T Cell Phenotypes in Upper Gastrointestinal Cancers

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Abstract

Background: Upper GI adenocarcinomas are increasing in prevalence. There has been limited research into the role of the immune system in the development and treatment of upper GI adenocarcinomas. Some studies have suggested that Th17 and Th22 cells may play a role in these cancers and could therefore be a potential area of further study.

Aims: This project focuses on developing multi-colour flow cytometry panels that can accurately identify the diversity of T helper cell phenotypes and use these techniques to further explore the role these cells may have in the development and disease process of upper gastrointestinal adenocarcinomas.

Methods: Blood samples from healthy donors and patients with either gastric adenocarcinoma or oesophageal adenocarcinoma would be collected and matched peripheral blood mononuclear cells (PBMC) isolated. Experiments were performed to develop an optimized flow cytometry panel that could identify the subtype populations of CD4 cells Th17 and Th22 by varying the duration of activation and the freeze / thawing of the PBMCs. Also a flow cytometry technique was developed to optimize the identification of these populations. Healthy donor PBMCs and upper GI cancer PBMCs were then analysed to determine the relative frequency of Th17 and Th22 cells within the different patient and healthy donor cellular populations.

Results: The optimal activation period to identification of Th17 and Th22 cells was 16 hours. Analyses of these populations in healthy donor PBMCs found a significant positive correlation between the relative frequency of Th17 and Th22 cells, Th17/Th1 and Th22 cells, and also Th17/Th1 and Th17 cells suggesting a possible relationship between these phenotypes. Comparisons between healthy donor PBMCs and patients with upper GI cancers observed an increase in the relative frequency of Th17, Th22 and Th17/Th1 cell populations but not Th17/Treg cells in patients. There was also evidence of an increase in the populations of Th22, Th17/Th1 and Th17/Treg cells with increasing disease stage. However, the converse was seen in the PBMCs of patients with gastric cancer as the percentages of Th22 and Th17 populations decreased with increasing disease stage suggesting a negative correlation.

Conclusion: These results show that the phenotypes Th22 and Th17 were accurately identified using a ten-fluorochrome flow cytometry panel in both healthy donors and cancer patients. There was also a relationship between Th17 and Th22 cells in both gastric and oesophageal cancers, however, the role they play maybe very different in the two different types of adenocarcinoma potentially suggesting a different pathophysiological process.

Declaration

The author of this thesis declares that no portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning

Copyright Statement

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Chapter 1 Introduction

1.1 Upper Gastro-Intestinal Cancers

1.1.1 Epidemiology Of Upper GI Cancers

Oesophageal cancer is the eighth most common cancer in men in the UK and over the last 30 years the incident rates have increased significantly for both men and women with an increase of more than 65% over the last 30 years (1). It particularly affects men over the age of 60 with a step rise associated with age. Although it accounts for only 2% of cancer cases diagnosed in the UK it accounts for almost 5% of cancer related deaths with an average 5 year survival on average of only 20%, despite advances in chemo and radiotherapy (2). The change in incidence has also been reflected in the histological type of oesophageal cancer occurring in the population. There are two main histological types of oesophageal cancer; squamous cells carcinoma (SCC) and adenocarcinoma. Over the last few decades with a sharp rise in the incidence of adenocarcinoma compared to a relatively static incidence of SCC (3). There are several hypotheses for this change in incidence but current evidence suggests that changes in lifestyle (e.g. increased obesity and reduction in smoking) could account for different risk factors in these two different histological cancers. The majority of documented cases of adenocarcinoma are in the distal third of the oesophagus, which has been linked to the pre-cancerous condition Barrett's oesophagus. This is a metaplastic condition associated with a chronic inflammatory process often secondary to gastro-oesophageal reflux disease (GORD). Risk factors for GORD include abdominal obesity, alcohol intake and smoking all of which are risk factors for Barrett's (4, 5) and the prevalence of central obesity has rapidly increased over the last few decades.

A similar pattern had been observed for gastric cancers. It is now the tenth commonest cancer in men in the UK occurring predominately in males with a sharp peak in incidence rates at around the 7^{th} decade (6). However unlike oesophageal cancer the incident rate of gastric cancer has been falling over the last 30 years in the UK. However this appears to vary considerably worldwide, with Japan and the Far East reporting significant increases in the incidence of gastric adenocarcinoma (7). There are several hypotheses to account for this difference. In the UK it is felt to be due to several factors including improved diagnosis and eradication of *H.Pylori* (a gram-negative microaerophilic, spiral bacterium). *H.Pylori* is associated with a chronic inflammation process that is a significant risk factor or the development of gastric cancer. On average there is a 2 fold increased risk of developing gastric adenocarcinoma (8). One hypothesis for the rapid

increase in incidence rate in the Far East is thought to be multifactorial, due to a number of variants including dietary differences, genetic variation as well as possible variations in prevalence of *H.Pylori* (9).

1.1.2 Pathophysiology of Upper GI Adenocarcinoma

The development of adenocarcinoma of the oesophagus appears to develop in a stepwise process. Through an inflammation-stimulated hyperplasia, a metaplasic process of columnar epithelium replacing squamous epithelium occurs as a response to that chronic inflammation. The premalignant metaplastic condition termed Barrett's is made when columnar epithelium are identified extending more than 10mm above the squamocolumnar junction. Initially thought to be genetic is now well established as an acquired condition secondary to insult of the tissues most commonly from acid reflux. However there is increasing evidence to suggest that there may be a genetic component to the subsequent development to dysplasia and adenocarcinoma (10, 11).

It is still not fully understood how the sequale of metaplasia-dysplasia-adenocarcinoma occurs. Several studies have focused on the immunological response to chronic insult particularly from reflux comparing the cytokine environment of benign condition of reflux oesophagitis with that of Barrett's oesophagus. Several cytokines have been specifically linked to Barrett's oesophagus compared to oesophagitis of columnar cells. A study by Fitzgerald et al identified that biopsies from patients with Barrett's oesophagus had distinct pattern of cytokines associated with the T helper cell phenotype Th2 characterised by high levels of IL-10 and IL-4 with relatively low pro-inflammatory cytokines such as IL-1β, IL-8, and interferon-γ (IFN-γ) in comparison to reflux oesophagitis (12). A more recent study in 2011 focusing on the immunological profile of Barrett's oesophagus and how it changed over time with the on-going development of metaplasia was performed on rats. They also found a distinct immunological profile of Th2 cells (IL-4, IL-10, and IL-13) were significantly increased in Barrett's oesophagus as compared to those in non-Barrett's oesophagus, while there were no differences in the levels of pro-inflammatory cytokines (13).

The pathogenesis of gastric cancer has long been thought to be related to chronic inflammation linked to numerous risk factors including diet, alcohol intake and the presence of *H.Pylori*. There are two distinct histological types of gastric cancer that can arise from this process; intestinal-type and diffuse-type. The development of the more common intestinal-type is thought to proceed in a similar stepwise process to that of other gastro-intestinal cancers such as

oesophageal. Risk factors act on the gastric mucosal microenvironment over a prolonged time period resulting in changes to the mucosa in a 'precancerous' cascade; normal gastric mucosa is transformed by chronic atrophic gastritis and develops multifocal atrophy and intestinal metaplasia, followed by dysplasia and then invasive carcinoma (14).

As mentioned previously one of the most significant risk factors for the development of adenocarcinoma is the presence of *H.Pylori*. A meta-analysis in 2006 found that patients infected with *H.Pylori* were twice as likely to develop gastric adenocarcinoma. However it is estimated that 50% of the world's population are infected with *H.Pylori* while only 1-3% of these will develop gastric cancer suggesting a more multifactorial role of *H.Pylori* in the development on gastric cancer (15).

Studies have found that *H.Pylori* induces the phenotypic changes of chronic gastritis, mucosal atrophy, intestinal metaplasia, and dysplasia, which are characteristic for progression to intestinal-type gastric cancer (16). The exact pathogenic role *H.Pylori* plays in the development of adenocarcinoma is not fully understood and it is suggested that a combination of genetic susceptibility and chronic inflammation contributes to the increased risk. Polymorphisms in several genes, particularly linked with immunological genes, have been considered to increase the risk for the development of gastric cancer. The pro-inflammatory cytokines such as TNF-α and interleukin-1-beta (IL-1β) have been found to be increased in patients with *H.Pylori* in gastric mucosa and in those with genetic polymorphisms have a significant increased risk in the development of gastric cancer (17, 18). Further studies looking at the specific role the immunological response and development of gastric cancers have found several components of both the innate and adaptive immunity which may be contributing factors (15).

Current evidence appears to suggest an immunological role in the development of upper GI cancer precursors but the specific role they play is still unclear and particularly how the immunological response changes or influences the development of adenocarcinomas. The complexity of the human immune system and the role immune cells play in the pathogenesis of a variety of diseases is still on going challenge for clinicians and immunologists alike.

1.2. The Human Immune System

1.2.1 Overview

The human immune system is a complex physiological system. Generally divided into two areas; the adaptive (acquired) immunity and innate (natural) immunity. Each of the major subdivisions of the immune system has both cellular and humoral components by which they carry out their protective function. Although originally thought to be distinct processes our understanding in recent years has significantly expanded to appreciate that there is a complex relationship between these two areas (19).

1.2.2 Innate Immunity vs. Adaptive Immunity

Innate Immunity consists of a number of components that form a first line defence against non-specific organisms. These include anatomical barriers, humoral barriers and cellular barriers. The anatomical barriers are very effective in preventing colonization of tissues by microorganisms. However, when there is damage to tissues the anatomical barriers are breached and infection may occur. Trauma to these defensive barriers leads to acute inflammation; a process by which humoral factors play a key component in immune defence. The complement system is a key component of the acute phase reactive process to a pathogen. It increases vascular permeability, recruitment of other cellular defence mechanisms such as phagocytes and opsonisation of bacteria. Part of the inflammatory response is the recruitment of polymorphonuclear eosinophiles and macrophages to sites of infection. These cells are the first line of defence in the non-specific immune system (innate immunity). Macrophages, neutrophils, natural killer cells and dendritic cells make up most of the innate immune system. The adaptive immune system is mediated predominately by lymphocytes consisting of T and B lymphocytes. Although similar in structure and both originate from bone marrow stem cells, they perform very different protective functions in the body.

Although we talk of the innate and adaptive immunity as separate entities we now understand that these systems are closely linked. A specialized group of cells termed antigen-presenting cells (APCs) link the innate and adaptive immune systems by taking up and processing antigens so they can be recognized by T cells, and by producing cytokines. APCs enhance innate immune cell function and they are essential for activation of T cells. There are also natural killer T cells (NKT), which exhibit characteristics that place them at the border between innate and adaptive immunity. Unlike conventional T cells they do not rely on the MHC molecule for activation. They

also produce various cytokines, of which some may have opposite functions which may play a key function in immune surveillance of spontaneous and carcinogen induced cancers (20).

1.2.3 Lymphocytes

Lymphocytes are morphologically heterogeneous. Differences can be seen in nuclear to cytoplasmic (N:C) ratio, the nuclear shape and the presence or absence of azurophilic granules.

1.2.3.1 B Cells

These are mainly responsible for the production of antibodies which act on extracellular pathogens. The B cell receptor (BCR) is a membrane bound form of antibody (immunoglobulin or Ig) that B cells secrete following activation and differentiation. Due to the unique mechanism of genetic recombination a limited number of gene segments can produce a vast number of different receptors and thus antibodies (21).

1.2.3.2 T Cells

Some bacterial pathogens and all viruses replicate within cells where they cannot be detected by antibodies. T cells play a key role in the destruction of these pathogens through the cell-mediated immune response of adaptive immunity. It depends upon direct interaction of T cells with cells bearing the recognized antigen. There are a number of different types of T Cells however all T cells recognize antigens present on the surface of other cells using a specific receptor - the **T cell antigen receptor (TCR)** (figure 1.1).

1.2.3.3 TCR

There are two types of TCR;

- TCR α and TCR β; These are heterodimers of two polypeptides linked by a disulphide bond
- TCR γ and TCR δ; These are also polypeptide chains similar to α:β TCR but have different antigen-recognition properties.

The αβ or γδ heterodimers must associate with a series of polypeptide chains collectively termed the CD3 complex for the antigen-binding domains of the TCR to form a complete, functional receptor that is stably expressed at the cell surface and is capable of transmitting a signal upon binding to antigen (22).

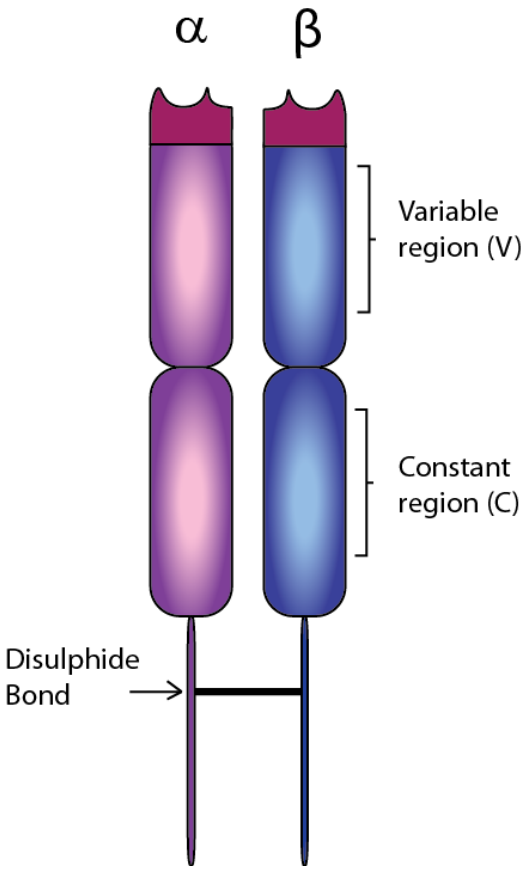


Figure 1.1 T Cell Receptor

The T cell receptor is a molecule found on all T cells allowing it to recognize specific antigens bound to the major histocompatibility complex (MHC) molecule. The most common form of this molecule is made up of two different protein chains α and β linked by a disulphide bond. The variable region of the molecule allows for specific antigen recognition. Adapted from (21)

1.2.3.4 Major Histocompatibility Complex

T cell receptors only recognize linear epitopes in the form of short peptides which are generated by degradation of intact proteins within the cell (a process termed antigen processing) expressed on the major histocompatibility complex (MHC). There are two forms of MHC molecules; class I molecules and class II. They differ by how proteins are sourced to make the molecule to display on the cell surface. MHC class I molecules binds epitopes of 8-11 amino acids ad MHC class II epitopes of 13-17. In both cases proteins from the cell are digested into short peptide fragments; however peptides derived from proteins produced within the cell are displayed on the cell surface through binding to MHC class I molecules, but peptides derived from proteins ingested from the extracellular environment by phagocytosis are presented by MHC class II molecules, which are present only on professional antigen presenting cells (23).

The two classes of MHC are recognized by different functional classes of T cell; CD 4+ T helper cells recognize antigens in association with the MHC class II molecules whereas CD8+ Cytotoxic cells recognize antigens associated with MHC class I molecules. A small proportion of αβ T cells express neither CD4 nor CD8; these 'double negative' T cells might have a regulatory function (21).

1.2.3.5 CD3 Complex

Although the TCR provides extensive variability in its antigen recognition it does not provide any signalling capability within the T cell. Studies showed that other molecules are required for the TCR to be expressed on the surface of a T cell. We now know that a number of molecules are required known as the CD3 complex.

The CD3 complex is made of polypeptide chains; CD3γ, CD3δ, CD3ε and CD3ζ (figure 1.2). These are also known as the invariant chains as they do not show any variation. CD3γ, CD3δ, and CD3ε are heterodymers but CDζ is slightly different in structure in that it has a smaller extracellular domain amongst other features (23).

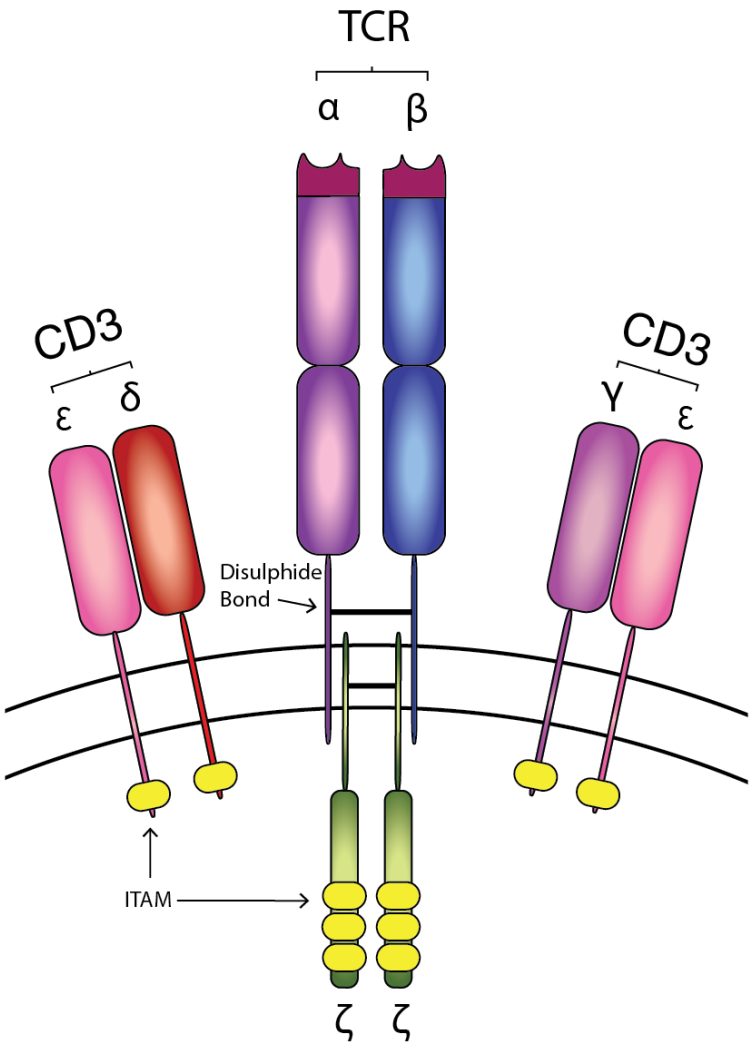


Figure 1.2 CD3 Complex

The CD3 complex is a series of invariant polypeptide bonds that allows intracellular signalling following activation of the TCR. The cytoplasmic component of the CD3 molecule contain immunoreceptor tyrosine based activation motifs (ITAMs) which are an essential component of T cell activation. Adapted from (21)

1.2.4 T Cell Differentiation and Proliferation

Once T cells have finished their development in the thymus they enter the circulation in the form of naïve T cells (figure 1.3). These are maintained in the circulation by periodically being stimulated by self MHC complexes and IL-7. Once they encounter its specific antigen, presented to it a peptide:MHC complex on the surface of an antigen-presenting cell (APC), they proliferate and differentiate in to armed **effector T cells** (priming). Dendritic cells which are found in abundance in the T cell areas of lymph nodes and spleen, are the most effective antigen-presenting cells for the initial activation of naive T cells (21).

Differentiation and proliferation occur through several key mechanisms including antigen presentation, costimulation and cytokine secretion. Initial studies in the 1980's identified the interaction of the peptide:MHC complex and the TCR as the primary component in the activation and differentiation of naïve T cells. However in 1996 Paul and Seddar described a complex process of interaction which expanded beyond the peptide:MHC complex. They suggested that other cytokines and other costimulatory pathways are also involved in priming T cells along with the peptide:MHC complex to form the immunological synapse (IS) (24). Early studies defined the function of the CD28 receptor as a stimulatory receptor for the activation of naive T cells (25) with the B7 family (B7.1 (CD80) and B7.2 (CD86))as its ligand on the APC (figure 1.4). One of the main functions of this costimulation is to augment and sustain T-cell responses initiated by antigen-receptor signalling by increasing the affinity of the IL2 receptors whilst stimulating the production of IL-2 by the T cell (26). IL-2 is essential for cell survival and differentiation.

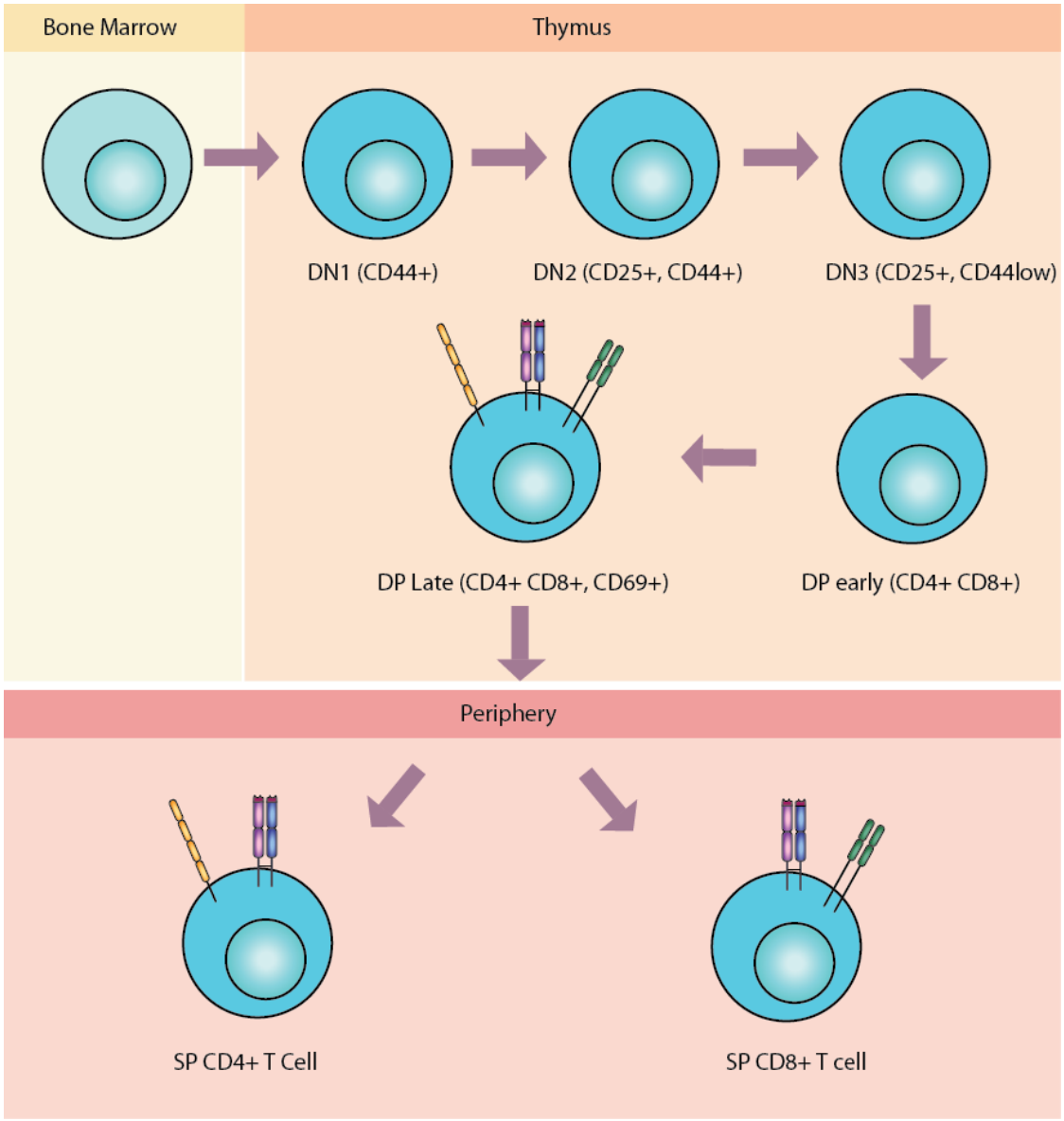


Figure 1.3. T Cell Proliferation

Thymocytes move through the medulla the thymus where there cytokine expression changes starting off as double negative (DN1) cells to become double positive (DP) for CD4 and CD8 expression. Following development in the thymus the thymocytes leave the thymus entering the blood stream as single positive (SP $CD4^+$ or $CD8^+$) T cells. Adapted from (23).

