E.J., Huitema, M.G., Limburg, P.C. & Kallenberg, C.G. (1994) Changes in levels of soluble T-cell activation markers, sIL-2R, sCD4 and sCD8, in relation to disease exacerbations in patients with systemic lupus erythematosus: a prospective study. *Annals of the rheumatic diseases,* 53, 235-239.

Swain, S.L., Hu, H. & Huston, G. (1999) Class II-independent generation of CD4 memory T cells from effectors. *Science,* 286, 1381-1383.

Symons, J.A., Wood, N.C., Di Giovine, F.S. & Duff, G.W. (1988) Soluble IL-2 receptor in rheumatoid arthritis. Correlation with disease activity, IL-1 and IL-2 inhibition. *Journal of immunology,* 141, 2612-2618.

Talal, N. (1994) Oncogenes, autogenes, and rheumatic diseases. *Arthritis and rheumatism,* 37, 1421-1422.

Tan, E.M., Cohen, A.S., Fries, J.F., Masi, A.T., McShane, D.J., Rothfield, N.F., Schaller, J.G., Talal, N. & Winchester, R.J. (1982) The 1982 revised criteria for the classification of systemic lupus erythematosus. *Arthritis and rheumatism,* 25, 1271-1277.

ter Borg, E.J., Horst, G., Limburg, P.C. & Kallenberg, C.G. (1990) Changes in plasma levels of interleukin-2 receptor in relation to disease exacerbations and levels of anti-dsDNA and complement in systemic lupus erythematosus. *Clinical and experimental immunology,* 82, 21-26.

Valencia, X., Yarboro, C., Illei, G. & Lipsky, P.E. (2007) Deficient CD4+CD25high T regulatory cell function in patients with active systemic lupus erythematosus. *Journal of immunology,* 178, 2579-2588.

Vallerskog, T., Gunnarsson, I., Widhe, M., Risselada, A., Klareskog, L., van Vollenhoven, R., Malmstrom, V. & Trollmo, C. (2007) Treatment with rituximab affects both the cellular and the humoral arm of the immune system in patients with SLE. *Clinical immunology,* 122, 62-74.

Vargas-Rojas, M.I., Crispin, J.C., Richaud-Patin, Y. & Alcocer-Varela, J. (2008) Quantitative and qualitative normal regulatory T cells are not capable of inducing suppression in SLE patients due to T-cell resistance. *Lupus,* 17, 289-294.

Veiga-Fernandes, H., Walter, U., Bourgeois, C., McLean, A. & Rocha, B. (2000) Response of naive and memory CD8+ T cells to antigen stimulation in vivo. *Nature immunology,* 1, 47-53.

Veis, D.J., Sorenson, C.M., Shutter, J.R. & Korsmeyer, S.J. (1993) Bcl-2-deficient mice demonstrate fulminant lymphoid apoptosis, polycystic kidneys, and hypopigmented hair. *Cell,* 75, 229-240.

William R. Heath & Francis R. Carbone. (2001) Cross-presentation in viral immunity and self-tolerance. *Nature Reviews Immunology* **1**, 126-134

Williams, W., Bhagwandass, A., Sargeant, L.A. & Shah, D. (2003) Severity of systemic lupus erythematosus with diffuse proliferative glomerulonephritis and the ineffectiveness of standard pulse intravenous cyclophosphamide therapy in Jamaican patients. *Lupus,* 12, 640-645.

Wright, W.E. & Shay, J.W. (2000) Telomere dynamics in cancer progression and prevention: fundamental differences in human and mouse telomere biology. *Nature medicine,* 6, 849-851.

Yanaba, K., Bouaziz, J.D., Matsushita, T., Magro, C.M., St Clair, E.W. & Tedder, T.F. (2008) B-lymphocyte contributions to human autoimmune disease. *Immunological reviews,* 223, 284-299.

Yoshie, O., Imai, T. & Nomiyama, H. (2001) Chemokines in immunity. *Advances in immunology,* 78, 57-110.

Zhang, B., Zhang, X., Tang, F.L., Zhu, L.P., Liu, Y. & Lipsky, P.E. (2008) Clinical significance of increased CD4+CD25-Foxp3+ T cells in patients with new-onset systemic lupus erythematosus. *Annals of the rheumatic diseases,* 67, 1037-1040.

Zheng, B., Han, S., Takahashi, Y. & Kelsoe, G. (1997) Immunosenescence and germinal center reaction. *Immunological reviews,* 160, 63-77.

Ziegler-Heitbrock, H.W. (1996) Heterogeneity of human blood monocytes: the CD14+ CD16+ subpopulation. *Immunology today,* 17, 424-428.

Ziegler-Heitbrock, H.W., Fingerle, G., Strobel, M., Schraut, W., Stelter, F., Schutt, C., Passlick, B. & Pforte, A. (1993) The novel subset of CD14+/CD16+ blood monocytes

exhibits features of tissue macrophages. *European journal of immunology,* 23, 2053-2058.

Ziegler-Heitbrock, L. (2007) The CD14+ CD16+ blood monocytes: their role in infection and inflammation. *Journal of leukocyte biology,* 81, 584-592.

Zonana-Nacach, A., Barr, S.G., Magder, L.S. & Petri, M. (2000) Damage in systemic lupus erythematosus and its association with corticosteroids. *Arthritis and rheumatism,* 43, 1801-1808.