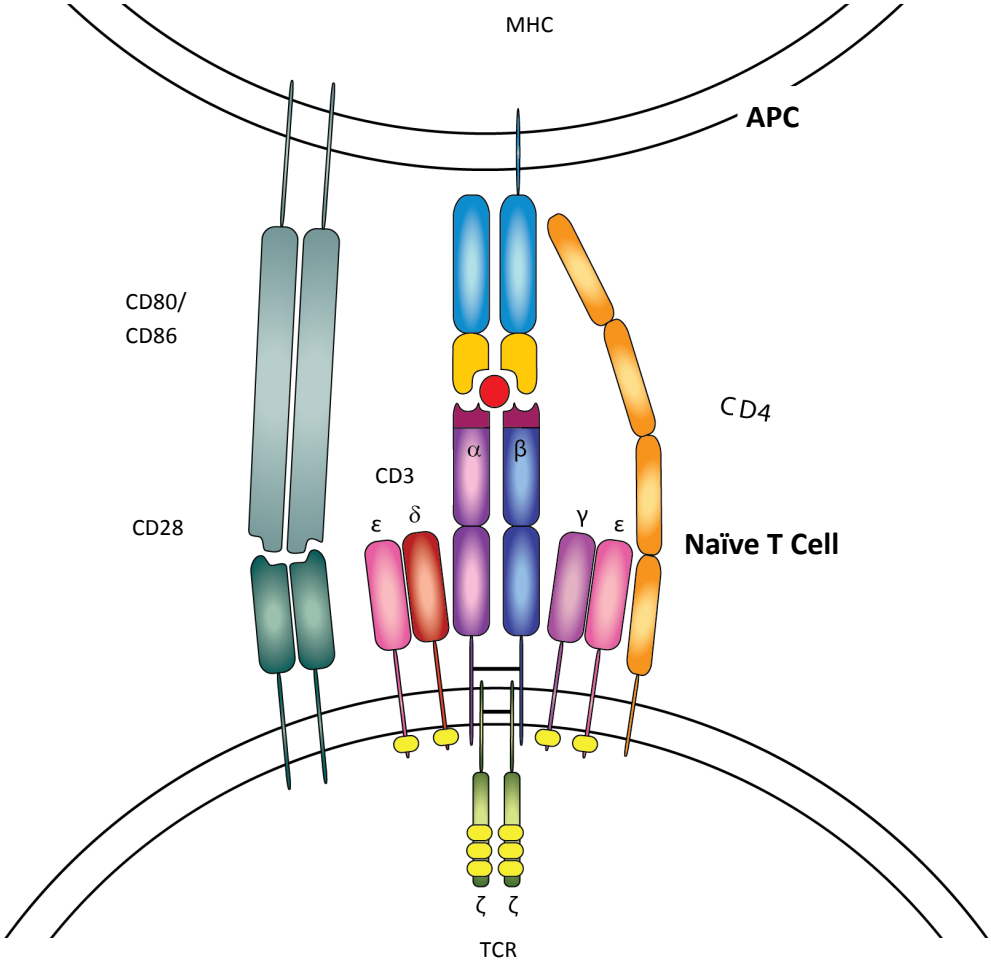


Figure 1.4 T Cell activation by the Immunological Synapse

T cell activation by the MHC molecule of the antigen presenting cell (APC) is a complex process requiring the presence of CD3 for intracellular signalling, and CD4 or CD8 molecules to stabilise the TCR/MHC interaction as well as costimulation of other ligands CD28/CD80 interaction. It is thought IL-2 is an important cytokine in this process. Adapted from (21).

1.2.4.1 CD4+ T cell

The cytokine melee of the interstitial environment controls the differentiation process of effector T cells. With the improved techniques of flow cytometry phenotyping has identified several subsets of T cells with a variety of different functions (figure 1.5). It was originally identified by Mossman and Coffman (1989) that there were 2 different classes of CD4+ T cells known as T helper cell 1 (Th1) and T helper cell 2 (Th2) (27). They were differentiable by the class of molecules on their surface and also the cytokines they produced. Th1 cells were proliferate produces of IFN-γ, IL-2, and some producing TNF-α. Th2 cells do not produce any IFN-γ; their main cytokine production is IL-4, IL-5 and IL-13. It was felt that Th1 cells were critical for immunity to intracellular microorganisms such as viruses and Th2 cells were critical for immunity to many extracellular pathogens such as parasites. Cytokines play an important role in their function but they are also a key component to their differentiation. Studies have shown that the main cytokines involved in differentiation of T cells into Th1 cells are IFN- γ, IL-12 and IL-18. In the case of IFN-γ, its effect may be to prevent the outgrowth of Th2 cells rather than to promote directly the selective development of Th1 cells (28). IL-12 appears to induce some IFN showing a positive feedback loop for Th1 cells as well. IL-4 has been demonstrated to have the greatest influence in driving Th2 differe3 IL-ntiation (29). However recent studies have shown that a number of other cytokines are involved in the differentiation of Th2 cells, including a number of members from the IL-1 family; IL-18 and IL-33 (30, 31).

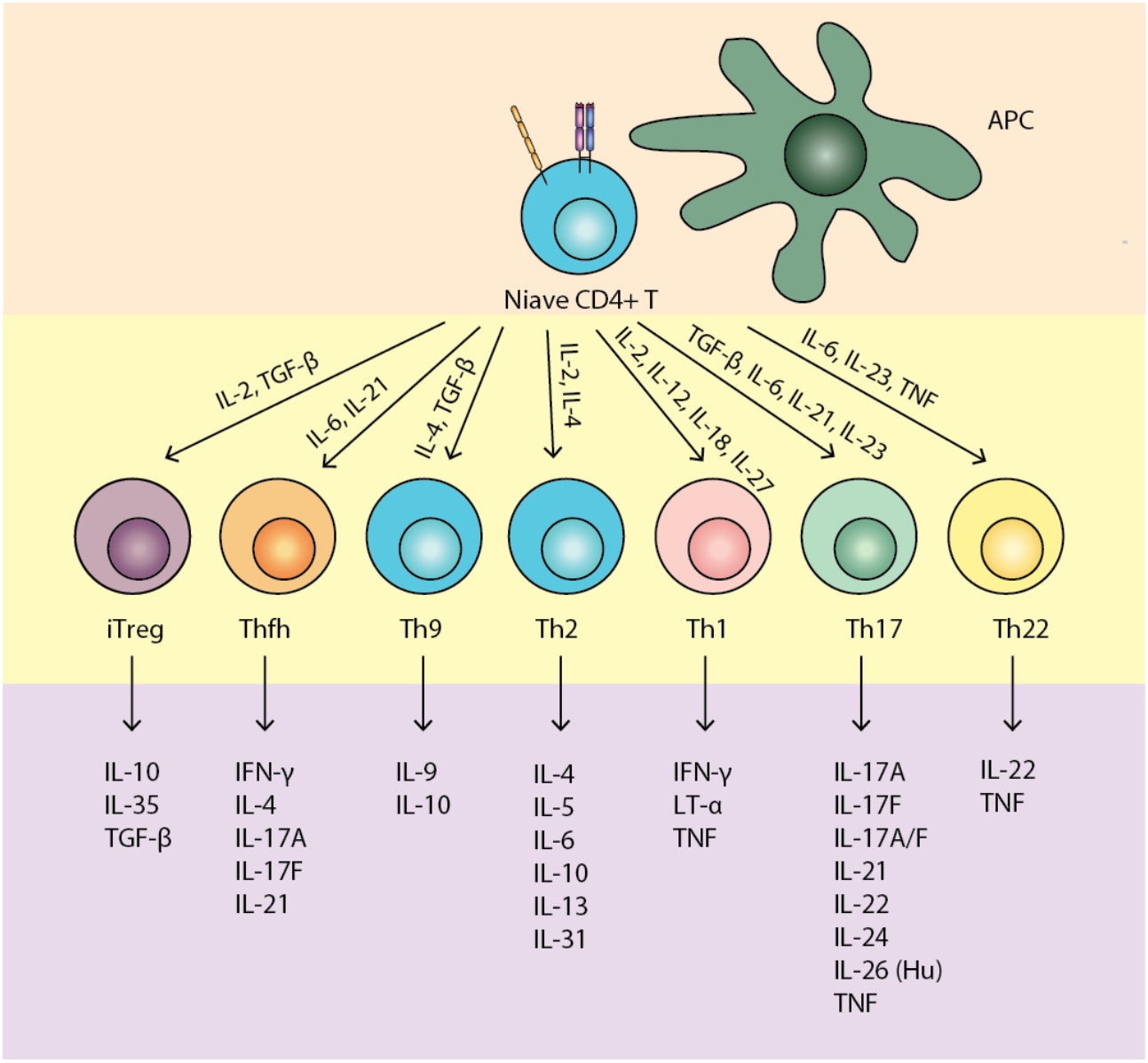


Figure 1.5 CD4+ T Cell Differentiation

T cell differentiation from naïve $CD4^+$ cells depends upon the cytokine environment in which the naïve cells are activated. Adapted from (21)

1.2.4.2 T Reg Cells

Another important subset of the adaptive immune system is the regulatory T cell. In 1995 Sakaguchi at el identified a subset of T cells which expressed $CD4^+$ and $CD25^+$ and account for 10-15% of circulating $CD4^+$ cells. They found that depletion of this population led to the generation of a spectrum of autoimmune diseases when transferred to immune-compromised recipients (32). It was later identified that these originated within the thymus and so were defined as natural regulatory cells. Further studies identified that a key feature of these Treg cells was the expression of a forkhead box transcription factor FoxP3 which interferes with the production of IL-2 (33). Thus its key function is to regulate and supress the immune system and studies have shown mutations in FoxP3 result in a deficiency of Treg cells which in humans this leads to the lethal auto-immune syndrome IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked) (34). Although Tregs supress IL-2 production studies have shown that IL-2 co-stimulation as well as continuous TCR triggering is essential to maintain Tregs in the periphery (35).

Apart from thymic produced regulatory T cells (nTreg) cells another subtype of regulatory T cells exist in the periphery known as induced regulatory T cells (iTreg) which originate from circulating naïve CD4+ FoxP3- T cells. In 2001 Yamagiwa et al identified that whilst TGF- β has suppressive role on T cell function it can also synergize with IL2 to also stimulate production of iTreg (36). They found that TGF- β had a stimulatory role on naïve $CD4^+$ $CD45RA^+$ T cells resulting in the development of $CD4^+$ $CD25^+$ $CD45RA^-$. Further studies support the importance of TGF-β and IL-2 in the co-stimulation of iTreg and these have also identified that iTreg cells express FoxP3 through direct stimulation by high levels of TGF-β (37-39). It is also suggested that in addition to direct activation of FoxP3 and promotion of cell survival in the presence of high amounts of TGF-β, IL-2 opposes differentiation of activated CD4+ T cells into T helper 17 (Th17) cells (40). Recent challenges in the analysis of iTreg cells in vivo have been identifying what population in peripheral blood have originated from the thymus as nTreg and what percentage have originated from naïve $CD4^+$ T cells. In 2010 Thornton et al identified that $FoxP3^+$ nTreg cells originating in the thymus were positive for Helios, a member of the Ikaros transcription factor family and that induced peripheral iTreg cells were negative for Helios. Therefore this marker could be used to identify what percentage of peripheral population were nTreg cells and iTreg cells (41).

Since the identification of the FoxP3 transcription factor a further subgroup of Treg cells have been identified which are $FoxP3^-$ known as type 1 regulatory cells Tr1 which can arise without the presence of nTreg but still have similar regulatory function. Initially identified as suppressor $CD4^+$ T cell by their unique cytokine production of high levels of IL-10, some TGF-β and IFN-γ, and

minimal levels of IL-2, IL-4 and IL-17, this identified them as a separate subset to the Th2 and Th17 cells (42). Although defined as FoxP3$^-$ they do however when initially activated express FoxP3 transiently but is not maintained and do not reach the high levels of nTreg and iTreg (43). The main driving force for the production of Tr1 cells is IFN-α, but not TGF-β, acting synergistically with IL-10 by immature dendritic cells (44). To date Tr1 cells still lack a defined cell surface signature in comparison to nTreg and iTreg cells.

It is well documented that deficiency in Treg function and frequency can have a significant impact on the human body leading to a range of autoimmune and inflammatory conditions. Mice studies looking at the role of FOXP3 found that those mice with mutations in this gene developed fatal lymphoproliferative disorder highlighting its importance for normal immune homeostasis (45).

1.2.4.3 TH17

Since Mossman and Coffmans work a significant number of subgroups of CD4+ cells have been identified. Further studies looking at cytokine production identified another subset of T helper cells; the Th17 cells. These were found to not produce the "classical" Th1/Th2 cytokines, these were predominately IL-17a, but also IL-17f, IL-21 and IL-22 producing CD4$^+$ cells. They were also identified by key transcription factors which are key to production and function of Th17 cells; signal transducer and activator of transcription 3 (Stat3), retinoid-related orphan receptor γt (ROR-γt), and nuclear receptor ROR-α (46). It is thought that the key function of Th17 cells is in the defence against extracellular pathogens at mucosal surfaces (e.g. bacteria and fungi) through an inflammatory process and have been implicated in the induction of several autoimmune diseases such as hyper-immunoglobulin E (IgE) syndrome (HIES), where recurrent infections are a feature (47). Although IL-17 is one of the main cytokines produced by Th17 and is an identifier of this phenotype it must be noted that Th17 cells are not the only producers of IL-17. It has been shown that NKT (48), CD8 cells (49) and Macrophages (50) all produce IL-17 in variable amounts.

Differentiation of Th17 cells from naïve CD4+ cells is still not fully understood. In 2006 Veldhoen et al identified that interleukin-6 (IL-6) in combination with transforming growing factor β (TGF-β) stimulate a naive CD4$^+$ T cell to become a T helper 17 (Th17) cells (51). It was felt that the IL6 inhibits TGF- β induction of FoxP3 transcription factor whilst stimulating RORγt$^+$ development which alongside IL-17 production helped to identify Th17 as complete new subset of CD4$^+$ helper cells. Development of Th17 also is dependent on the production by the T cell of the cytokine IL-21 which is also capable of suppressing TGFβ mediated induction of FoxP3 (52). It acts as a powerful positive feedback stimulant reinforcing the Th17 induction process and showing that Th17 development has the logic similar to that of Th1 and Th2 cells (53). Other cytokines linked

with Th17 included IL-23 which although not unique to Th17 cells has been found to aid in proliferation. IL-23 was found to consist of a unique p19 subunit and a p40 subunit similar to that of IL-12 thus having a similar function of stabilising the cell (54).

How much the of each of these cytokine contributes to the differentiation of Th17 cells is still relatively unknown and also how much TGF- β actually plays a role is still not clear. Another key question is do Th17 cells originate from a similar origin to TReg Cells that have undergone 'reprogramming' depending upon the microenvironment? $Foxp3^{+}RORγt^{+}IL\text{-}17^{+}$ has been identified in vivo in pro-inflammatory conditions suggesting an intermediary between Treg and Th17 cells. Studies have shown that $Foxp3^{+}$RORγt+ subsets are still fully functioning and under certain conditions can be driven towards IL-17 producing cells with TGF- β being the important factor as to the direction T cells differentiate(55). Increasingly, it is felt that unlike Th1 and Th2 cells which are relatively stable and terminally differentiated subsets, Th17 have a high degree of plasticity similar to Treg cells (figure 1.6) (56). Studies have suggested that Th17 cells vary in the production of IL-17 and have been shown to produce IFN-γ which may represent a redifferentiation into Th1 cells (57).

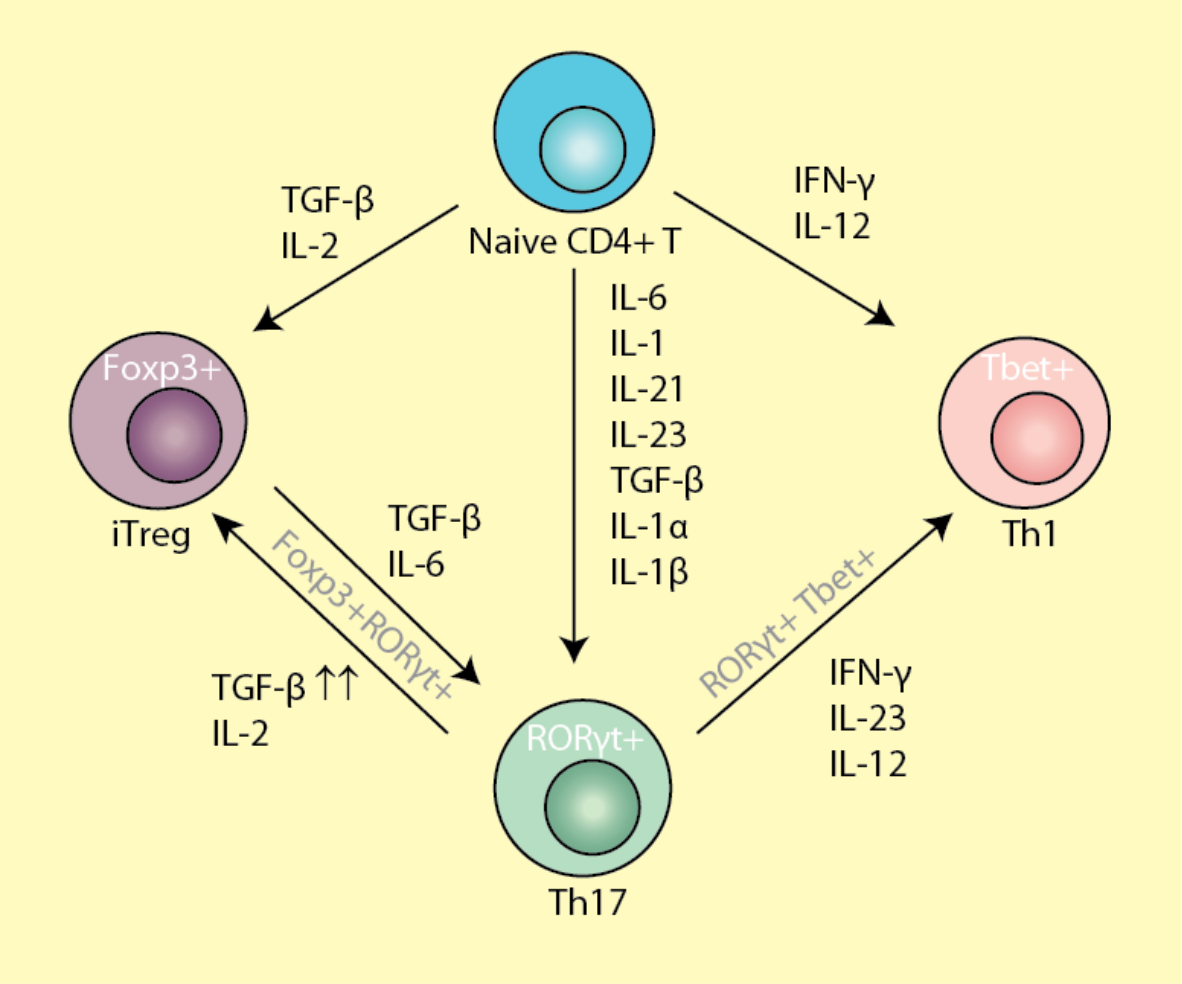


Figure 1.6 Th17 Plasticity

The differentiation of Th17 cells from naïve $CD4^+$ cells is more complex than was originally thought and that the Th17 cell may not be a definitive endpoint. They maybe 'reprogrammed' further depending upon the cytokine environment suggesting that Th17 cells have a plastic nature. Adapted from (56)

1.2.4.4 TH22

Although Th17 cells are a key producer of IL-22, the signature cytokine IL-17 and IL-22 are not always co-expressed (58, 59). IL-22 is another inflammatory cytokine stemming from the IL 10 family, acting mainly on non-haematopoietic cells such as epithelial cells and is therefore thought to be involved in early host defence against microbial pathogens (60). This unique attribute is a result of the IL-22R, which forms a complex with the IL-10R2 receptor expressed only on stromal cells and not on haematological cells and is therefore an important mediator of the interaction of the immune system with its non-haematological environment (61). In 2009 Duhen et al found that there IL-22 could be produced without interferon IFN-γ (Th1), IL-4 (Th2), and IL-17 (Th17) suggesting that there was another subtype T helper cells, Th22. They also found that they're differentiation was dependent upon IL-6 and TNF-α (62).

1.3 Immunological Response and Cancer Development

1.3.1 Immunological tumour defence

The idea that the human immune system effectively protects humans against pathogens could also play a role in tumour defence was first hypothesised more than 100 years ago. However it was in the mid 1950s when MacFarlane Burnet and Lewis Thomas first formalised the hypothesis of "cancer immunosurveillance' suggesting that the immune system had a significant role in tumour defence (63). Early studies exploring the way tumours are influenced by our immune system in mice found that transplanting a tumour from one mouse into another induced a variable immune response to that tumour (64). They also identified that mice that were T cell efficient did not have this same protective role of the immune system suggesting the importance of T cell mediated response to tumours (65). This suggested the idea of the existence of "tumour specific antigens" which provides a key cornerstone in the cancer immunosurveillance theory. For the immune system to detect tumours they must be identifiable.

It wasn't until the 1990s that the idea of immunosurveillance really developed where studies examining the role of IFN-γ in the development of methylcholanthrene (MCA)-induced sarcoma formation. Studies found that mice treated with neutralizing monoclonal antibodies specific for interferon- γ (IFN-γ) in which tumours where transplanted found that these tumours grew more rapidly (66). Further observations found that immunodeficient mice who were IFN-γ-insensitive lacking the IFNGR1 ligand-binding subunit of the IFN-γ receptor were significantly more sensitive than wild-type mice to tumour induction by MCA (67). Further understanding of the human immune system and particularly of innate immunity suggested that the process of tumour cell detection was more complex and involved more than just lymphocytes of the adaptive immune system. Further studies of natural killer cells identified that these may have a significant role in tumour cell defence (68). However immunosurveillance may not be the most appropriate way of describing this process as we know that the immune system exerts both host-protecting and tumour sculpting effects on developing tumour therefore the term Immunoediting is often used (69).

1.3.2 Immunoediting

It has subsequently been hypothesised that there are a number of dynamic phases to immunoediting; the elimination, equilibrium and escape phases (69). The elimination phase encompasses the original hypothesis of immunosurveillance in which the human immune system

recognises and destroys potential tumour cells, which have developed as a result of failed intrinsic tumour suppressor mechanism (figure 1.7). Studies on mice have shown that the immune system, particularly T cells, may play a significant role in tumour development but the process of how the innate and adaptive immune system does this is still debated (70, 71). Building on early studies Shankaran et al in 2001 found that a significant number of mice lacking recombination-activating gene-2 (RAG-2) an essential component in the maturation of function T cells, B cells and NKT cells (72) developed spontaneous gastrointestinal malignancies with aging (73).

Dunn et al has theorised that the initiation of the "antitumor immune system" occur when growing tumours disrupt local tissue through angiogenesis or invasive tissue growth which results in the production of local inflammatory response which together with local cytokines initiate a local immune response (74).

However tumour cell variants may not be wholly eliminated and this an equilibrium phase may be entered where the immune system balances net growth of the tumour. Resulting in the cancer entering a dormancy phase, which could help explain the long latency period from initial transformation event to escape phase and development of malignant disease. Tumour dormancy suggests tumours that remain present in patients for are controlled by local mechanisms such as blood supply limitation or control by the immune system or cellular dormancy where cells enter a phase of quiescence, however they eventually progress locally or form distant metastases (75). In contrast to evidence for the elimination phase evidence for the equilibrium phase had been largely inferred from clinical observation. However In 2007 Koebel et al supported the evidence for the equilibrium phase through studies of cohorts of wild-type C57BL/6 or 129/SvEv were injected with MCA to stimulate the development of sarcoma. Those mice with locally stable disease were then treated with monoclonal antibodies to deplete $CD4^+$/$CD8^+$ and neutralize IFNγ, following which significant numbers (60%) then when on to develop progressively growing sarcomas (76). In contrast they identified that tumour overgrowth did not occur when monoclonal antibodies were used that deplete natural killer cells suggesting the adaptive immune system plays an important role in preventing MCA-induced sarcoma growth (76). More recent studies have supported this theory of an immune-mediated equilibrium suggesting that this could be the longest of the three phases where there is continuous eradication of tumour cells and continuous emergence of resistant tumour cell variants by immune selection pressure (77).

Over time, different genetic variants of the tumour cells may develop which may prevent them being eradicated by the immune system. Essentially “escaping” immune detection and elimination. The theory of tumours evading the immune system has been well documented. A review by Browning in 1992 suggested various mechanisms as to how tumours subsequently evade the immune system (78). One of the most documented principle theories is that the loss of the MHC class I protein expression by tumours plays a considerable role (79). It has already been identified that adaptive immunity plays a considerable role in tumour suppression and eradication and therefore supports the theory that the adaptive immune system, particularly T lymphocytes are also a key component in immunoediting. Also when tumours fail to respond to IFN-γ through mutations or deletions of the IFN-γ receptor gene, tumour cells fail to upregulate the MHC class I protein and fail to develop key components of antigen presenting (TAP1, TAP2). Therefore this down regulation of MHC class I proteins results in increase tumour growth and potential dissemination (80). Several studies have shown loss of MHC class I protein is observed frequently in a number of cancers including colorectal, melanoma and renal cancers (81).

Another key theory, which has been exhaustively reviewed but is still not fully understood is the role of Treg cells in cancer escape. As previously mentioned Tregs play a significant component in immunological regulation and can supress the function and proliferation of $CD4^{+}CD25^{-}$ and $CD8^{+}$ T cells. High levels of Treg cells have been found as infiltrates in several cancer types, including breast and lung (82), along with an altered cytokine environment with increased levels of IL-10 and TGF-β suppressing IFN-γ production (83). A number of studies have suggested that IL-10 is produced directly by the certain tumour cells or by tumour infiltrating macrophages (84). However the relationship between IL-10 and cancer progression is still debateable. Some studies have shown direct correlation between tumour growth as such as human melanoma lines however certain cancers such as thyroid cancer IL-10 does not appear to be associated with cancer progression (84, 85).

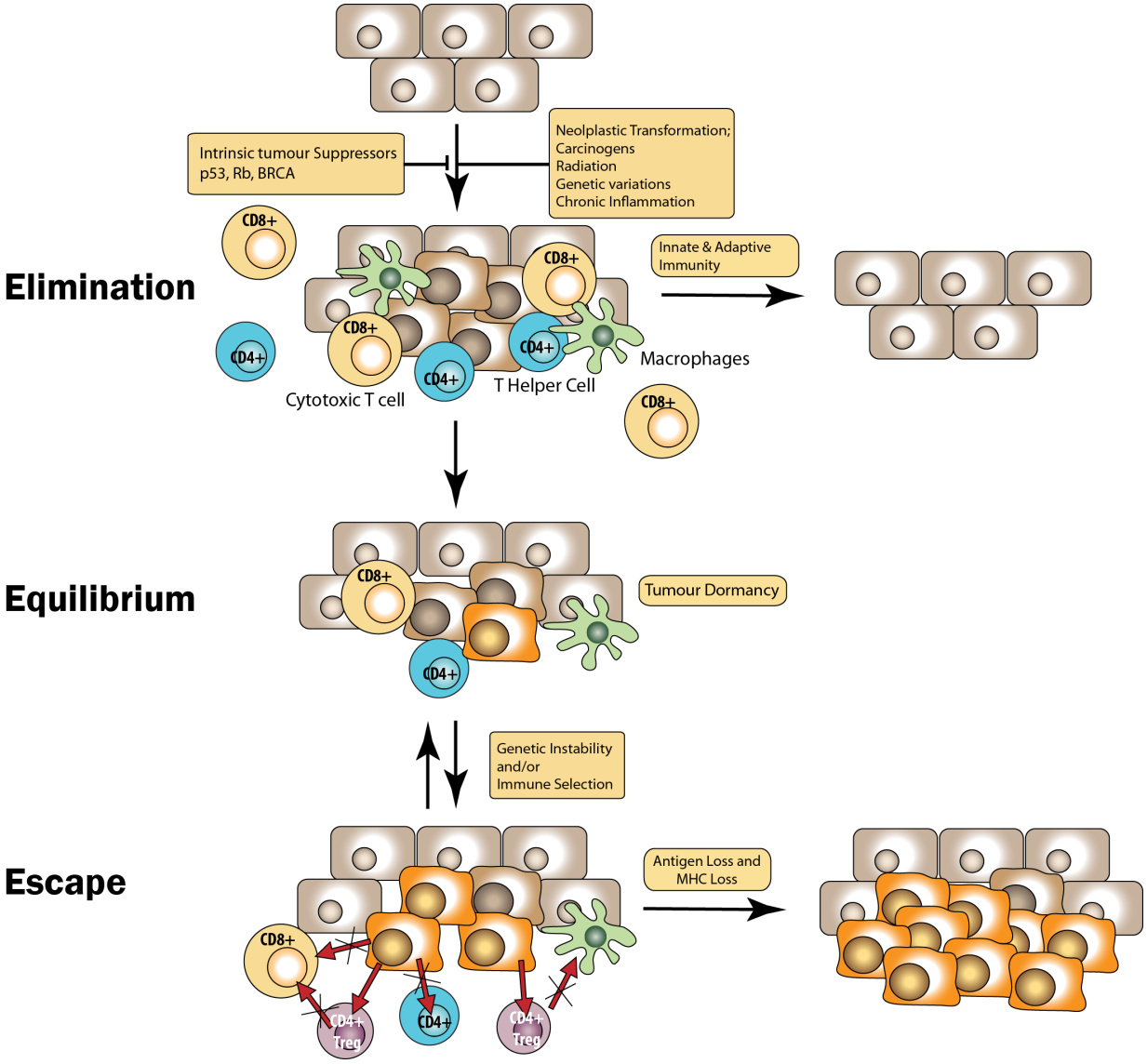


Figure 1.7 Immunological Control

One common hypothesis for how tumours evade the immune system is that of immunoediting. The suggestion is that 3 phases occur leading to tumours escaping immunoloigcla control;

Elimination: Following the failure of intrinsic cellular controls both the adaptive and the innate immne system play a role in eliminaiting tumour cells

Equilibrium: However tumour cells may not be completely eliminated and an equilibrial phase is entered where a blance between formation of tumour cells and elimination occurs.

Escape: Eventually through a process of selection, variants in the tumour may evolve that result in evasion of the immune system.

Adapted from (74, 80)

1.3.3 Tumour Infiltrating Lymphocytes

Much of the research and our understanding of the relationship the immune system has with tumour development and progression comes from mouse models. However the question arises as to whether the same process that we see in the tight controls of mouse studies happen in the complex immunological system of humans. We know from clinical case series and longitudinal studies that patients who are immunodeficient such as AIDS have a significantly increased risk of developing malignancies most often associated with virus e.g. Kaposi's sarcoma from the herpes virus or urogenital cancers from human papilloma virus (86). Although a significant number of AIDS related cancers are secondary to virus not all can be explained by viral immunology such as increased frequency of lung cancers in AIDS patients (87).

There are also numerous studies reporting the increased risk of de novo cancers in patients following organ transplant who are subsequently immunosuppressed. A recent study review of patients following renal transplant found there was a significantly increased risk of developing urological tumours following transplantation (88, 89).

Further supporting immunological involvement in human cancers is the numerous studies focusing on tumour infiltration by lymphocytes. One the earliest studies to suggest the association of TILs and prognosis was Murray et al who found that in a large cohort of colorectal patient, those patients who had higher levels of inflammatory cells in their tumours had a better prognosis that those with no tumour infiltrates (90). Over the last few decades studies into various types of cancers (cervical, breast and renal cancer) have also identified a relationship between tumour infiltrating lymphocyte involvement and prognosis, both positive and negative outcomes (91-93).

1.3.3.2 Phenotypes of TILS

These studies identified that it wasn't sufficient to simply quantify the lymphocyte involvement in a tumour but it was important to identify the specific phenotypes of lymphocyte involved as multiple studies have also shown that the relationship isn't always favourable and that in some cases there was a negative association with decreased survival rates. Curiel et al in 2004 identified that increased numbers of tumour infiltrating lymphocytes were associated with poor prognosis. When they phenotyped the cells involved they found significantly increased numbers of $CD4^+CD25^+FoxP3^+$Treg cells compared to $CD4^+CD25^-$ and $CD8^+$ cells in patients with ovarian carcinoma compared to healthy donors (94). This has subsequently been found by a number of further studies including colorectal cancer and melanoma (95, 96).

1.3.3.3 Th17 cells in cancer

Several particular phenotypes of $CD4^+$ cells have become increasingly linked with prognosis in a wide variety of cancers. The role of Th17 cells in cancers has been particularly examined as it has become increasingly being recognized as a key player in inflammation, autoimmunity and allergic reactions and potentially cancers (97). This may suggest that there is an association between chronic infection and inflammation and tumourgenesis. The understanding of how Th17 of involved in tumour immunology is still poorly understood. Early mouse models have suggested that IL-17 played a key protective role in colonic cancer development as IL-17 deficient mice had a greater rate of tumour growth and metastatic development compared to control mice (98). However they could not convincingly identify that the IL-17 produced in the control mice was from Th17 cells as there was also decreased IFNγ^+ NK and IFNγ^+ tumour-specific T cells in the tumour draining lymph nodes and at the tumour sites. Conversely studies have also shown that decreased levels of Th17 in mice which were IL-17R−/− injected with prostate and melanoma cancer and lymphoma cells lines was associated with decreased tumour growth. They correlated this result pre-treating mice with IL-17 which was associated with increased tumour growth in mice inoculated with lymphoma cell lines (99). Certainly in the controlled environment of mouse models studies have shown that Th17 appears to have a significant role in tumour development whether it be protective in cancers such as colonic cancer or involved in tumour growth such as prostate cancer.

Human in vitro studies have also shown this variability in the roe of Th17 in cancers. Several recent studies have been linked Th17 cell levels with improved prognosis and slow tumour growth. A recent study evaluating the phenotypes of TILS in prostate cancer found that IL-17 producing CD4+ cells were inversely proportional to tumour grade. They hypothesised that Th17 cells may have in certain cancers a protective antitumour role. In an attempt to further understand the tumour microenvironment, in ovarian cancer Kryczek et al focused on the relationship between IL-17 and other lymphocytes. They found that high numbers of IL-17 producing $CD4^+$ cells correlated with the number of IFNγ$^+$ $CD4^+$ T cells, IL-17^+ IFNγ$^{+,}$ and IFNγ$^+$ $CD8^+$ T cells and inversely correlated with the frequency of immunosuppressive $FOXP3^+$ cells. They also found that high levels IL-17 in TIL s and PBMCs were directly associated with stage of disease and prognosis in ovarian cancer suggesting that IL-17 might have a protective role in ovarian cancer (100).

However a number of studies have found the inverse and that in certain cancers they are linked with poor prognosis. A study by He et al in 2011 found that in pancreatic cancer higher levels of

IL-17 associated with $CD4^+$ cells were found in both TILs of pancreatic cancer patients and PBMCs. Also they found that IL-17 levels correlated to disease stage and survival (101) . They summarised that these high levels of IL-17 were secondary to high levels of Th17 cells however as previously mentioned several other phenotypes produce IL-17 including NKT cells.

As mentioned several studies suggest a protumour effect from Th17 cells mediated by IL-17 which has been seen in both mouse and human models. It is thought that IL-17 may have an effect on angiogenesis and thus tumour growth through the induction of a wide range of angiogenic factors such as VEGF, PGE2, keratinocyte-derived chemokine, and nitric oxide from fibroblasts which was seen in studies on colonic cancer (102) and hepatocellular carcinoma (103).

Other theories suggest that overall Th17 may have a net anti-tumour effect. One particular area of study suggests that the net effect of IL-17 depends upon local interaction with other effector cells particularly IFN-γ producing effector T cells (Th1), NKT, and CTL (104). Another suggestion is that Th1-type chemokines CXCL9 and CXCL10 are induced by TH17 through interaction with IFN-γ, resulting in further recruitment of effector cells (100).

It is clear that Th17 cells have an ambiguous role to play in cancer and the variability of the results of these studies highlight the complexity of the tumour microenvironment and may represent the plasticity of Th17 cells (figure 1.8).

Th22 cells as reported are a relatively new distinct subset of CD4+ helper cells and as such little is known or understood about their functioning role in cancers. More recently studies are starting to show that Th22 cells appear to have a similar inflammatory role to Th17 cells and therefore may have an important role together in cancer pathogenesis. Certainly studies have shown that Th22 and IL-22 are linked with various inflammatory conditions such as Crohns disease (105).

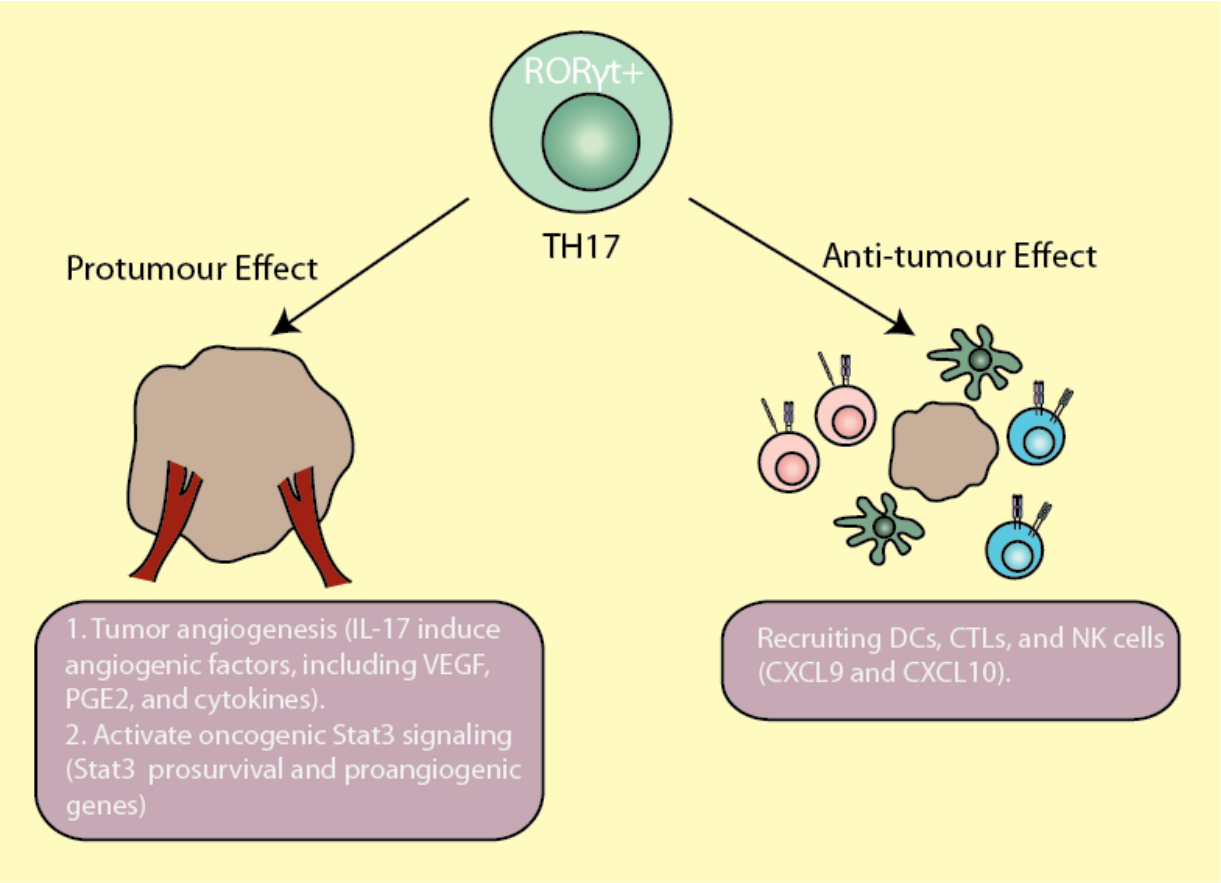


Figure 1.8 The role of Th17 in the tumour microenvironment

The role of Th17 in tumour development appears to be varied displaying both protumour and anti-tumour effects. This may highlight the plastic nature of Th17 cells.

1.4 Upper Gastrointestinal Tumours – Gastric and Oesophageal Cancer

1.4.1 TILS in Upper GI cancers

Over the last decade there has become increasing interest in immunology of these cancers with the hope of increasing survival and improving prognosis. One particular area of increasing interest is the role tumour infiltrating lymphocytes play in the pathophysiological process of oesophageal and gastric adenocarcinoma.

Currently there is very little evidence to show the role of tumour infiltrating lymphocytes in oesophageal adenocarcinoma. An early study in 1996 by Rockett et al looked at general lymphocyte involvement in oesophageal cancers including squamous cell carcinoma (SCC) and adenocarcinoma (AC). Using anti CD25 as a marker for activated lymphocytes they found that there were significantly more TILs in squamous carcinoma compared with adenocarcinoma hypothesising that this may be a reason why the survival rates and disease prognosis is much better in SCC of the oesophagus compared to AC (106). A more recent study by Zingg et al in 2010 examined the role of TILs in survival and prognosis of oesophageal adenocarcinoma particularly focusing on Treg cells. Through immunohistochemistry methodology they examined specifically for Anti-CD3, anti-CD4, anti-CD8, anti-CD25 and anti-FOXP3. Their results found no correlation between $CD4^{+}CD25^{-}$, $CD8^{+}$ or $CD4^{+}CD25^{+}FoxP3^{+}$ and prognosis. There were however significant limitations to this study in both methodology and interpretation of the results (107).

The evidence supporting the role of tumour infiltrating lymphocytes in gastric cancer is far more extensive. Certainly there are several studies, which support the hypothesis that TILs in gastric cancer can be used as an independent prognostic factor for overall survival. In 2008 Lee et al found that on immunostaining for CD3, CD8, CD20 and CD45RO found that patients who had high densities of CD3, CD8 and CD45RO had significantly improved prognosis (108). Further studies looking at specific phenotypes in gastric cancer found that CD8+FoxP3+ and CD4+FoxP3+ densities correlated with increases in tumour size and grade suggesting poor associated outcomes however they did not look specifically at survival (109). Other studies looked at the ratio between Tregs and $CD8^{+}$ cells and found that densities of single populations didn't correlate significantly with overall survival however that higher $Foxp3^{+}$ Tregs/$CD8^{+}$ ratio was an independent factor for poor outcome and overall survival (110).

Although several studies have shown poor outcomes associated with certain TIL populations several other studies have found contradicting results. Kim et al in a recent study found that

increased TIL populations were associated with improved prognosis where density of $FoxP3^{+}$ populations and $CD8^{+}$ populations could be used as independent factors of prognosis. They also looked at specific ratios of populations and found $CD8^{+High}$/$FoxP3^{+High}$ group had best overall survival, whereas the $CD8^{+Low}$/$FoxP3^{+Low}$ group showed the worst survival (111).

The evidence currently is very ambiguous and it is difficult currently to make any clear conclusion on the role of TILs in gastric cancers. One of the key limiting factors in the methodology of several of theses studies is the reliance on a single marker, $FoxP3^{+,}$ for Treg cells but as previously mentioned it is now felt that not all regulatory T lymphocytes express FoxP3 (Tr1 cells) also there are populations of nonsupressive T cell populations which transiently express FoxP3 and therefore may not be adequate alone to identify Treg cells (112).

1.4.2 TH17 and TH22 in Gastric Cancer

Several recent studies in gastric cancer have focused on Th17 and Th22 in gastric cancers. In 2012 Zhuang et al examined the phenotype profile of TILs in gastric cancer. They specifically focused on IL-22 producing CD4 cells. Their results highlighted the complexity of the tumour microenvironment. They identified that $IL\text{-}22^{+}CD4^{+}$ were associated with tumour progression with significantly higher levels of $IL\text{-}22^{+}CD4^{+}$ in higher tumour grades increasing exponentially. They also interestingly found that higher levels were associated with improved 21 month survival. When they explored the phenotype of these IL-22 producing T helper cells they highlighted several different phenotypes contributing to the microenvironment. These phenotypes were defined by their cytokine pattern. There were 2 particular phenotypes of interest that they identified; Th22 defined as $IL\text{-}22^{+}IL\text{-}17^{-}IFN\gamma^{-}\ CD4^{+}$ and another group of T Helper cells defined as $IL\text{-}22^{+}IL\text{-}17^{+}IFN\ \gamma\ ^{+}CD4^{+}$. It was suggested that this last phenotype could be a transient population of Th17 cells supporting the hypothesis of the plastic nature of Th17 cells. These were both increased significantly in tumour populations. However $IL\text{-}22^{+}\ IL\text{-}17^{+}IFN\gamma^{-}\ CD4^{+}$ and $IL\text{-}22^{+}\ IL\text{-}17^{-}\ IFN\gamma^{+}\ CD4^{+}$ were not significantly increased in tumour populations. When they examined survival prognosis with specific IL-22 producing phenotypes they found that only Th22 ($IL\text{-}22^{+}IL\text{-}17^{-}IFN\gamma^{-}\ CD4^{+}$) were associated with improved survival. Interestingly when they explored phenotypes of circulating T cells in PBMC populations they found no difference between any specific population and normal donors (113).

Another recent study did however find that circulating populations of CD4+ lymphocytes were increased in gastric cancer patients compared to normal donors. They identified CD4+IL-17+ were

increased but not $CD4^{+}IFN\text{-}\gamma^{+}$. On further defining sub-populations they identified that there was also a significant increase in $CD4^{+}IL\text{-}22^{+}IL\text{-}17^{-}INF\text{-}\gamma^{-}$ (which they define as Th22 cells),

$CD4^{+}IL\text{-}22^{+}IL\text{-}17^{+}INF\text{-}\gamma^{-}$ (Th17), $CD4^{+}IL\text{-}17^{+}IFN\text{-}\gamma^{+}$ (Th17/Th1) cells in gastric cancer patients compared to donors but they also found another population of cells $CD4^{+}IL\text{-}22^{+}IL\text{-}17^{-}INF\text{-}\gamma^{+}$. However Th1 cells were not increased in gastric cancer patients. Circulating Th22 cells and Th17 were also found to directly correlate with stage with highly significantly increased levels of both subgroups in higher grades of tumours (114) .

Interestingly when they looked at survival they found the converse of Zhaung et al's analysis of TIL populations in gastric cancers in that high levels of Th22 and Th17 were associated with poor 21 month survival.

Tumour infiltrating lymphocytes clearly seam to play a role in gastric cancer but as of yet is unclear in oesophageal adenocarcinoma. However the data currently is confusing and contradicting. Some papers have also suggested that not only is the phenotype of lymphocytes important but also the pattern of distribution of the TILs in gastric cancer is important in predicting prognosis (115).What is clear from the studies is that the microenvironment of oesophageal cancer and gastric cancer is relatively unexplored and poorly understood.

1.5 Aims of the Research Project

Upper gastrointestinal adenocarcinomas are associated with poor prognosis and often limited options for treatment. This project aims to explore the relationship of the immune system with the development and prognosis of these cancers. By investigating how particular areas of the adaptive immune system, particularly T lymphocytes, respond in upper GI cancers we can further our understanding of the complex relationship of the tumour microenvironment. Evidence has shown us that the immune system is a complex process and there appears to be a role in tumour development but that relationship is still relatively poorly understood. This project also aims to clarify the role different phenotypes of T lymphocytes play in upper GI cancers with a prospect of being able to use these as potential biomarkers of disease and disease progression. This may also lead to development of potential immunotherapies for these cancers, such as adoptive T cell therapies.

The specific aims of the project are as follows;

1. Improve upon existing techniques using flow cytometry to more accurately phenotype particular T cell subsets, Th17 and Th22, by using more specific and additional parameters.
2. Compare peripheral blood mononucleocytes (PBMCs) of normal donor populations with upper GI cancer patients to assess how immunological profiles change in the peripheral circulation of this patient group.
3. Compare PBMCs of upper GI cancer patients pre and post treatment to observe any immunological changes in T cell phenotypes following treatment.
4. Analyse tumour infiltrating lymphocytes of tumour specimens from oesophageal and gastric cancer patients to phenotype populations with a particular focus on Th17 and Th22 phenotypes.

Further possible areas to explore could include;

1. Analysis of phenotypes of T cells in PBMCs of a variety of cancer patients with adenocarcinomas; e.g. colorectal and breast, and observe how these compare with upper GI cancer patients
2. Compare the immunological phenotypes of T cells in premalignant conditions such as Barrett's oesophagus and *H.Pylori* induced gastritis with the TILs of the relevant adenocarcinomas.

Chapter 2 Material and Methods

2.1 Blood Sample Collection

Blood was taken from healthy donor volunteers and patients following full ethical approval according to the deceleration of Helsinki and documented consent. Blood was collected in EDTA (ethylenediaminetetraacetic acid) bottles (BD Vacutainer®, Oxford, UK) to prevent coagulation. Patient samples were collected directly from patients identified in upper GI oncology outpatient clinics when patients first presented prior to chemotherapy or surgical intervention. The samples were collected in EDTA vacutainers. These were kept at room temperature and were analysed within 30 minutes of collection.

2.2 PBMC isolation

Peripheral blood mononuclear cells (PBMC) were isolated from fresh blood samples by Ficoll density centrifugation. Blood samples were diluted 1:1 with phosphate buffered saline (PBS) and layered over 25ml of Lymphocyte separation medium (PAA Laboratories GmbH, Pasching, Austria). Samples were then centrifuged at 400g for 20 minutes with no brake to allow a density gradient to form. The opaque layer containing PBMCs above the lymphocyte separation medium were then collected and the plasma layer discarded. The PBMCs were then washed with PBS for 10 minutes at 800g with brake. Samples were counted using trypan blue exclusion dye (Sigma-Aldrich, Dorset, UK). These samples were then either activated directly or cryopreserved.

2.3 Cryopreservation

PBMCs isolated from fresh blood samples were counted and diluted with freezing medium (Fetal Calf Serum plus 5% concentration of DMSO) to give a concentration of 1×10^7 per ml. These were frozen in 1ml aliquots in cryofreeze bottles at 70^oC then thawed when required.

2.4 T Cell Activation

PBMCs for analysis were activated with PMA (50 ng/ml), ionomycin (1 µg/ml) and brefeldin A (1 µl/ml) to prevent cytokine secretion at a concentration of 1×10^6 cells /ml in T cell media (1% glutamine, 1% penicillin/streptomycin and 10% FBS) for a variable amount of time depending upon the experiment.

T-cell activation is normally triggered by the interaction of a cell surface receptor to its specific ligand molecule. This results in a cascade effect through activation of various tyrosine kinases and effectors which result in the regulation of the IL-2 gene. Once stimulated IL-2 production continues T cell activation and proliferation.

This process of activation can be artificially stimulated by using PMA which acts as a lymphocyte mitogen via the PKC pathway. It is thought to be a better mitogen activator of T lymphocytes than other alternative lymphocyte mitogens such as phytohemagglutinin (PHA) (116)

In addition Ionomycin is often used as another mitogenic compound. Ionomycin is a Ca2+ ionophore which induces an increase in Ca2+ mobilisation across cell membranes (117). It has been shown that together with PMA ionomycin becomes a very potent activator of T cells by synergistically enhancing the activation of PKC (118).

BFA is important to add during this stage of activation as it disrupts intracellular transport of proteins via the golgi-complex and thus allows intracellular cytokines to accumulate intracellularly thus allowing improved flowcytometry detection (119).

2.5 T cell population analysis - Flow cytometry

Flow cytometry was used to identify T cell populations. For the analysis of surface markers and intracellular cytokines using multiple fluorochromes the BD LSRFortessa™ cell analyser was used for flow cytometry (BD biosciences, California, USA) allowing up to 18 different fluorochromes to be analysed. BD FACSDiva™ software (BD biosciences, California, USA) was used for calibration and collection of the flow cytometry results. Further analysis was performed on FlowJo v.10 software (Flowjo data analysis software, Oregon, USA).

To identify the lymphocyte population in the PBMCs the size of cells (side scatter (SSC)) and degree of granulation (forward scatter (FSC) were gated upon to allow subset analysis of that population only (Figure 2.1). For further analysis of the subset populations surface marker fluorochromes were used identify the surface markers CD4+ and CD8+ and depending upon the cytokine profile required different fluorochromes were used for each cytokine required. Due to the complexity of the use of multiple fluorochromes manufacturers advice was used in the choice of particular fluorochromes to create a panel of specific fluorochromes to allow the detection of lymphocyte subset populations. BD FACSelect™ multicolor panel designer (BD biosciences, California, USA) and BioLegend's Fluorescence Spectra Analyzer (Biolegend, California, USA) were

used to aid in fluorochrome selection. Advice from both manufacturers was used in the final selection of the fluorochromes. To prevent inaccurate population selection the fluorochromes with the highest stain index were chosen. This helped to prevent potential errors to occurring due to cell autoflouroscence, spectral overlap (spillover) from other fluorochromes and electrical noise. The brightest fluorochromes were defined by their stain index which allows accurate discrimination between negative and positive populations. The stain index is described as the difference between the width of 2 SD of the negative population (W) and the distance between the positive and negative populations (D). The background noise will affect the width of a negative population (W) (figure 2.2). Therefore a bright fluorochrome is often described as one with a high stain index reducing the risk of false positives from background noise (120).

Prior to running patient samples through the flow cytometer calibration was performed. Accurate calibration was important to prevent potential sources of error through spectral overlap. Spectral overlap (spillover) is a significant issue in these experiments due to the large number of fluorochromes required for each experiment. Therefore flow cytometry beads (manufacturer) were used during the setup of the compensation for each fluorochrome. This provided a strongly positive result for each fluorochrome and a strongly negative result allowing a more accurate setup of compensation and adjustment of the spectral overlap (spillover).

For each fluorochrome 2 FACS LSR tubes were used. In one tube 2 drops of postive control beads were placed and the other 2 drops of negative control were placed. In each tube 2 µl of the appropriate fluorochrome were added. 100 µl of FACS buffer (PBS and 5% FCS) was then added to each tube. This was kept in the dark to prevent degradation of the fluorochromes by UV light for 30 minutes. Following this 1ml of FACS buffer (PBS and 5% FCS) were added to each tube and centrifuged for 3 minutes at 400G. The supernant was then removed and the pellet resuspended in 200 µl of FACS buffer. The compensation was performed using BD FACSDiva™ software (BD biosciences, California, USA). This was performed automatically by the software and adjusted manually for clearly defined positive and negative detection of the fluorochromes. Single stained cells were also used to check the compensation. It was not necessary to compensation with the same tissue that will be analysed as the compensation is dependent on the fluorochrome not the cell type (121).

Following compensation normal donor and patient samples were then prepared for flow cytometry. Depending upon the experiment patient samples or normal donor samples were prepared fresh or from frozen samples. Patient and normal PBMCs were stained for both surface markers and intracellular markers. A standard 96 well plate was used and cells were diluted in

FACS buffer (PBS and 5% FCS) to give a concentration of 1 x 10^6 cells / well. To address any further issues of false positives or false negatives despite compensation, controls were used on every sample run through the flow cytometer. Two forms of controls were used; fluorescence-minus- one (FMO) control and negative controls. FMOs were all the antibodies together in one sample except one fluorochrome which is required for gating providing a negative control (122). The negative controls and FMOs were used then during the gating process to identify those cells that stained positively for the fluorochrome particularly where the populations were small. The negative gate identified by the FMO and negative control would be transferred to the all test sample results and those samples that were outside this gate were defined as staining positively.

Prior to surface staining an FC block was used at 1 µl/100µl as well as a live/dead discriminator. The live/dead discriminator varied depending upon the experiment. FC block was used to help reduce background staining. Many cell types, including B lymphocytes, NK cells, granulocytes, monocytes, macrophages, and platelets express FC receptors and some antibodies bind to Fc portions of these receptors therefore a block is required. The cells are then incubated on ice for 30 minutes following which a wash is performed with FACS buffer. Surface staining is then performed with appropriate fluorochrome depending upon the experiment. This was then left for a further 30 minutes on ice. Following which another wash is performed.

For intracellular staining cells must be permeabilised to allow fluorochromes to enter the cell membrane. This is performed with a permeabilisation kit (Bioleged FOXP3 Fix/Perm Buffer Set). 100 µl of 1X BioLegend's FOXP3 Fix/Perm was added to each well. This was then incubated at room temperature in the dark for 30 minutes. A wash is then performed with PBS following which the cells are incubated in 200 µl of 1X BioLegend's FOXP3 Perm buffer for 15 minutes at room temp in the dark. A wash was then performed and a further 100 µl of 1X BioLegend's FOXP3 Perm buffer is added to each well. Intracellular staining was then performed adding 1 µl of each fluorochrome to each appropriate well. The cells were incubated at room temperature in the dark for 30 minutes following which a wash was performed. The cells were then resuspended in 200 µl of PFA 1% following which they were then transferred to FACS bottles for analysis.

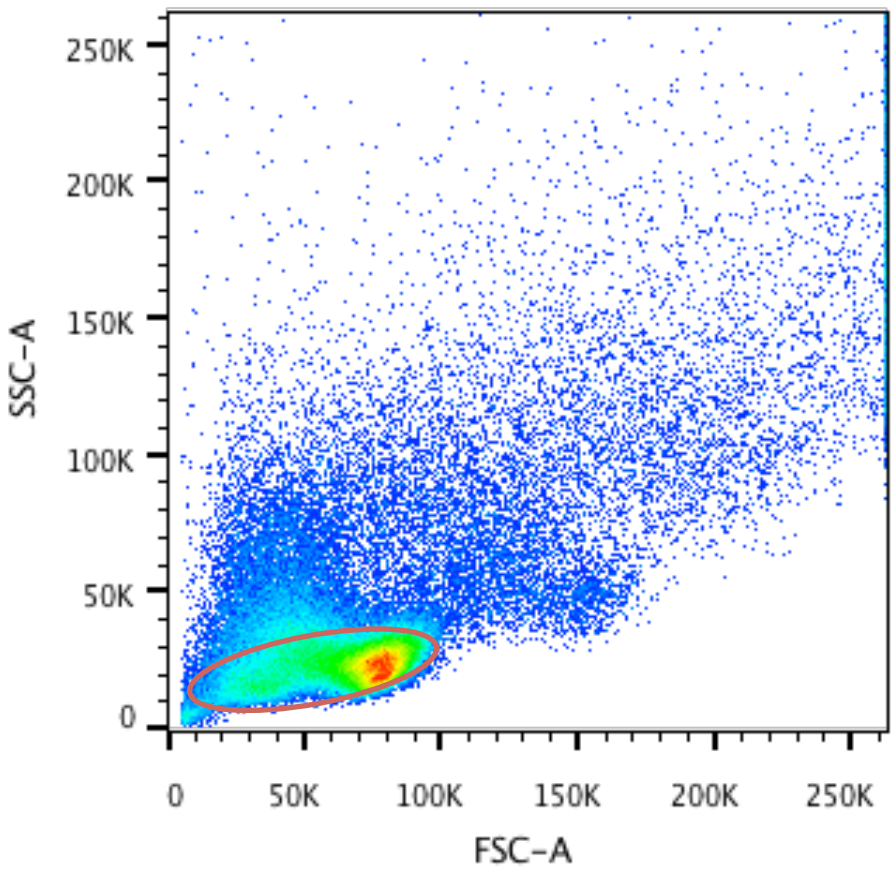


Figure 2.1 Typical forward scatter/side scatter (FSC/SSC) dot-plot.

Dot plot showing the different lymphocyte populations typically identified by side scatter and forward scatter measurements.

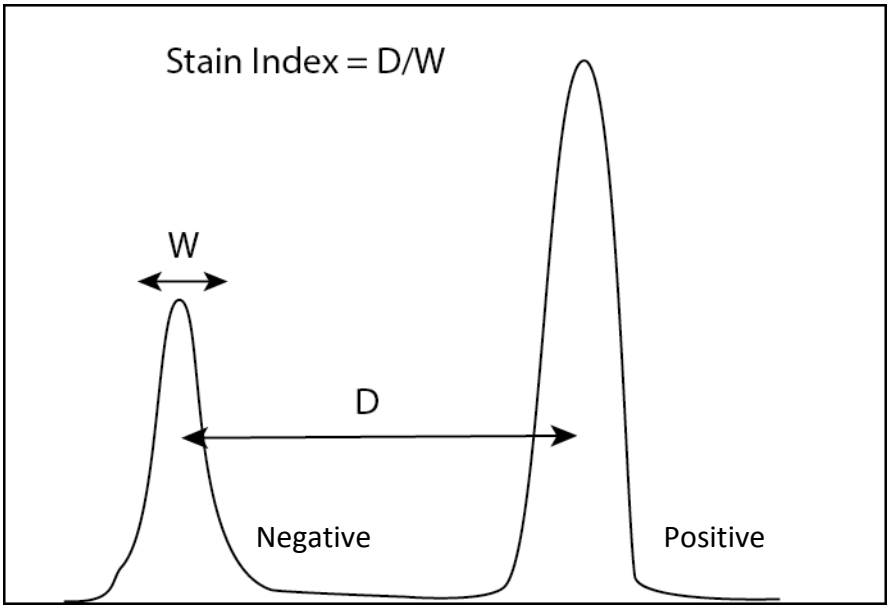


Figure 2.2 Stain Index.

Histogram depicting the antigen positive and antigen negative populations of a fluorochrome highlighting the definition of stain index. Adapted from "Selecting reagents for multicolour flow cytometry with BD™ LSR II and BD FACSCanto™ systems" by Maecker H and Trotter, J. Nature Methods 5, (2008).

Chapter 3 Optimal Methodology for assessing T helper cell phenotypes Th17 and Th22 in a PBMC population

3.1 Introduction

For the detection of intracellular cytokines it is unclear whether there is an optional length of activation is required for the measurement of T cell function. However prolonged activation is also associated with significant levels of cell death thus a balance is required to achieve optimal activation with minimal cell death. Brefeldin A has been shown to be associated with cell toxicity and death when used for periods longer than 4 hours however it is necessary for intracellular staining. To allow batch analysis of patient samples thereby reducing inter-experiment variations, freezing patient samples would be practical. However the effect of freezing on PBMCs and the ability to detect Th17 and Th22 cells remains unknown. To define Th22 and Th17 cells these were identified based on their surface markers and intracellular cytokine production without a batched array setting.

3.2 Optimising the duration of activation and methodology for sample handling

To compare both the optimal duration period of activation and the effects of freezing on normal donor PBMCs, experiments were designed comparing the effects of these processes on detection of Th17 and Th22 cells. Healthy donors PBMCs were isolated using a standard ficoll density centrifugation. Half the samples were cryopreserved at a concentration of 1 x 10^7cells / ml and stored at -70^0C. Fresh samples were then also divided into 2 flasks and suspended in T cell medium at a concentration of 1x10^6 cells /ml. Within one Flask T cells were activated immediately by the addition of PMA (50 ng/mL) and Ionomycin (1 µg/mL), including Brefeldin A (1ul/ml). This was left to activate overnight for approximately 16 hours. The second flask was activated the following morning for a period of 4 hours. Frozen samples after a one weak storage period were defrosted in a water bath of 37^{o}C and then suspended in T Cell suspension medium (TSM) at a concentration of 1x10^6 cells /ml and divided into 2 flasks.

Following activation, PBMC were washed with PBS and then divided into 8 Wells of a 96 U well plate at a concentration of 1x10^6 cells /100ul. This provided 6 FMO samples for controls to allow accurate gating of populations, 1 unstained sample also used as an additional control for

accurate gating of cell populations during flow cytometry analysis and 2 experimental multicolour samples.

To identify Th17 and Th22 subtypes in the healthy donor PBMCs, the surface markers CD4+ and CD8+ were used to define the T helper cells. Then they were also stained for the intracellular cytokines IL-17, IL-22, INFγ and IL-2 which are markers of Th22 and Th17 phenotypes. The fluorochromes chosen for this experiment to create Panel 1 (Figure 3.1) are as follows. A wide range of fluorochromes were across different lasers to reduce spillover as well as a wide spread across spectrum again to prevent spillover into other channels (Figure 3.2).

Figure. 3.1

Flow cytometry Panel Design Number 1

	CD4	IL17α	IL-22	CD8	INFγ	IL2	Surface protein or Cytokine
PANEL 1	Brilliant Violet 421	FITC	PE	APC	PerCP/CY5.5	CY7	Fluorochrome
	Violet **F**	Blue **F**	Blue **E**	Red **C**	Blue **B**	Blue **A**	Laser on LSR II

Spectral Analyzer Results for Panel Number 1

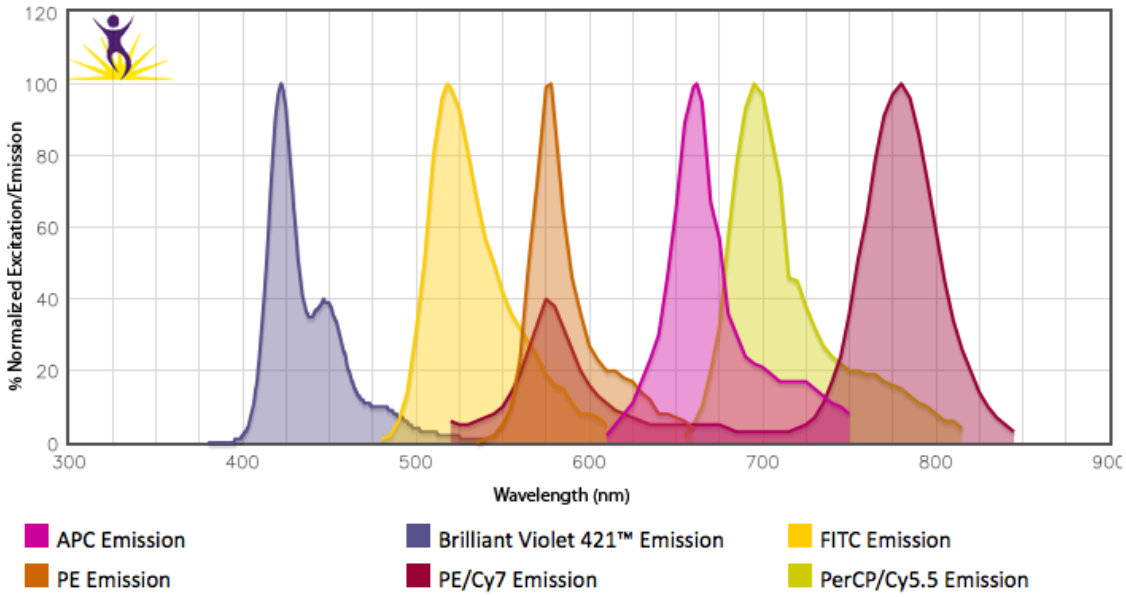
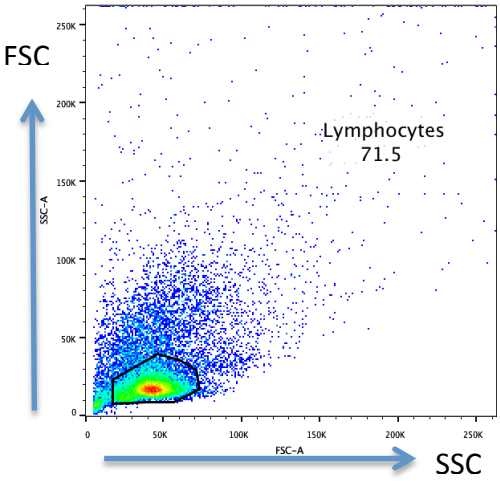


Figure 3.1 Flow cytometry Panel Design

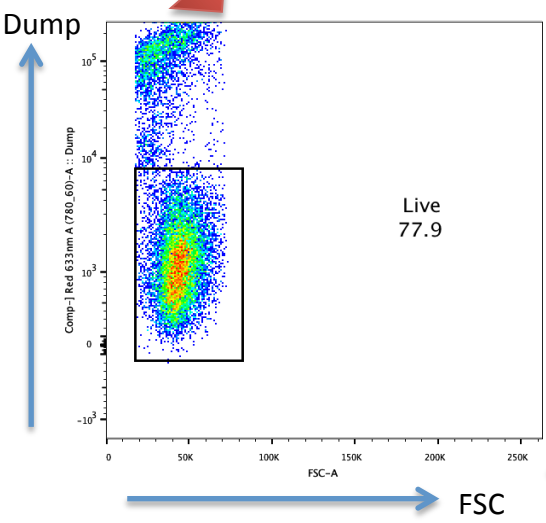
Spectral analysis using Biolegend online Spectral Analyzer Tool shows fluorochrome choice over a wide range of spectra with little overlap. The fluorochromes were specifically chosen to try and reduce spillover into other channels of the flow cytometer. By preventing spillover it allows for more accurate detection of populations and reducing the risk of false positives. Also another technique to reduce false positives used was choosing different lasers for each fluorochrome and thus detection of each cytokine or surface marker.

Figure 3.2

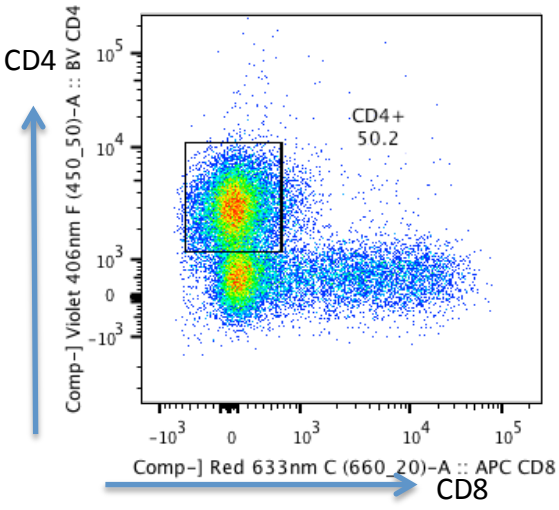
Gating protocol



a) Lymphocyte Population



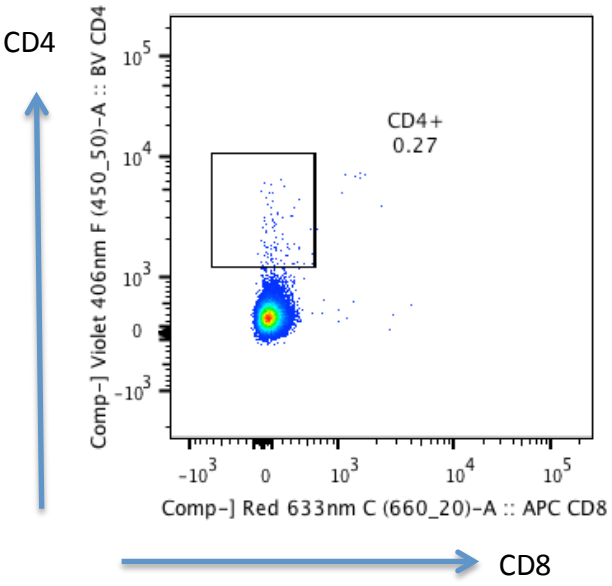
b) Live/Dead



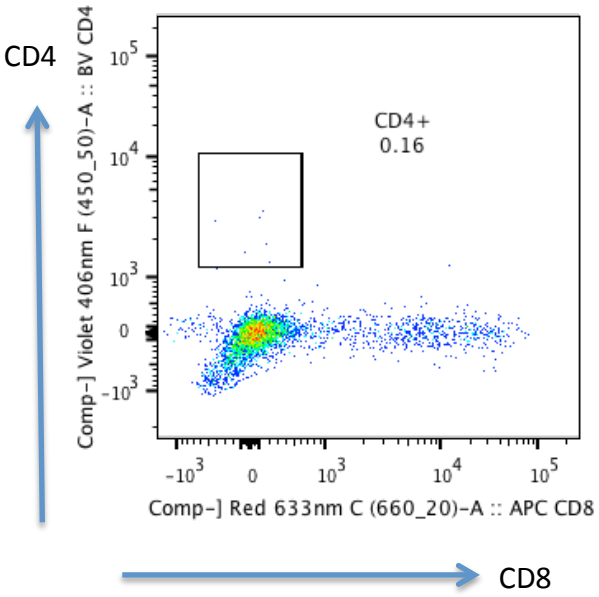
c) $CD4^+$ T Cell Population

Controls

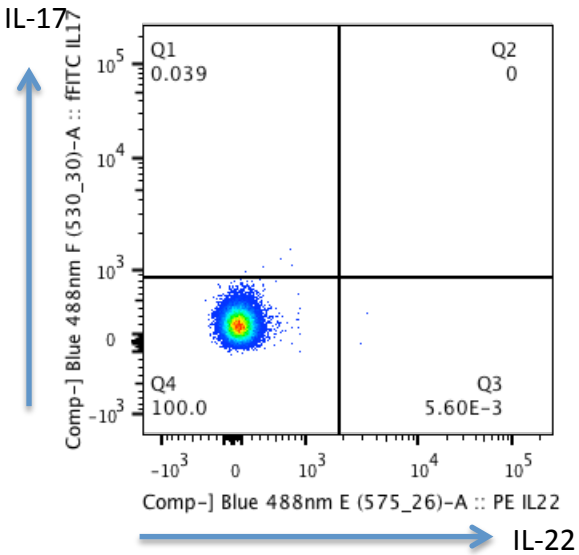
d) Negative Control to define $CD4^+$ T Cell Population



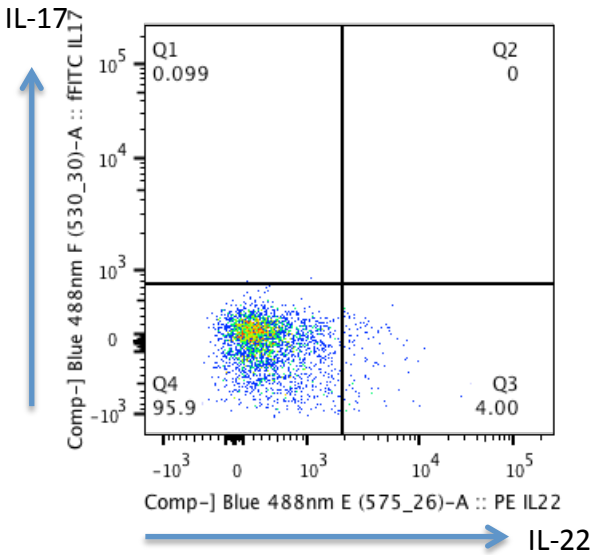
e) FMO BV421 ($CD4^+$ fluorochrome) to define $CD4^+$ T Cell Population



f) Negative Control to define IL-17^+ and IL-22^+ T Cell Population



g) FMO FITC (IL-17^+ fluorochrome) to define IL-17^+ producing T Cell Population



h) FMO PE (IL-22$^+$ fluorochrome) to define IL-22$^+$ producing T Cell Population

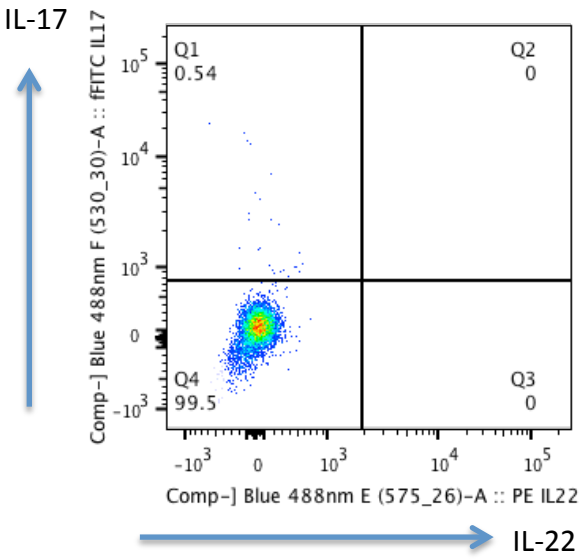


Figure 3.2 Gating technique for analysis of CD4$^+$ T Helper cell lymphocyte populations and IL-17 and IL-22 producing sub-populations from PBMC using flow cytometry

Analysis was performed by FlowJo software. Lymphocyte populations were defined using FSC and SSC sizes. This population was then gated for further analysis of live or dead cells. Live discriminator (labelled Dump) was used to reduce auto-fluorescence from dead lymphocyte. Live cells did not stain positively and therefore the negative population was gated for further analysis. CD4$^+$ population was defined using both a negative control as well as an FMO control for BV421 (Fluorochrome for CD4$^+$). The same technique was used to define positive and negative gates for IL-22 and IL-17. In graph g) Q1 defines the positive quadrant and in graph h) Q3 defines the positive quadrant to be used in further analysis.

Figure 3.3

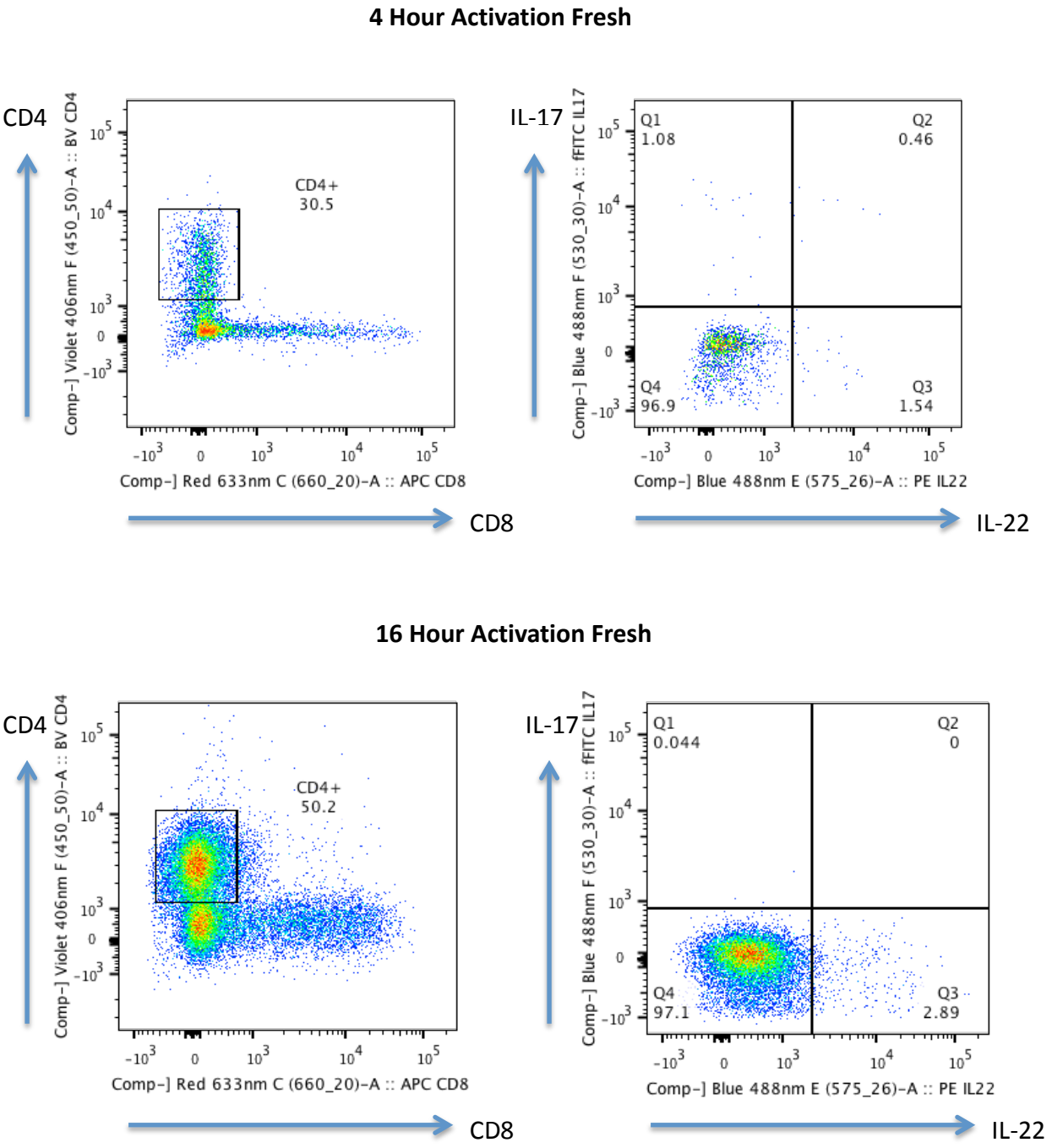
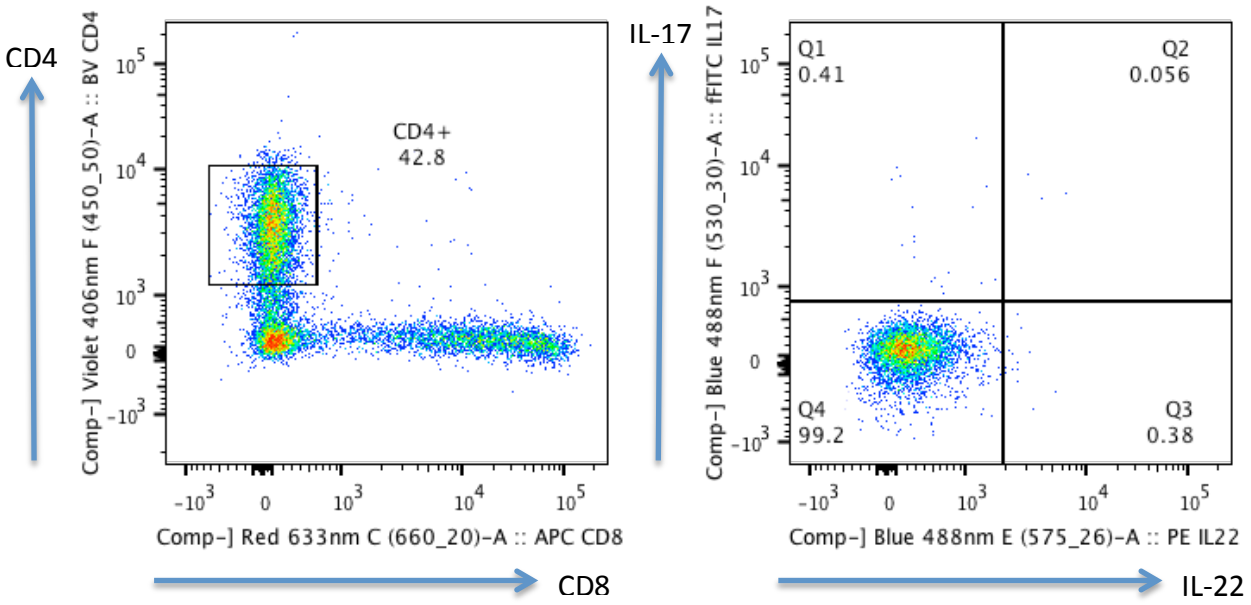


Figure 3.3 Fresh Samples of PBMC analysed with flowcyometry following surface and intracellular staining using Panel Number 1 optomised for detection of IL-17 and IL-22. *The PBMC cells were immediately activated following lymphocyte separation for either 4 hours or 16 hours. Following activation they were then stained using standard methodology. Although total number of populations were different between 4 hour activation and 16 hour activation the percentage populations were analysed. Analyses was performed on FlowJo to detect populations of IL-22 and IL-17 producing populations. First step of defining lymphocyte populations was*

performed using FSC and SSC sizes. This population was then gated for further analysis of live or dead cells. Live discriminator (labelled Dump) was used to reduce auto-fluorescence from dead lymphocytes. Live cells did not stain positively and therefore the negative population was gated for further analysis. $CD4^+$ population was defined using both a negative control as well as an FMO control for BV421 (Fluorochrome for $CD4^+$). The same technique was used to define positive and negative gates for IL-22 and IL-17

Figure 3.4

4 Hour Activation Frozen



16 Hour Activation Frozen

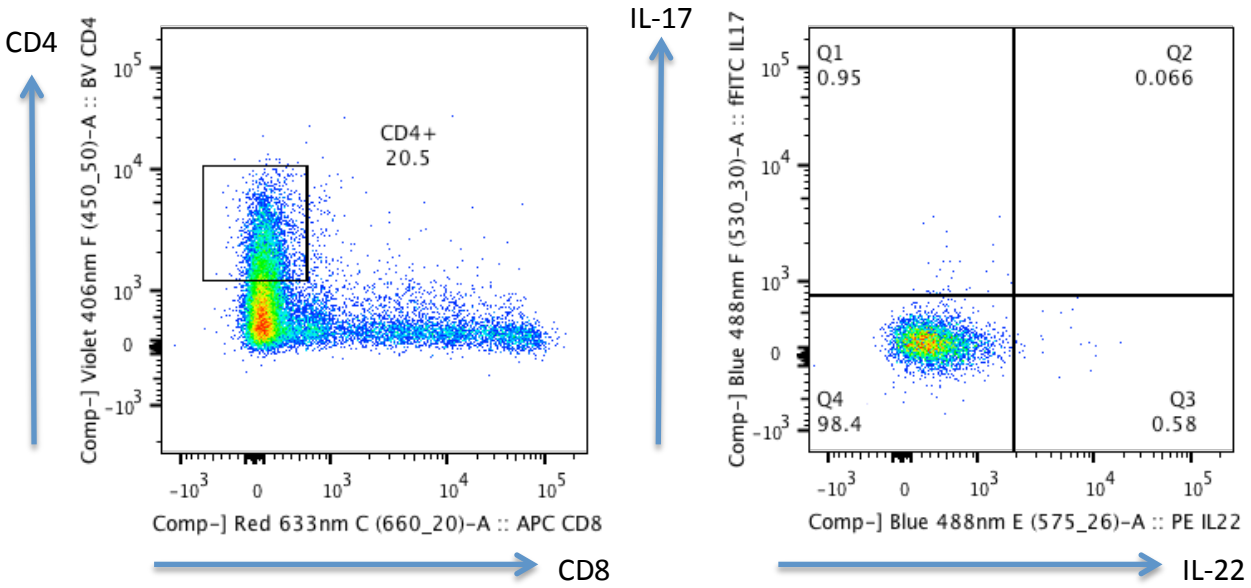


Figure 3.4 Frozen Samples of PBMC analysed with flowcyometry following surface and intracellular staining using Panel Number 1 optomised for detection of IL-17 and IL-22. *Donor PBMC cells were frozen using standard freezing technique. These were then thawed 1 week later and following thawing the cells were activated for either 4 hours or 16 hours. They were then stained using standard methodology. Although total number of populations were different between 4 hour activation and 16 hour activation the percentage populations were analysed. Analyses was performed on FlowJo to detect populations of IL-22 and IL-17 producing*

populations. First step of defining lymphocyte populations was performed using FSC and SSC sizes. This population was then gated for further analysis of live or dead cells. Live discriminator (labelled Dump) was used to reduce auto-fluorescence from dead lymphocytes. Live cells did not stain positively and therefore the negative population was gated for further analysis. $CD4^+$ population was defined using both a negative control as well as an FMO control for BV421 (Fluorochrome for $CD4^+$). The same technique was used to define positive and negative gates for IL-22 and IL-17.

Figure 3.5

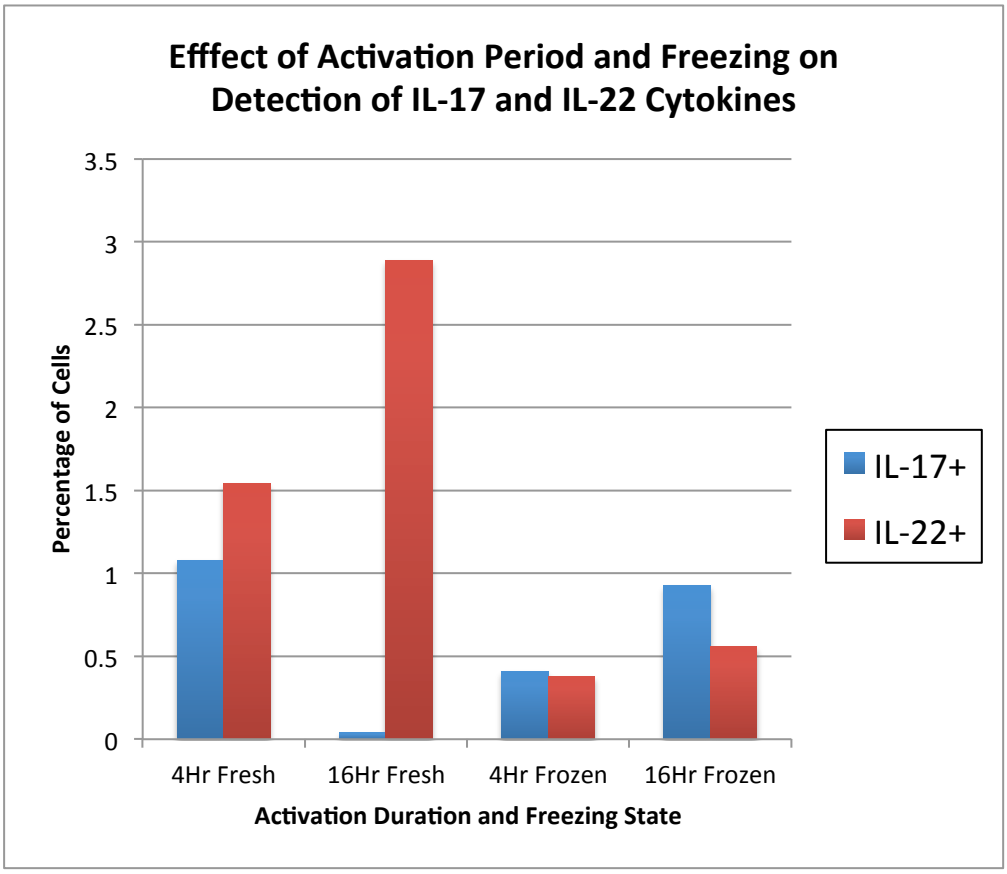


Figure 3.5 Percentage of cytokines IL-22 and IL-17 produced by $CD4^+$ T Helper cells after activation for either 4 hours or 16 hours following either freezing or activation fresh.

The gated populations of $CD4^+IL\text{-}17^+$ and $CD4^+IL\text{-}22^+$ for both fresh and frozen samples and 4hr and 16hr activation were assessed. The percentages of these populations defined by analyses on Graphpad FlowJo V6 were then compared in figure 3.5 to determine optimal period of activation as well as the effects on detection these populations following the freezing process.

3.2.1 Panel Number 1 Results: Detection of IL-17 and IL-22 following analysis of fresh samples, frozen samples and variation of duration of activation.

Results from figures 3.2, 3.3 and 3.4 identify some of the challenges with developing an optimal methodology of activation and sample handling. Detection of IL-17$^+$ cytokine appeared to be low with an average percentage of less than 1%. This is slightly lower than expected for normal donor population in the literature (123-125). Also, freezing seemed to have a significant affect on the detection or production of IL-22$^+$ (figure 3.4) However a longer duration of activation appeared to improve IL-22$^+$ detection (figures 3.3 and 3.4).

On review of fluorochromes used it was identified that a relatively dim fluorochrome FITC was being used to detect a relatively low frequency population. Therefore the panel would need to be redesigned taking this into consideration. Also further review of the literature identified that to be able to more specifically identify the population of Th17 and Th22 further cytokines may need to be used rather than just IL-17 and IL-22 which are also produced by a number of other cells and also potentially other CD4$^+$ cells.

3.3 An experiment to identify an improved flowcytomtery panel for the detection of IL-17 and IL-22 in healthy donors and optimising the duration of activation and methodology for sample handling

The initial experiment identified some key issues in flow cytometry panel design such as choice of bright fluorochromes for known low density surface markers or intracellular cytokines. In the next experiment a brighter fluorochrome for IL-17 and for IL-22 was used. Brilliant violet 605 was chosen for IL-17 as this is a very bright stable fluorochrome and PE was kept for IL-22, as this is already a bright fluorochrome. As CD4 surface markers are relatively high density compared to the other cytokines it was decided to use a dimer fluorochrome for this surface marker, therefore FITC was chosen. In addition further review of the literature identified that other T helper cells may produce some amounts of IL-22 therefore to be more specific in the ability to identify Th22 subsets TNFα was added to the panel as evidence suggests that this is produced in high amounts by Th22 and therefore makes a good cytokine marker of this phenotype (126, 127). Also IL-10 was added to the panel design. Again on review of the literature the phenotype of Th17 has a plastic nature on depending upon the microenvironment may have either a pro-inflammatory role or a regulatory role (128). The challenge is therefore defining this population. In an attempt

to be more specific in defining Th17 subtypes IL-10 was added which is known to have regulatory function (129). As well as INFγ already in the panel design it may be possible to more specific on the role of Th17 in upper GI cancers by further understanding the nature of the IL-17 producing cells.

As with all flow cytometry panel design another challenge was making sure that the fluorochromes chosen were still spread across different lasers and filters as well as trying to spread out the wavelengths as much as possible. The aim is to try and reduce spillover into other channels and therefore reduce the chance of false positives. The panel chosen (figure 3.6) shows the varied laser and filter choice. The spectral analysis (figure 3.6) also shows a wide variation in wavelength with as minimal possible overlap. However as the number of fluorochromes increases it is invariable that some overlap will occur but this is where laser choice is essential to allow accurate compensation to eliminate this potential area for error.

3.3.1 Panel Number 2 Results: Detection of cytokines IL-17, IL-22, IL-10, TNFα and INFγ following analysis of fresh samples, frozen samples and variation of duration of activation.

Following the changes in panel design and addition of further cytokines IL-10 and TNFα detection of cytokines appears to be optimal after 16 hours of activation following freezing (figures 3.7d and 3.8). Fresh samples activated for 16 hours appear to have similar cytokine production however IL-22, IL-17 and TNFα production seem to be markedly improved on average (figures 3.7b and 3.8).

Following the change of fluorochromes particularly now using a dimmer fluorochrome FITC for CD4 detection has now resulted in significantly lower percentages of CD4+ detectable (p=0.001) (figures 3.7 a-d). Evidence suggests that long periods of activation with PMA and Ionomycin and the permeabalisation process result in down regulation of the CD4 surface marker resulting in difficulties identifying this marker which is key if we are to accurately identify the T Helper Cell populations and subtypes (130). This is clearly seen in the difference of average CD4+ surface marker detected for 4hr activation period fresh (and 16hr activation period fresh (mean = 34.65 vs mean = 8.82, p=0.001 using Chi Square) seen in figure 3.8.

Figure 3.6

Flow cytometry Panel Design Number 3

	CD4	IL-17	IL-22	CD8	INFγ	IL-2	IL-10	TNFα	
PANEL 1	Brilliant Violet 421	FITC	PE	APC	PerCP/ CY5.5	PE/CY7			Surface protein or Cytokine
	Violet **F**	Blue **F**	Blue **E**	Red **C**	Blue **B**	Blue **A**			
PANEL 2	FITC	BV605	PE	V500	PerCP/ CY5.5	PE/CY7	BV421	APC	Fluorochrome
	Blue **F**	Violet **B**	Blue **E**	Violet **E**	Blue **B**	Blue **A**	Violet **F**	Red **C**	Laser on LSR II

Spectral Analyzer Results for Panel Number 2

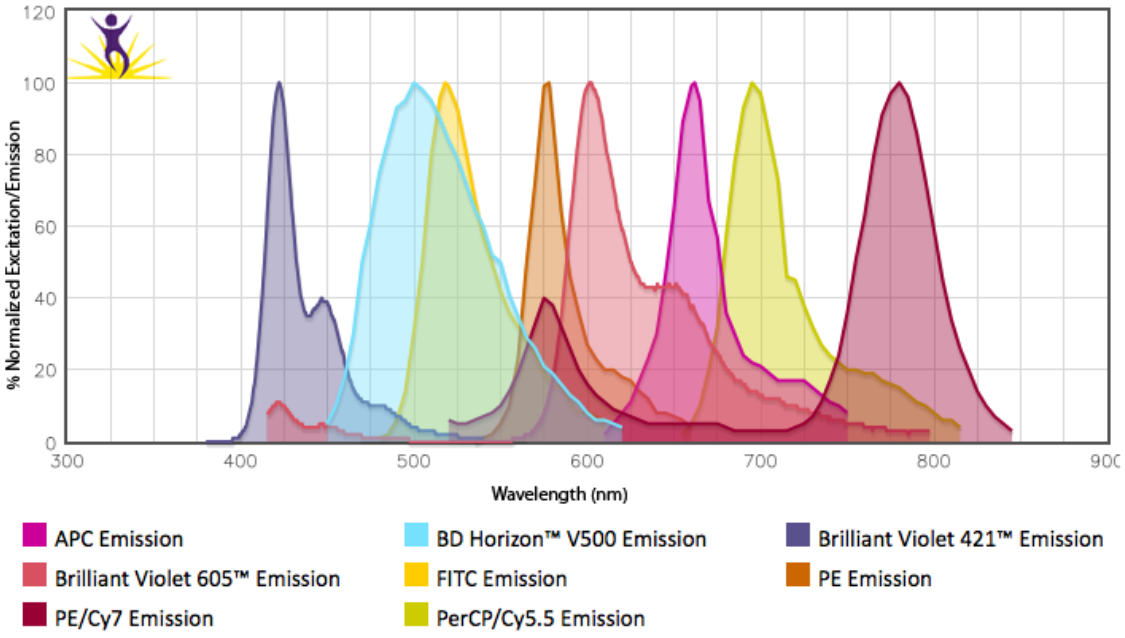


Figure 3.6 Flow cytometry Panel Design Number 2

Spectral analysis using Biolegend online Spectral Analyzer Tool shows fluorochrome choice over a wide range of spectra with little overlap. The fluorochromes were specifically chosen to try and reduce spillover into other channels of the flow cytometer. By preventing spillover it allows for

more accurate detection of populations and reducing the risk of false positives. Also another technique to reduce false positives used was choosing different lasers for each fluorochrome and thus detection of each cytokine or surface marker.

Figure 3.7 (a)

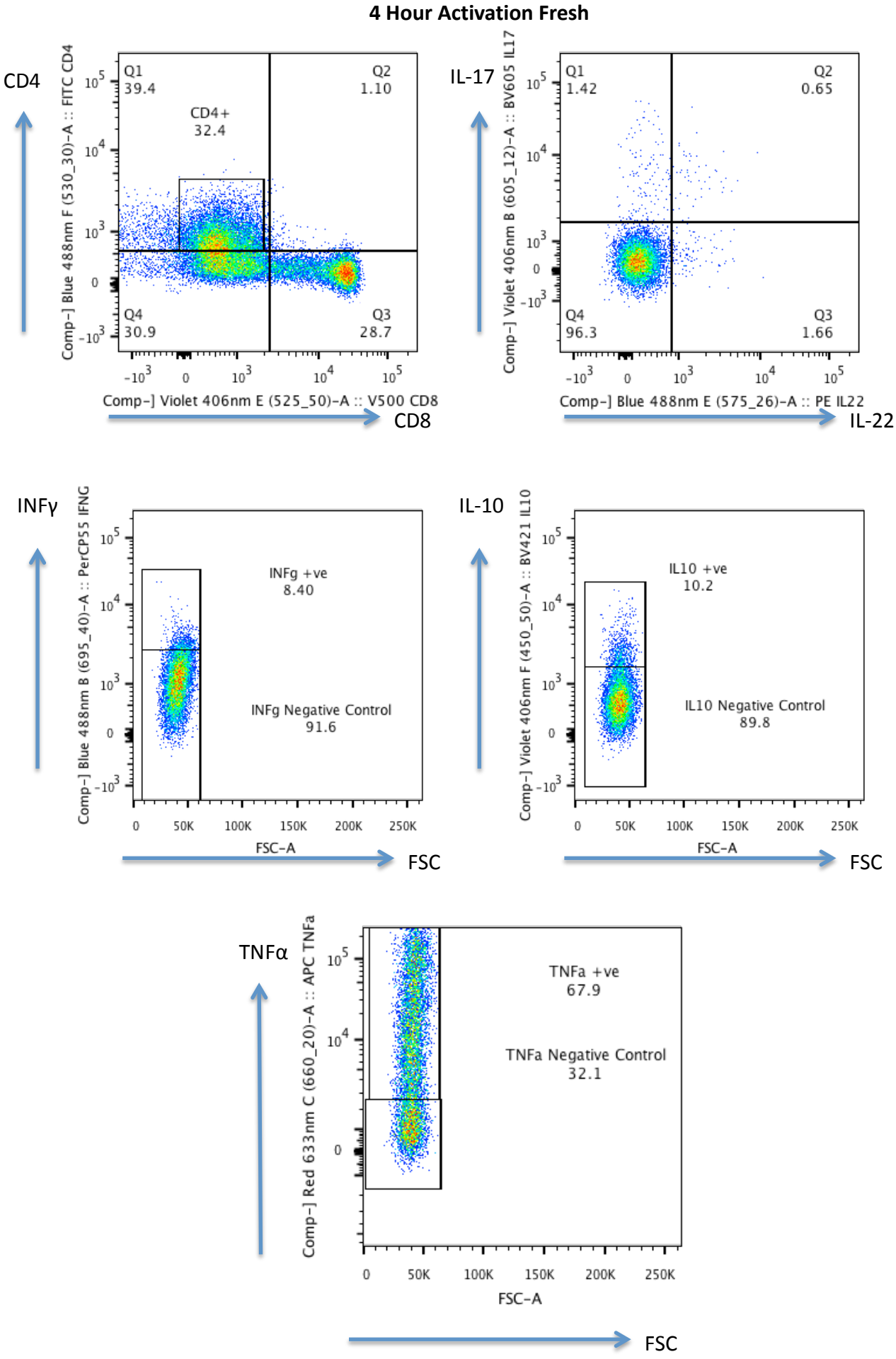
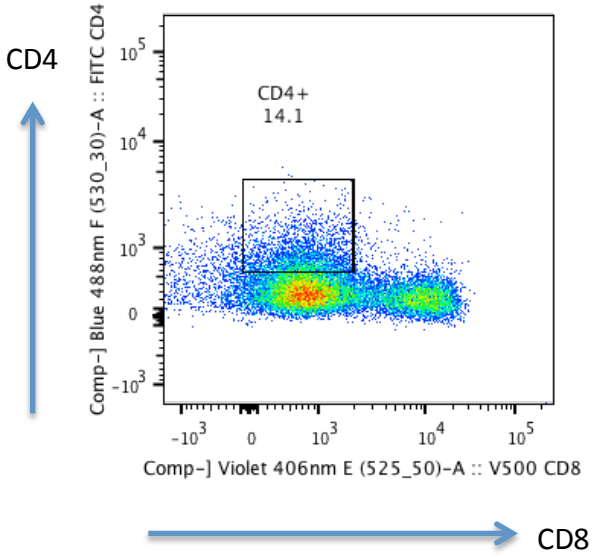
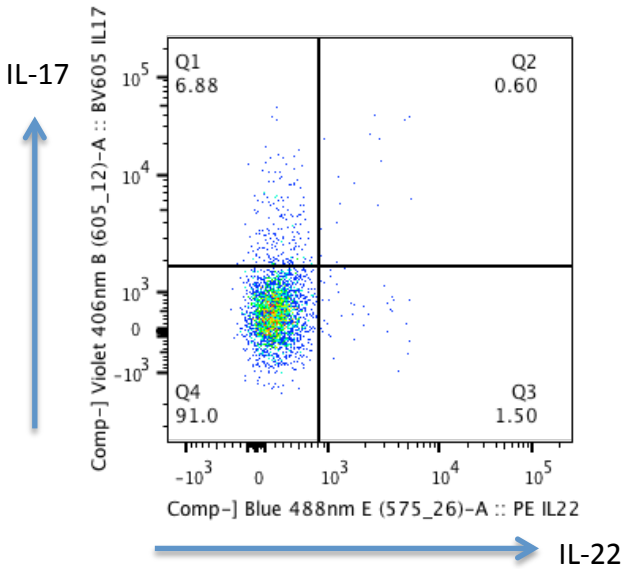
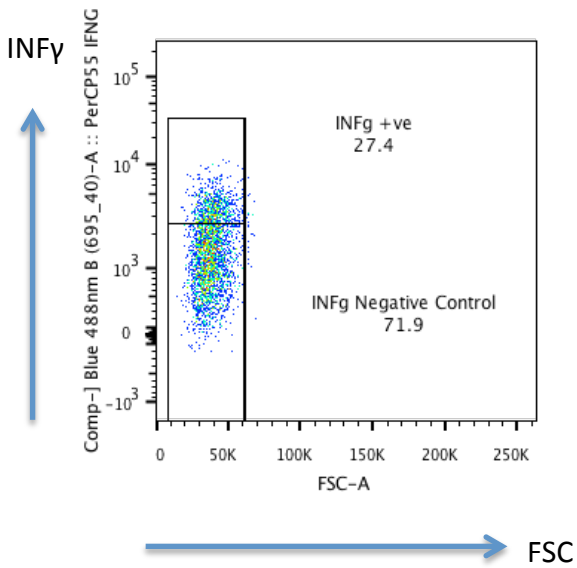

4 Hour Activation Fresh
CD4
Q1
39.4
Q2
1.10
CD4+
32.4
Q4
30.9
Q3
28.7
Comp-] Blue 488nm F (530_30)-A :: FITC CD4
Comp-] Violet 406nm E (525_50)-A :: V500 CD8
CD8
IL-17
Q1
1.42
Q2
0.65
Q4
96.3
Q3
1.66
Comp-] Violet 406nm B (605_12)-A :: BV605 IL17
Comp-] Blue 488nm E (575_26)-A :: PE IL22
IL-22
INFγ
INFg +ve
8.40
INFg Negative Control
91.6
Comp-] Blue 488nm B (695_40)-A :: PerCP55 IFNG
FSC-A
FSC
IL-10
IL10 +ve
10.2
IL10 Negative Control
89.8
Comp-] Violet 406nm F (450_50)-A :: BV421 IL10
FSC-A
FSC
TNFα
TNFa +ve
67.9
TNFa Negative Control
32.1
Comp-] Red 633nm C (660_20)-A :: APC TNFa
FSC-A
FSC

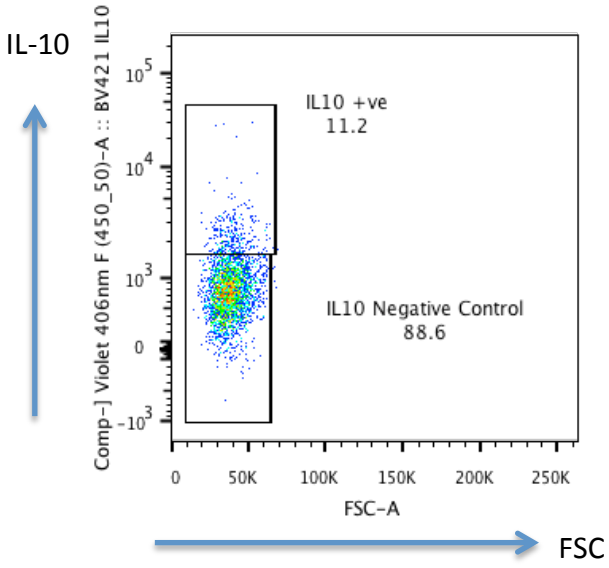
Figure 3.7 (b)

16 Hour Activation Fresh









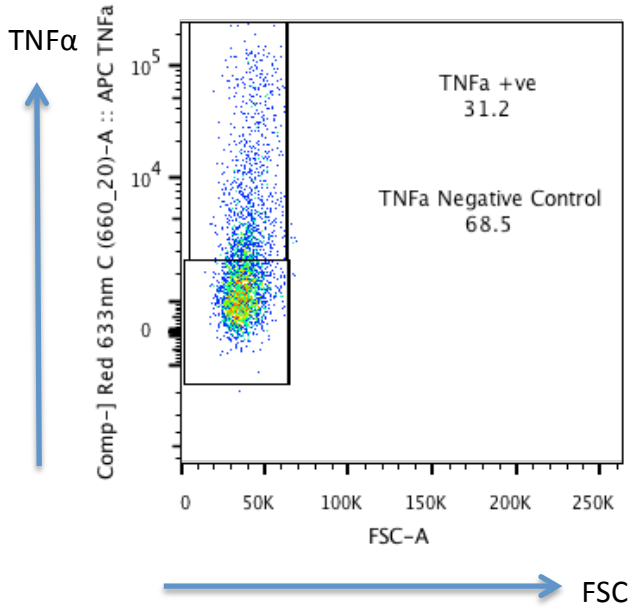
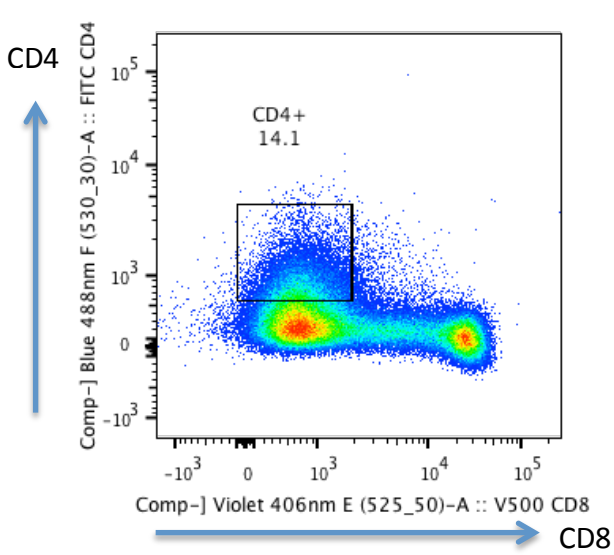
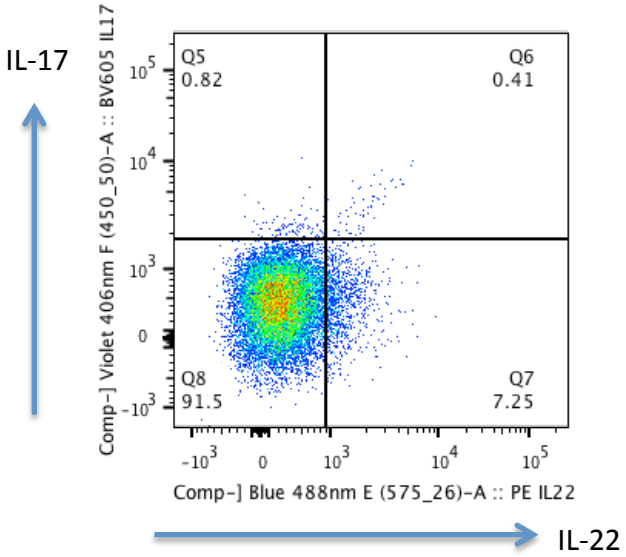
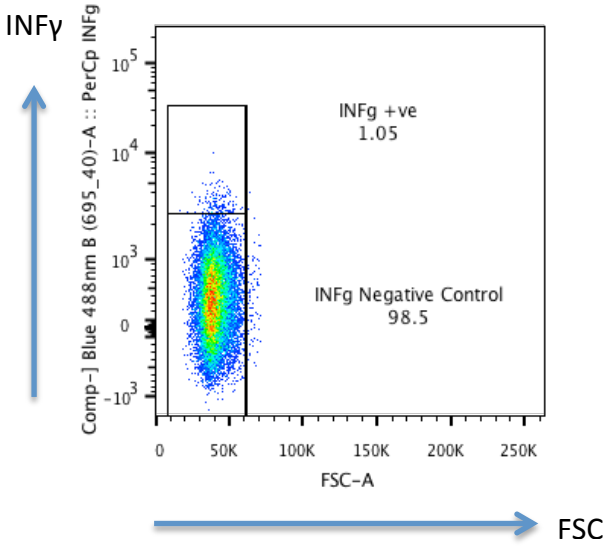


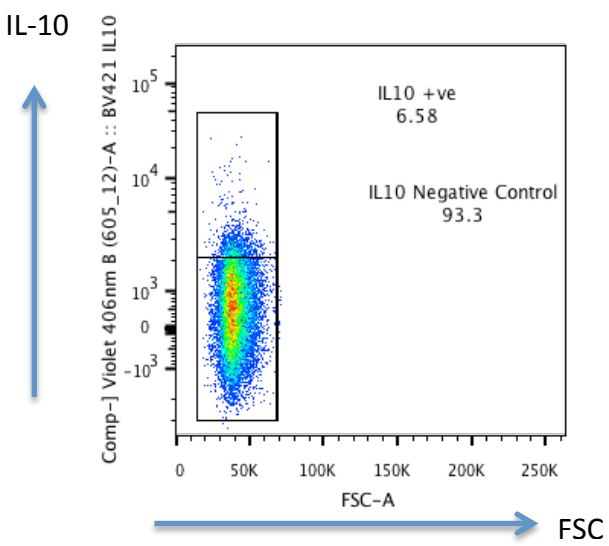
Figure 3.7 (c)

4 Hour Activation Frozen









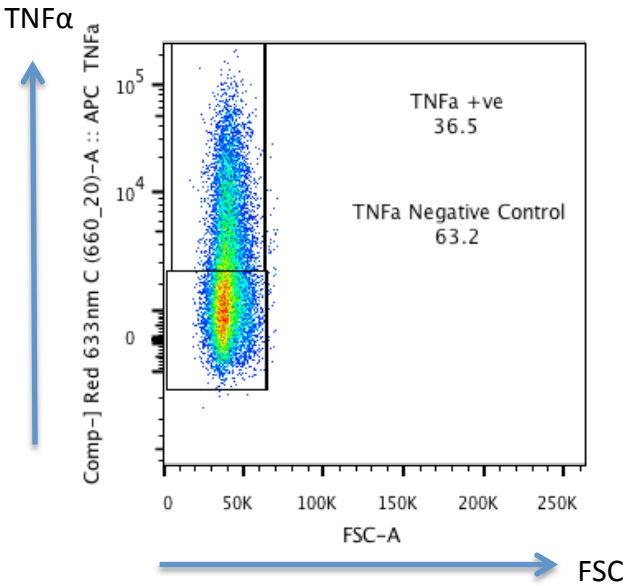


Figure 3.7 (d)

16 Hour Activation Frozen

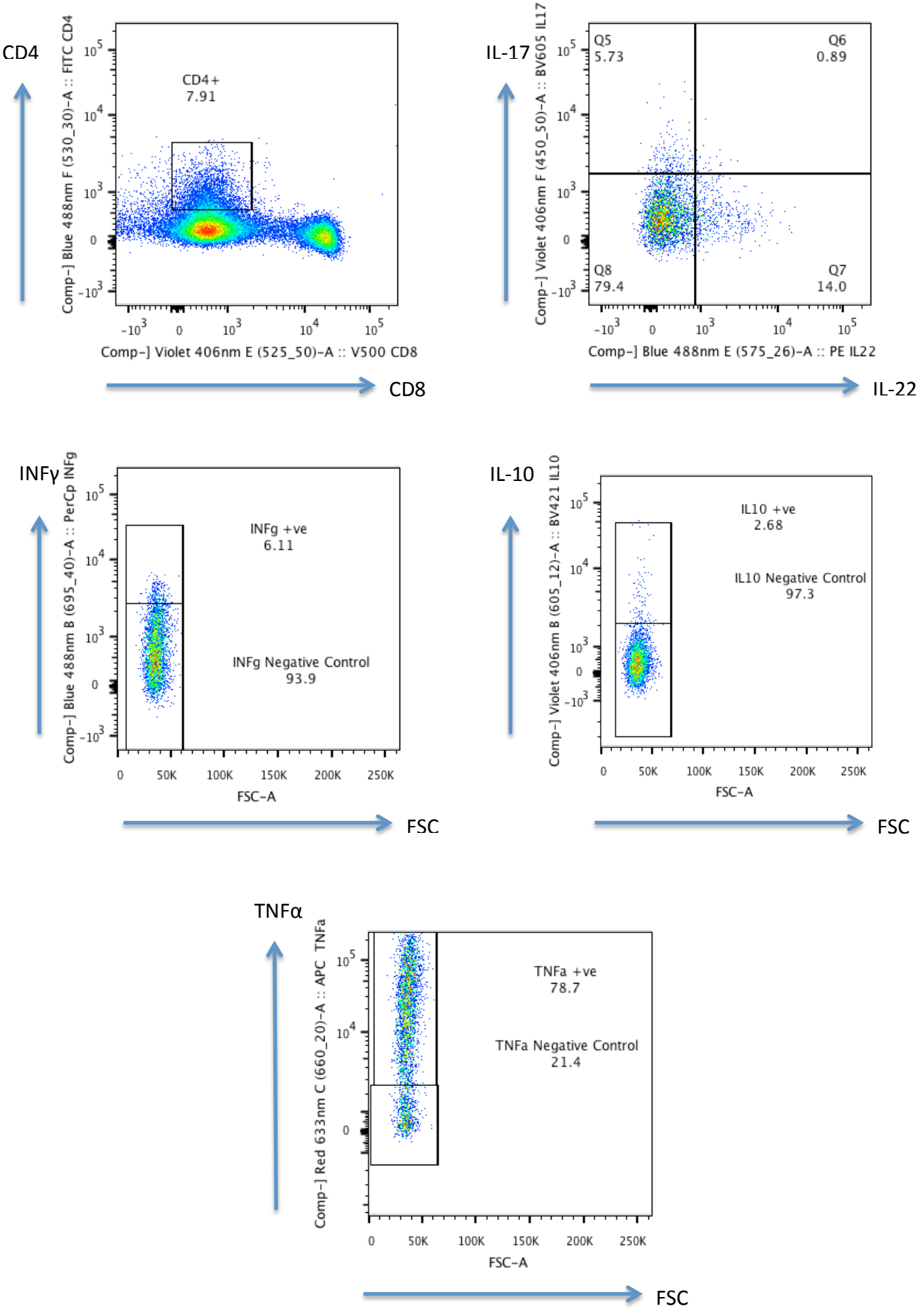
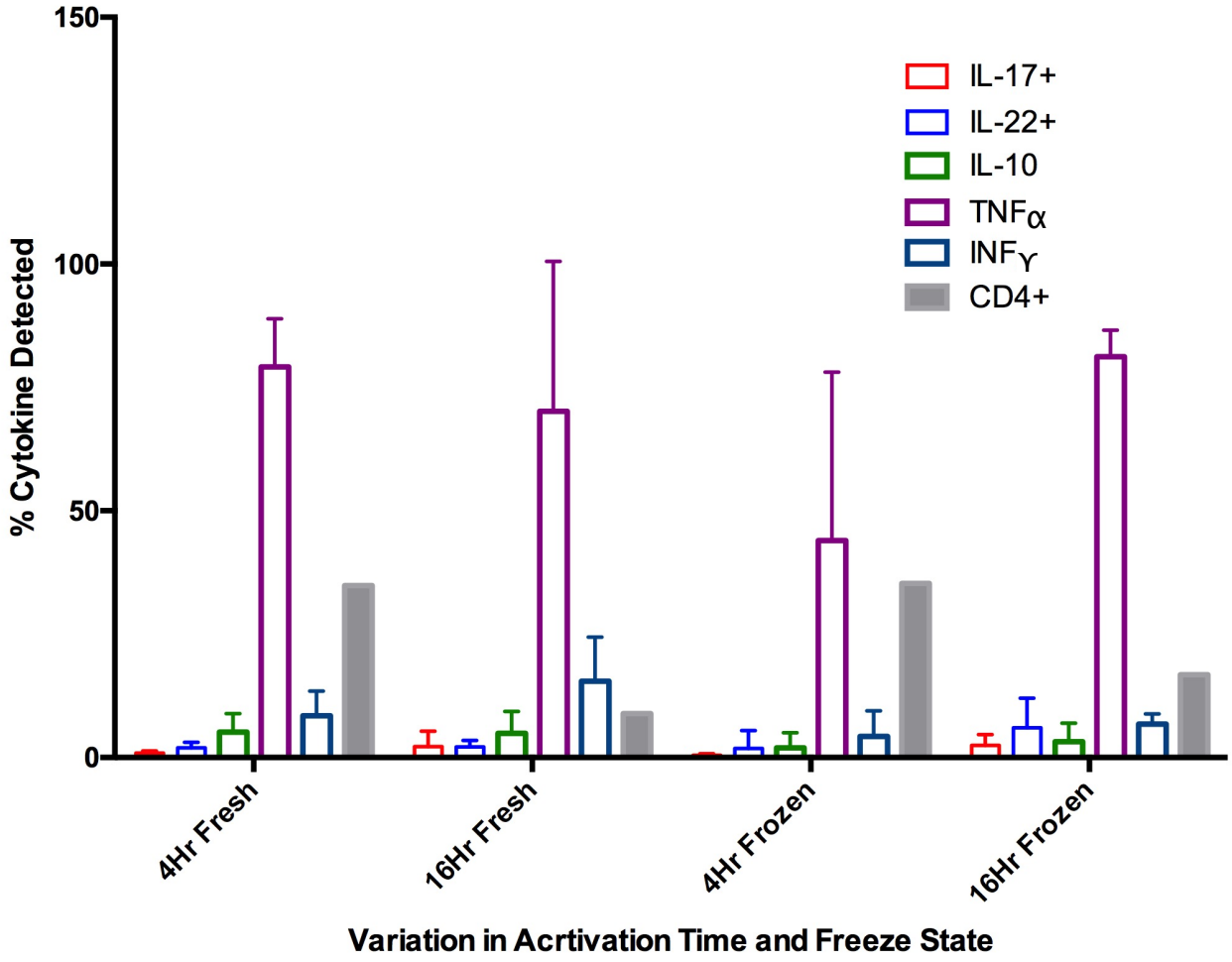


Figure 3.7 (a-d) Gating analyses showing percentage populations detected of CD4$^+$, IL-17$^+$, IL-22$^+$, INFγ $^+$, IL-10+, and TNFα$^+$ following variations of activation duration and effects of freezing on the samples.

Donor PBMC cells were frozen using standard freezing technique. These were then thawed 1 week later and following thawing the cells were activated for either 4 hours or 16 hours. They were then stained using standard methodology. Although total number of populations were different between 4 hour activation and 16 hour activation the percentage populations were analysed. Analyses was performed on FlowJo to detect populations of IL-22, IL-17, INFγ, IL-10, and TNFα producing populations. The first step of defining lymphocyte populations was performed using FSC and SSC sizes. This population was then gated for further analysis of live or dead cells. Live discriminator was used to reduce auto-fluorescence from dead lymphocytes. Live cells did not stain positively and therefore the negative population was gated for further analysis. CD4$^+$ population was defined using both a negative control as well as an FMO control for BV421 (Fluorochrome for CD4$^+$). The same technique was used to define positive and negative gates for IL-22, IL-17, INFγ, IL-10, and TNFα. The results of the detection of the different subtype populations can be seen in figures 3.7 (a-d) which shows the detection of each of the populations for both 4hr activation and 16hr activation as well as analyses on either fresh or frozen samples.

Figure 3.8

Mean and SD of cytokine production by CD4+ T Helper Cells following activation of varying time period as well as varying freeze state.



Figures 3.8 Variation of IL-10, IL-17, IL-22, TNFα and INFγ cytokines and CD4 surface marker produced following activation of PBMCs for 4 hours or 16 hours from fresh samples and frozen samples from healthy donors.

Healthy donor samples were activated either immediately after lymphocyte separation or following freezing for 1 week then thawed and immediately activated. Activation periods were also varied from 4 hours to 16 hours. Percentage of each cytokine was measure using a standard gating technique defining lymphocytes, live/dead population and then gating on CD4+ population defined by FMO's and negative control. Positive and negative gates for each cytokine was defined by FMOs for that cytokine and negative controls. The experiment was then repeated 4 further times with healthy donors and the data analysed using PRISM Graph Pad Statistical software. A mean and standard deviation of each cytokine production was collated.

3.4 Effect of Freezing, activation and permeabilisation on lymphocyte population and expression of CD4 and CD8 surface markers

Following the previous experiment, it was felt that to be able to continue to accurately define the CD4 population a further experiment would be needed to assess the effect of the freezing process, 16 hour activation and permeabalisation have on CD4 and CD8 expression. AS the previous experiment had identified the optimal detection of all key cytokines was following freezing and 16 hour activation it was important to assess the effect this had on the lymphocytes. Also permeabalisation is essential for intracellular cytokine detection but it was also felt important as to how this affected CD4 and CD8 expression. Therefore normal donor samples were analysed using flow cytometry for CD4 and CD8 surface markers using the following pathway (figure 3.9).

3.4.1 Results of effects of Freezing, activation and permeabilisation on lymphocyte population and expression of CD4 and CD8 surface markers

From analysis of a single donor it is clear that permeabilisation and activation have an effect on both lymphocyte shape and surface protein expression however freezing lymphocytes then thawing for analysis seems to have little effect. However it is important to note that analysis of a single donor may have an impact on the ability to reproduce the results.

Figures 3.9 and 3.10 show the variation in lymphocyte shape following permeabilisation. The lymphocytes become smaller with significantly lower FSC. SSC however remains the same. This suggests that the permeabilisation process reduces the size of the lymphocytes. Jacob et al found a similar change to cell morphology following similar permeabilisation technique with saponin which solubilises cholesterol in the membrane. However this change in size does not impact on the ability to identify the lymphocyte population.

Permeabilisation does not seem to have a significant effect on both $CD4^+$ and $CD8^+$ expression seen in figures 3.11-3.12. There is some down-regulation but this appears to be marginal. Activation however appears to have a significant effect on $CD4^+$ expression whilst only minimal effect on $CD8^+$ expression. This is in keeping with evidence in the literature.

This experiment has highlighted significant issues with this technique of intracellular flow cytometry which requires activation and permeabilisation in the detection of intracellular cytokines. The key issue being the accurate identification of the $CD4^+$ T helper cell population which is the focus of this study. On review of the literature and earlier results it was identified

that the fluorochrome used for $CD4^+$ staining may not be suitable and also to be more confident on the population identification a further surface marker of $CD3^+$ may be necessary (120).

Figure 3.9

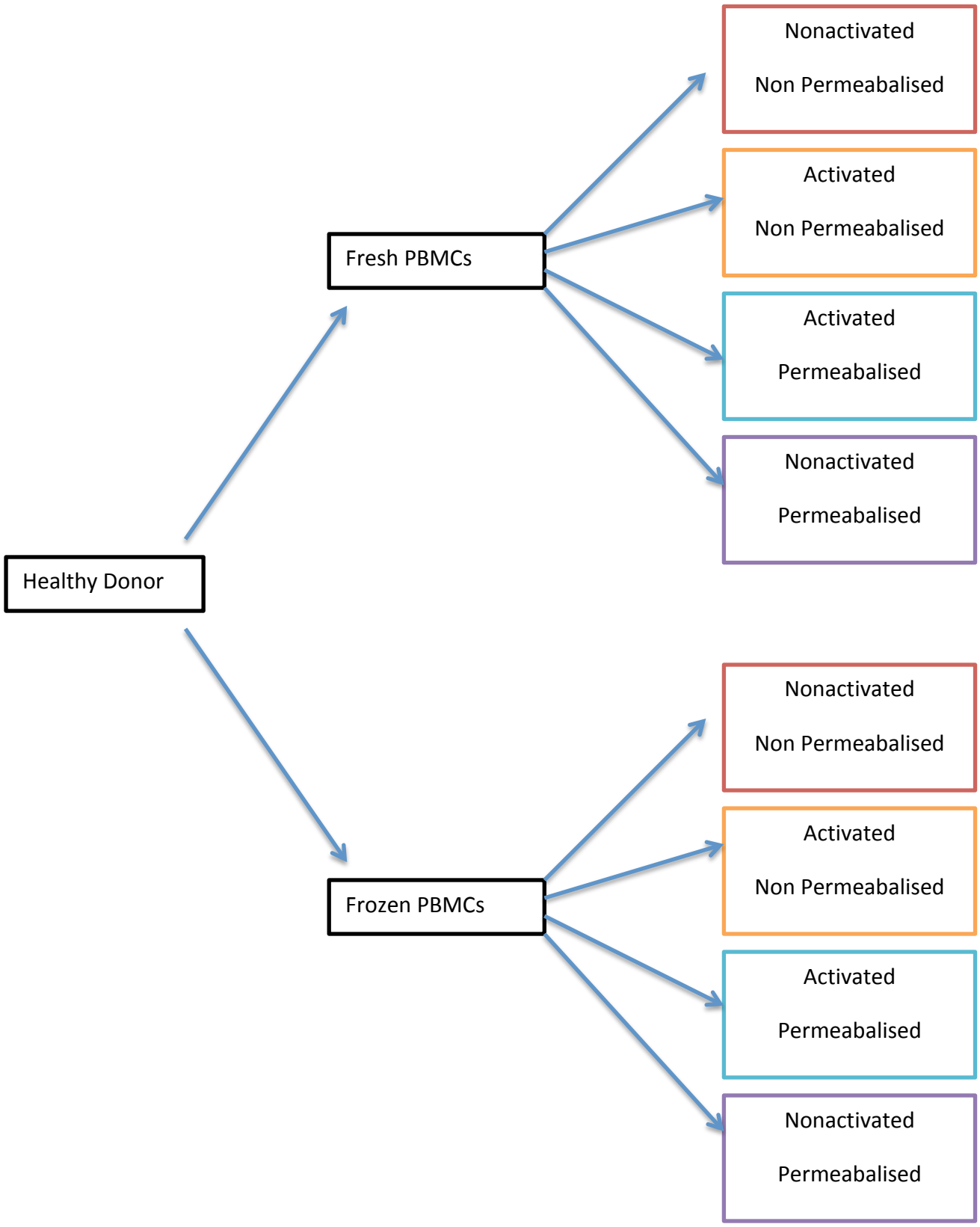


Figure 3.9 Flow diagragm showing details of experiment 3.3 examining the effects of freezing, activation and permeabalisation on lymphocyte population and expression of CD4 and CD8 surface markers

Healthy donor PBMCs were split into 2 groups with activation and permeablisation being perfomed on either fresh PBMC samples or frozen PBMC samples.

Figure 3.10

Fresh Samples PBMC - Lymphocyte Shape on FSC and SSC

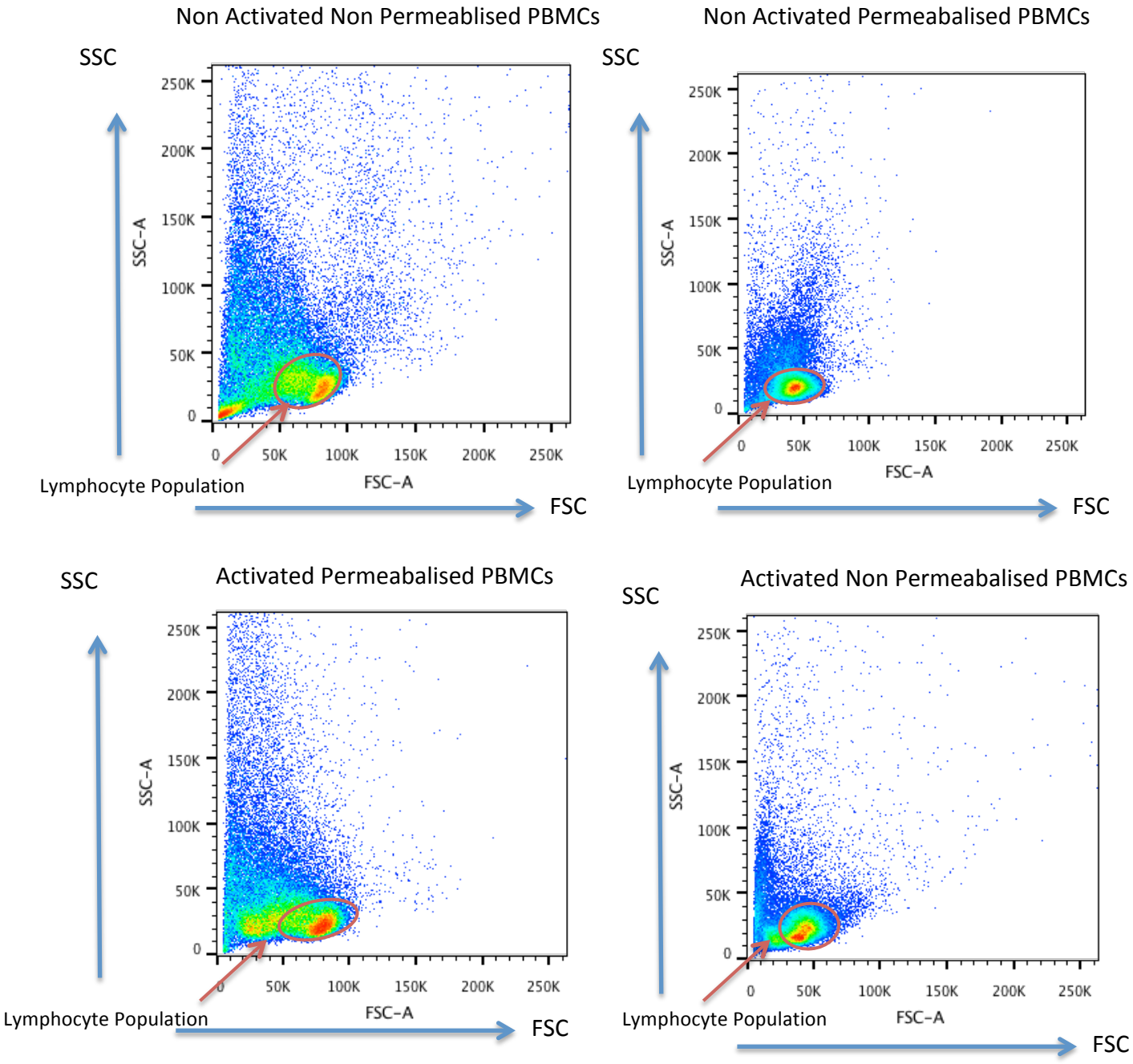


Figure 3.10 Effects of permeablaisation and activtion of FRESH PBMC normal donnor samples on lymphocyte size

Size is defined by forward scatter (FSC). Data is from sample normal donor activated or left unactivated for a duration of 16 hours using standard techniques immediately after lymphocyte seperation. A standard permeabalisation and surface staining technique was used. The highlighted lymphocyte population identifies the effect permeabilisation has on the gating and thus identification of the lymphocyte population based on FSC.

Figure 3.11

Frozen Samples PBMCs – Lymphocyte Shape on FSC and SSC

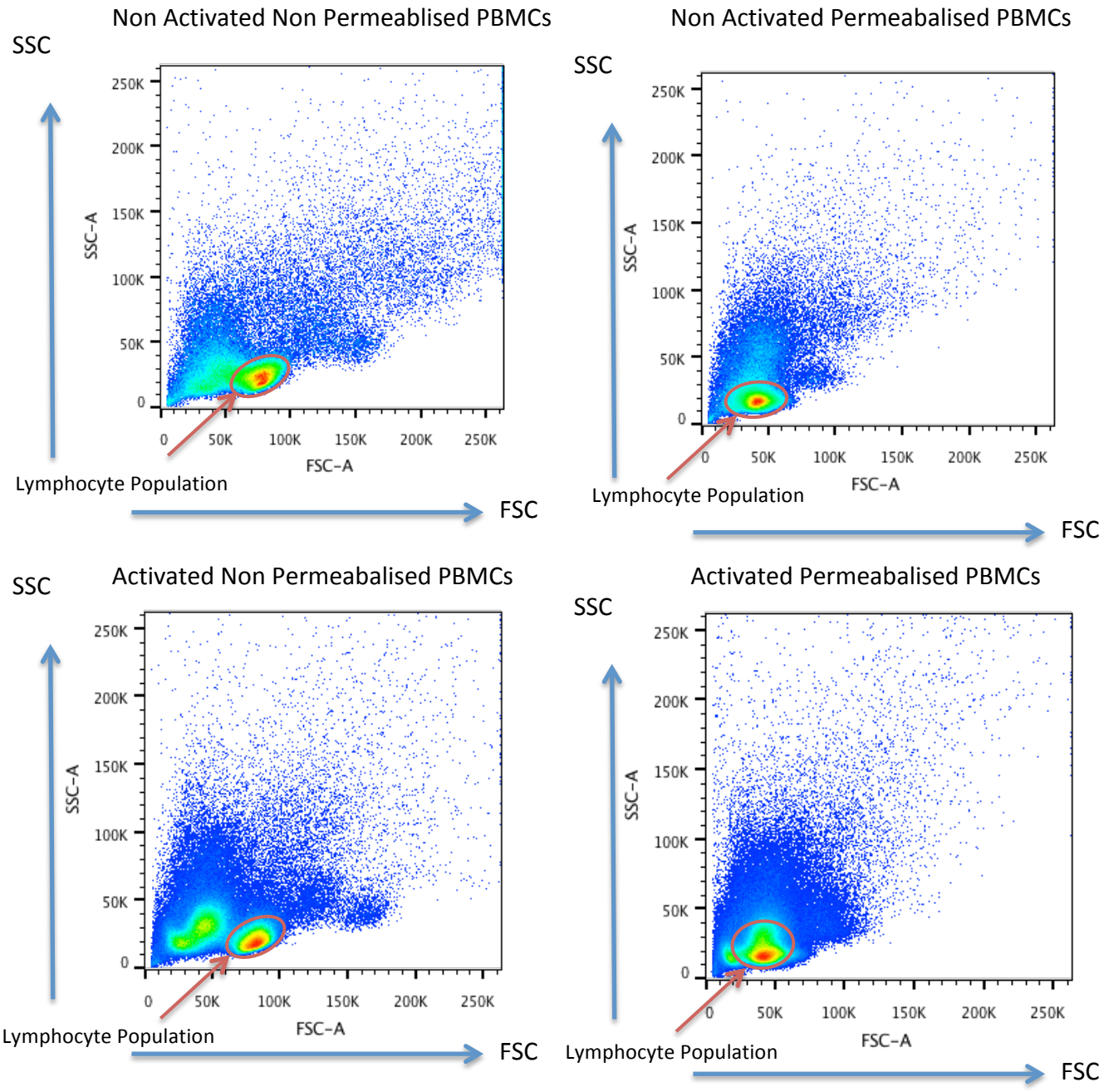


Figure 3.11 Effects of permeabilisation and activtion of FROZEN PBMC normal donnor samples on lymphocyte size

PBMCs were activated or left unactivated following standard freeze process and thawed 1 week later. Size is defined by forward scatter (FSC). Data is from sample normal donor activated for a duration of 16 hours using standard techniques. A standard permeabalisation and surface staining technique was used. The highlighted lymphocyte population identifies the effect permeabilisation has on the gating and thus identification of the lymphocyte population based on FSC.

Figure 3.12

Fresh Samples PBMC – CD4 Expression and CD8 Expression

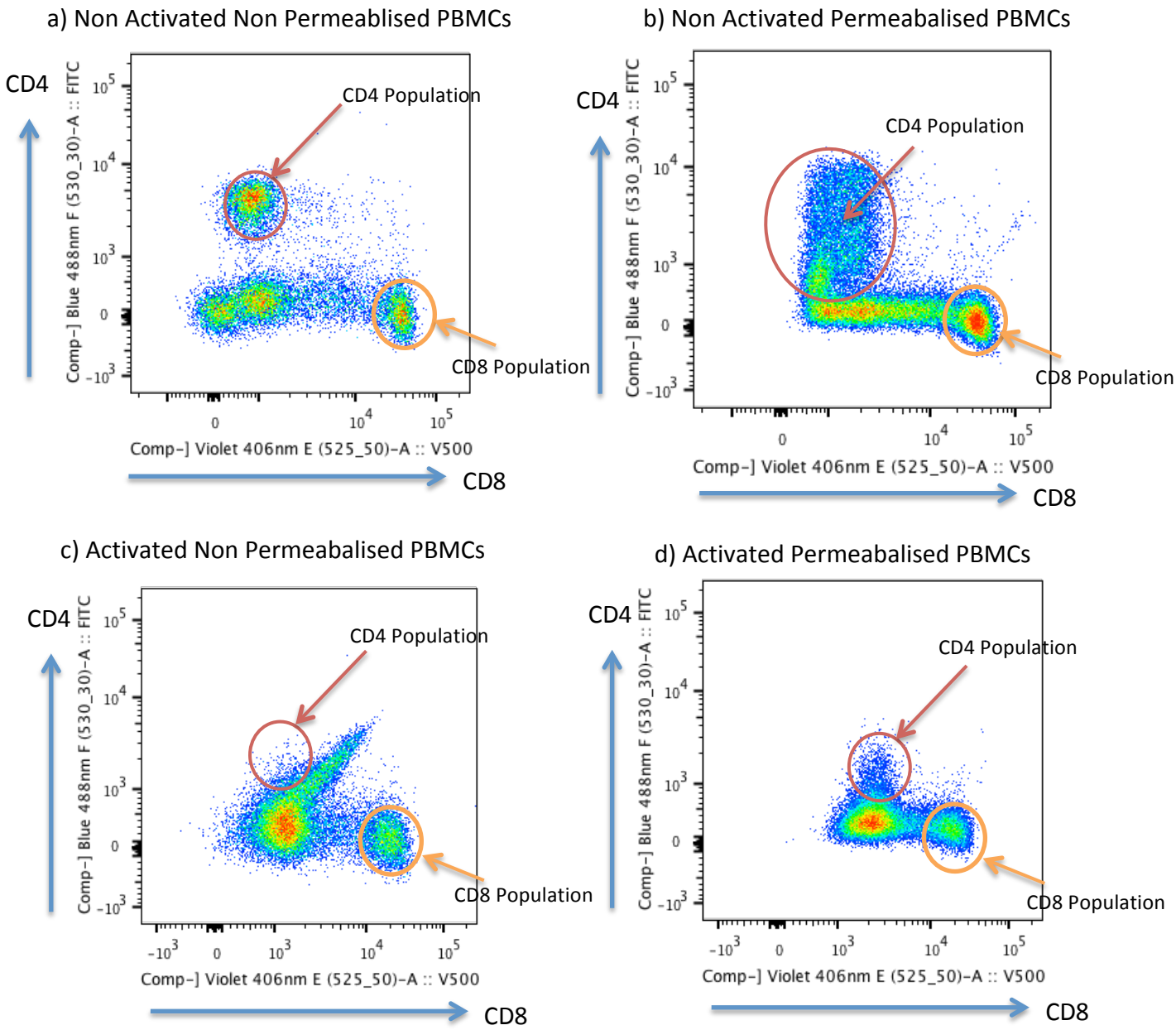


Figure 3.12 Effects of permeabilisation and activtion of FRESH PBMC normal donnor samples on CD4 and CD8 expression

PBMCs from a healthy donor were activated or left unactivated immediately following lymphocyte seperation. Activation was for a period of 16 hours using a standard technique. Populations of $CD4^+$ and $CD8^+$ following gating on lymphocyte groups as defined previously, were analysed. A standard permeabalisation and surface staining technique was used. The highlighted populations are $CD4^+$ and $CD8^+$. The graphs from a – d highlight the effects of activation and permeabilisation on the detection of the $CD4^+$ population however $CD8^+$ populaiton is unaffected. Activation and permeabilisation result in alower ercentage popluation detected when using the flourocrhome FITC.

Figure 3.13

Frozen Samples PBMC – CD4 Expression and CD8 Expression

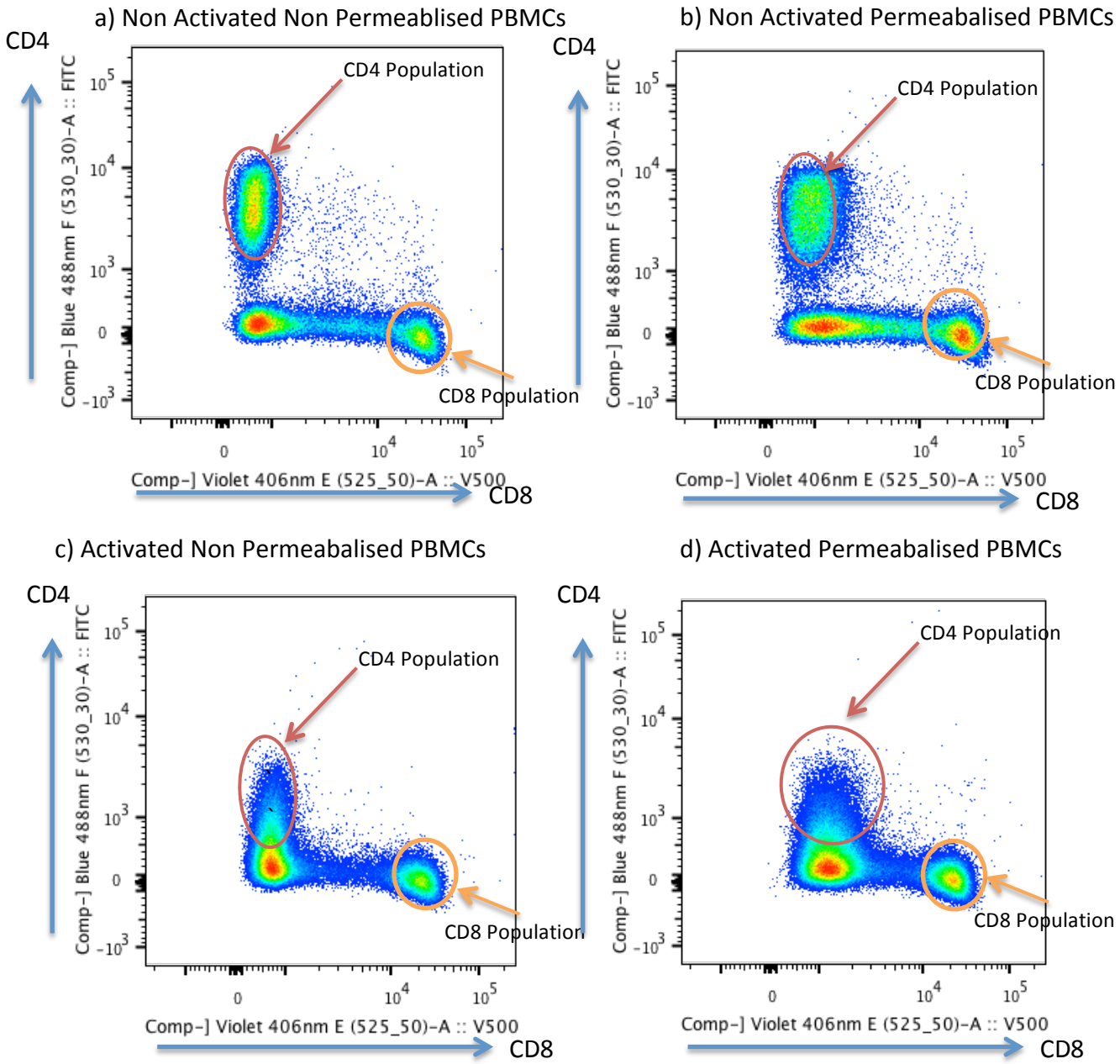
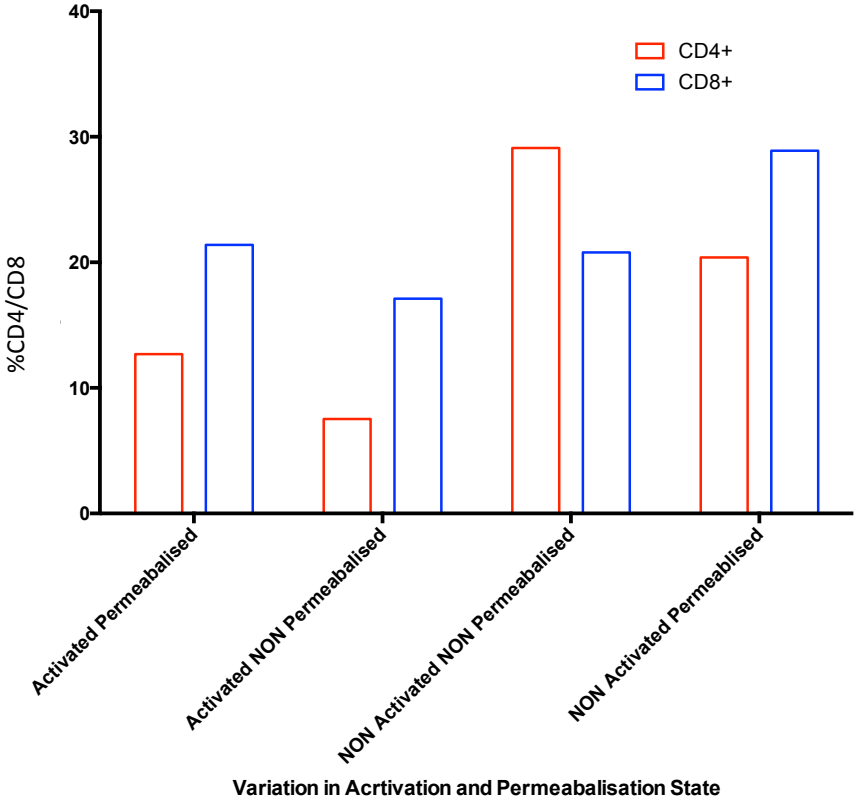


Figure 3.13 Effects of permeabilisation and activtion of FROZEN PBMC normal donnor samples on CD4 and CD8 expression

PBMCs were activated or left unactivated following standard freeze process after lymphocyte seperation then thawed 1 week. Populations of $CD4^+$ and $CD8^+$ following gating on lymphocyte groups as defined previously were analysed. Data is from a healthy donor sample activated for a duration of 16 hours using standard techniques. A standard permeabalisation and surface staining technique was used. The highlighted populations are $CD4^+$ and $CD8^+$. The graphs from a – d highlight the effects of activation and permeabilisation on the detection of the $CD4^+$ population however $CD8^+$ populaiton is unaffected. Activation and permeabilisation result in a lower percentage popluation detected when using the flourocrhome FITC.

Figure 3.14

a) **Fresh PBMCs (n=1)**



b) **Frozen PBMCs (n=1)**

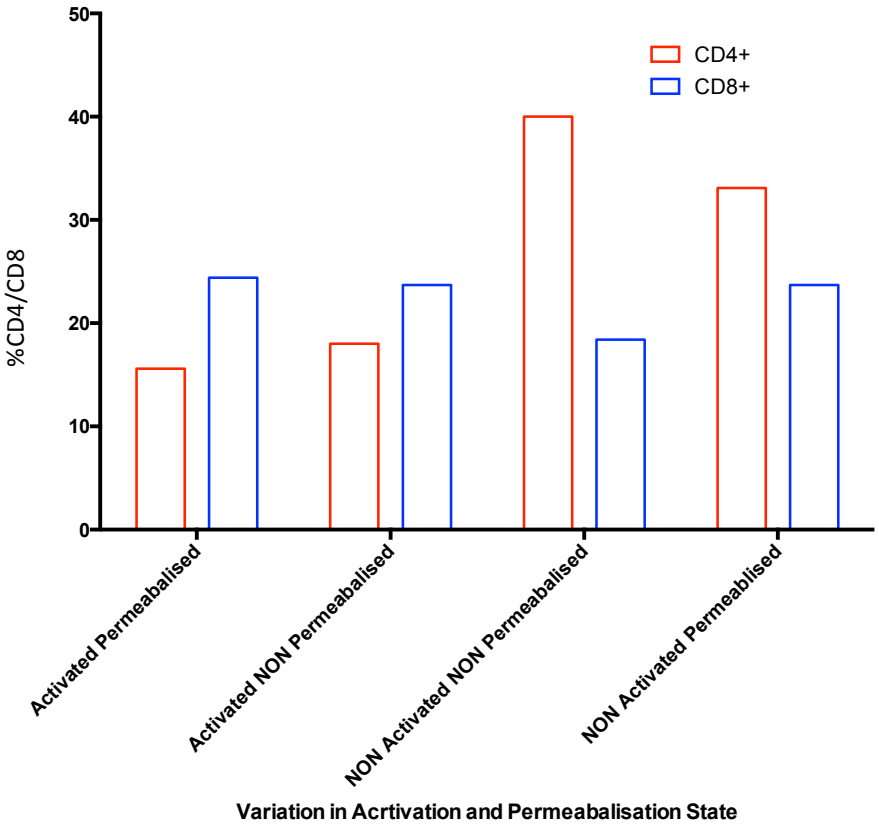


Figure 3.14 Effect of Freezing, activation and permeabilisation on lymphocyte population and expression of CD4 and CD8 surface markers

Graph a) and b) compare the differences of $CD4^+$ and $CD8^+$ surface protein expression following variations in freeze state (analysis performed on fresh PBMCs or Frozen PBMCs following thawing), activation (non activated or activated) and permeabilisation (permeabilised or non permeabilised). A Healthy donor PBMCs were analysed following a standard freeze technique, permeabilisation process and activation process (n=1). Analyses were performed on FowJo and percentage of surface marker CD4 and CD8 were measure using a standard gating technique defining lymphocytes, live/dead population. FMO's and negative controls were also used to allow accurate gating of $CD4^+$ and $CD8^+$ populations. Subsequent population percentages were analysed using PRISM Graph Pad Statistical software. The graphs highlight that following activation and permeabilisation the percentage and thus detection of populations of $CD4^+$ are significantly decreased however $CD8^+$ populations are unaffected.

3.5 Experiment to address identification of CD4+ population following activation and permeabilisation for intracellular flow cytometry

As a result of previous experiments figure 3.2 and 3.3 a further experiment was designed to try and improve detection and identification of T helper cell populations ($CD4^+$). It was decided to try a brighter fluorochrome in place of the previous dim fluorochrome FITC for $CD4^+$ staining. This fluorochrome was previously noted to be recommended for high density molecules such as surface markers CD4 or CD8 however following the activation and permeabilisation process this surface marker is significantly down-regulated therefore the density of this is reduced and a brighter fluorochrome is now needed. To allow compensation and panel setup to remain the same as previous experiments a fluorochrome was identified that would replace FITC as a surface marker whilst still using the same laser and filter as FITC. Alexa Fluor®488 (AF488) was identified as a suitable alternative with near exact excitation spectrum as FITC but significantly brighter making it more suitable for low density molecules. An experiment was therefore designed comparing FITC with Alexa Fluor®488 as surface markers for the identification of the $CD4^+$ population. Normal healthy donor PBMCs were used from frozen samples as the results from experiment 3.3 (figure 3.3) had very little effect on CD4+ expression. Samples were then thawed and immediately activated for 16 hours. Following which they were then surface stained with either FITC fluorochrome as well as V500 fluorochrome (CD8 antibody) or Alexa Fluor®488 with V500. They were then permeabilised as per standard protocol. The experiment was repeated on 3 healthy donor PBMC samples (n=3) (figure 3.15).

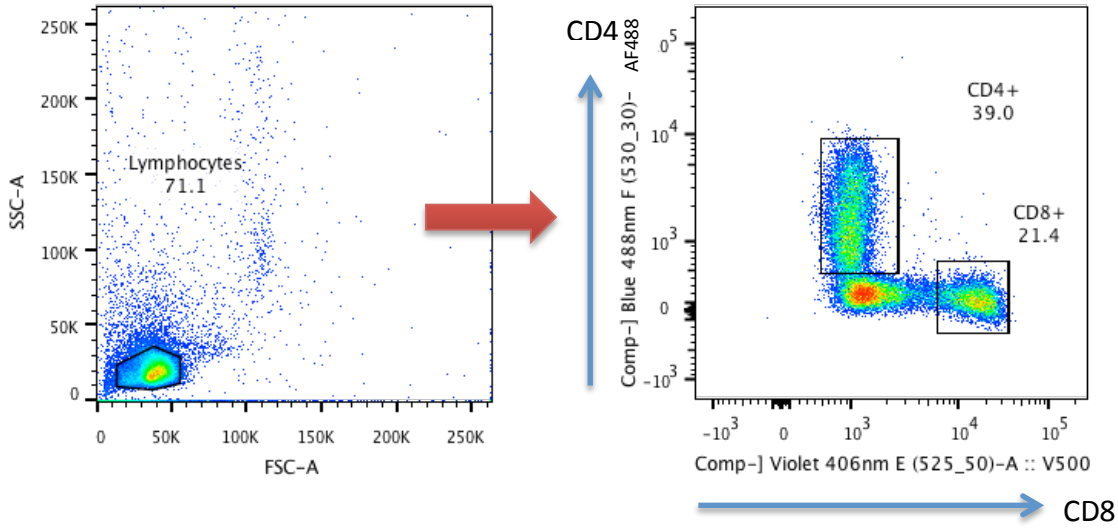
3.5.1 Surface staining results; FITC vs AF488 for CD4+ surface protein expression

The results set out in figures 3.14 – 3.16 clearly show that there is a significant improvement in the detection of the CD4+ surface protein with FITC fluorochrome and AF488 (19.6% vs 35.1%, P=0.0138) when activated for 16 hours and permeabilised using a standard technique following surface staining. Despite down-regulation of the CD4+ surface protein the CD4+ population is still identifiable as a distinct population using AF488 in the current flow cytometry panel configuration.

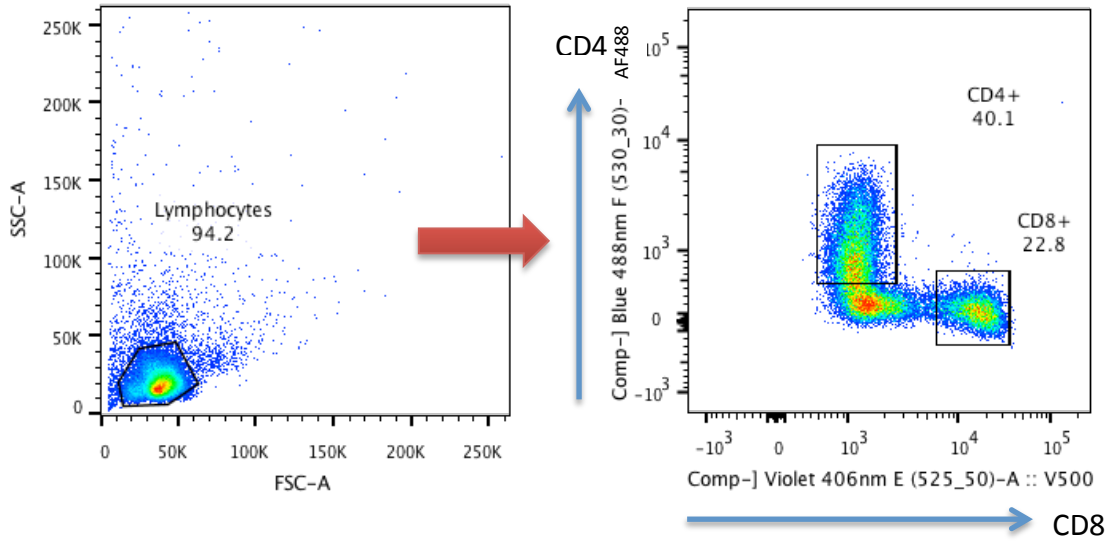
Figure 3.15

Frozen Samples PBMC – CD4 Expression and CD8 Expression

a) HD 1 - AF488 CD4$^+$



b) HD 2 - AF488 CD4$^+$



c) HD 3 - AF488 CD4$^+$

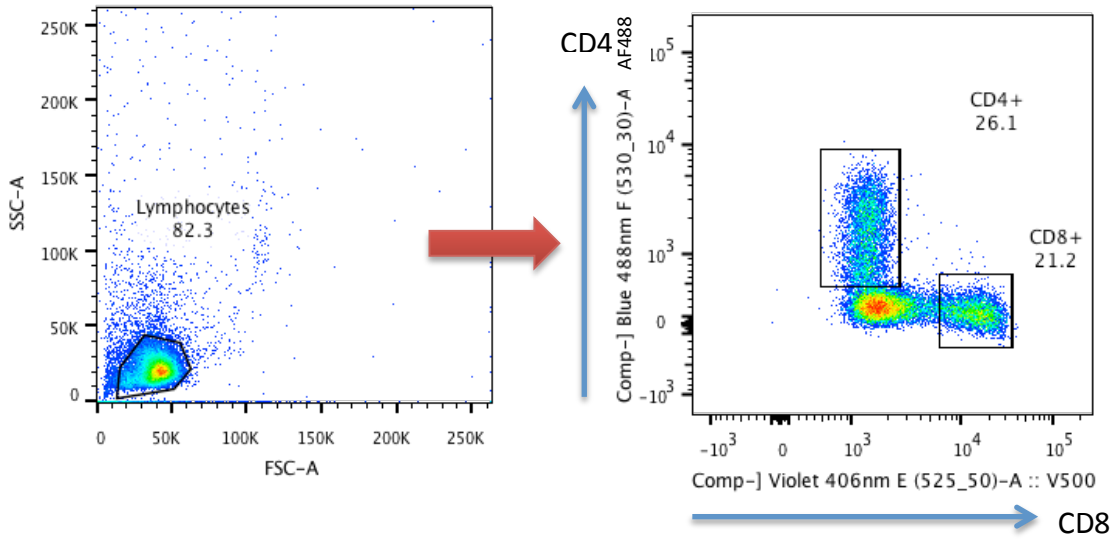
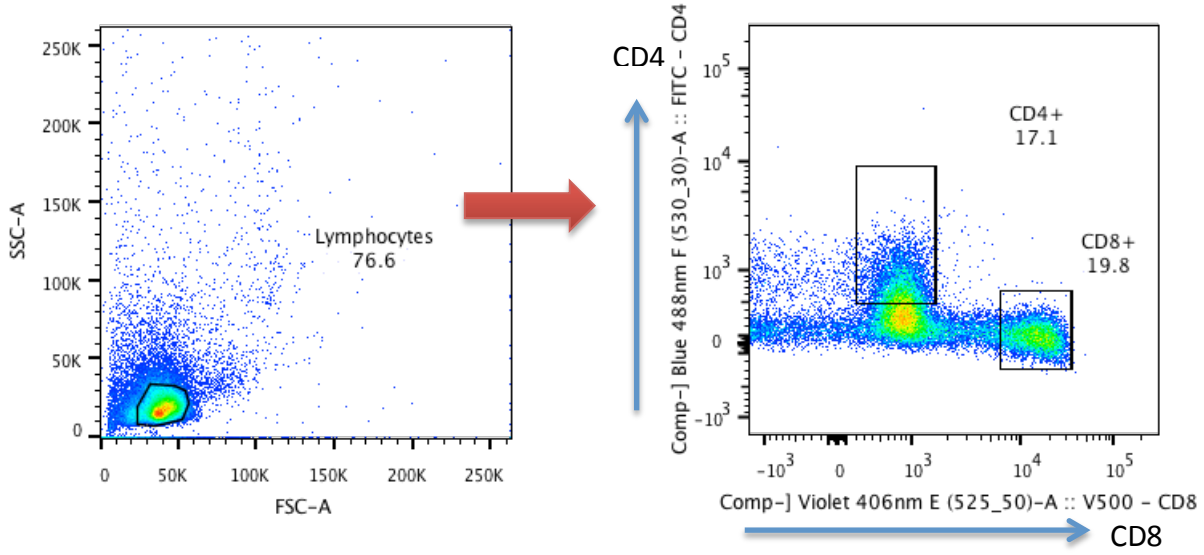


Figure 3.15 Effects of permeablaisation and activtion of FROZEN PBMC normal donnor samples on CD4 and CD8 expression using the Flourocrhome AF488

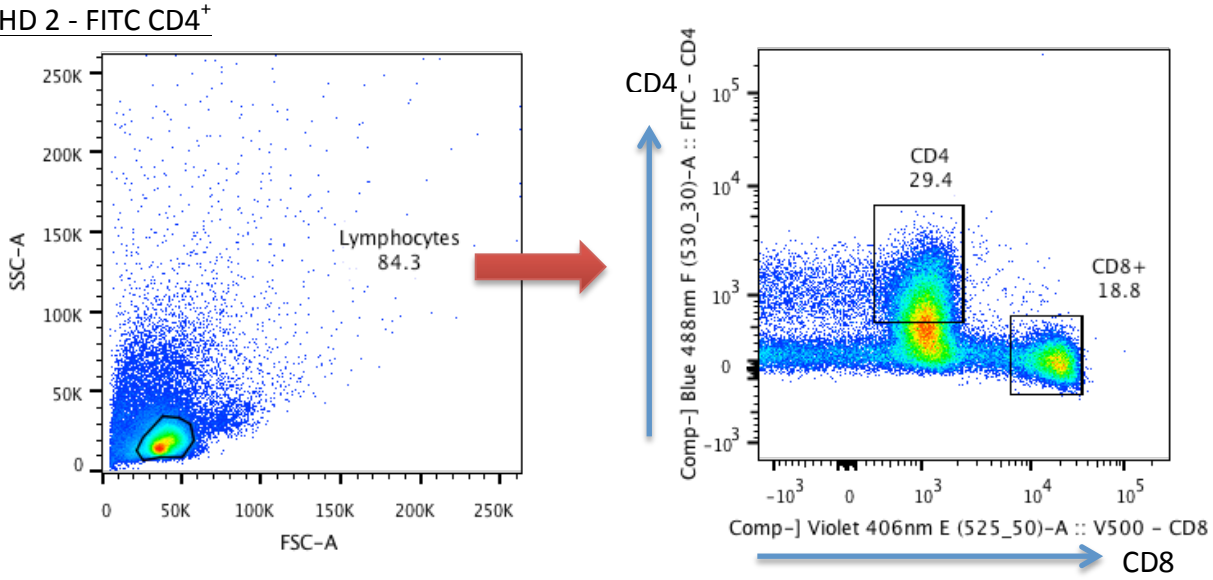
Healthy donor PBMCs were activated or left unactivated following standard freeze process after lymphocyte seperation then thawed 1 week later. Activation was for a period of 16 hours using a standard technique. Populations of $CD4^+$ and $CD8^+$ were analysed following gating on lymphocyte groups as defined previously. A standard permeabalisation and surface staining technique was used. The fluorochrome profile for CD8 was V500 but AF488 was used for CD4 populations. This experiment was repeated 3 times on further healthy donor samples (n=3). The highlighted populations are $CD4^+$ and $CD8^+$. Graphs a-c show the consistent detection of the $CD4^+$ population using the flourocrhome AF488 for the detection of $CD4^+$ populations.

Figure 3.16

a) HD 1 - FITC $CD4^+$



b) HD 2 - FITC $CD4^+$



c) HD 3 - FITC $CD4^+$

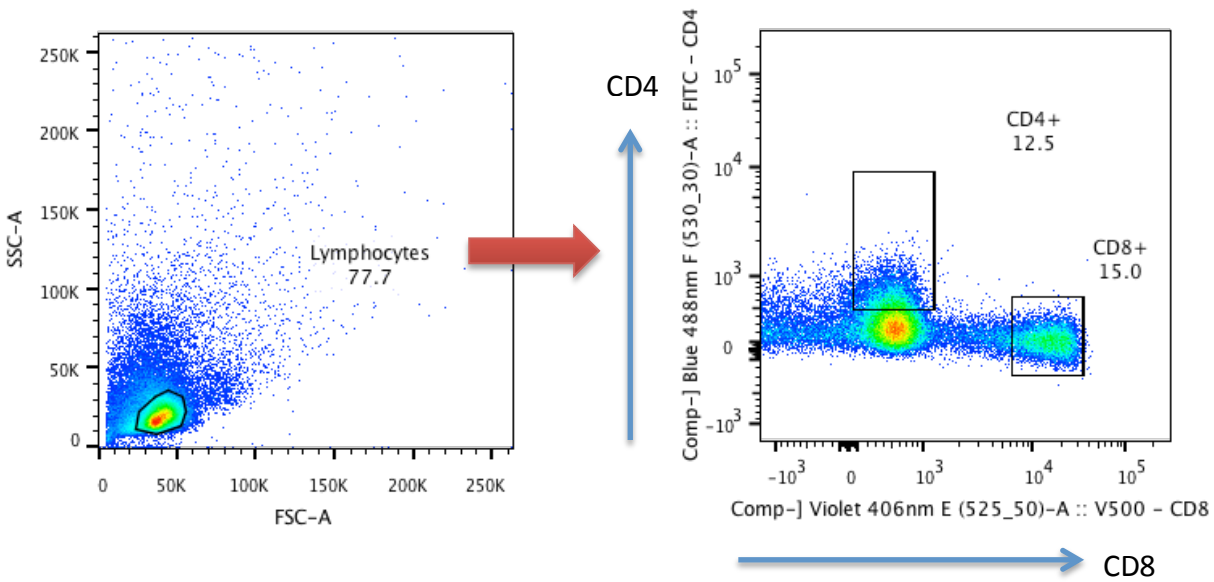


Figure 3.16 Effects of permeablaisation and activtion of FROZEN PBMC normal donnor samples on CD4 and CD8 expression using the Flourocrhome AF488

Healthy donor PBMCs were activated or left unactivated following standard freeze process after lymphocyte seperation then thawed 1 week later. Activation was for a period of 16 hours using a standard technique. Populations of $CD4^+$ and $CD8^+$ were analysed following gating on lymphocyte groups as defined previously. A standard permeabalisation and surface staining technique was used. The fluorochrome profile for CD8 was V500 but FITC was used for CD4 populations. This experiment was repeated 3 times on further healthy donor samples (n=3). The highlighted populations are $CD4^+$ and $CD8^+$. Graphs a-c show the consistent difficulties in the detection of the $CD4^+$ population using the flourocrhome FITC for the detection of $CD4^+$ populations.

Figure 3.17

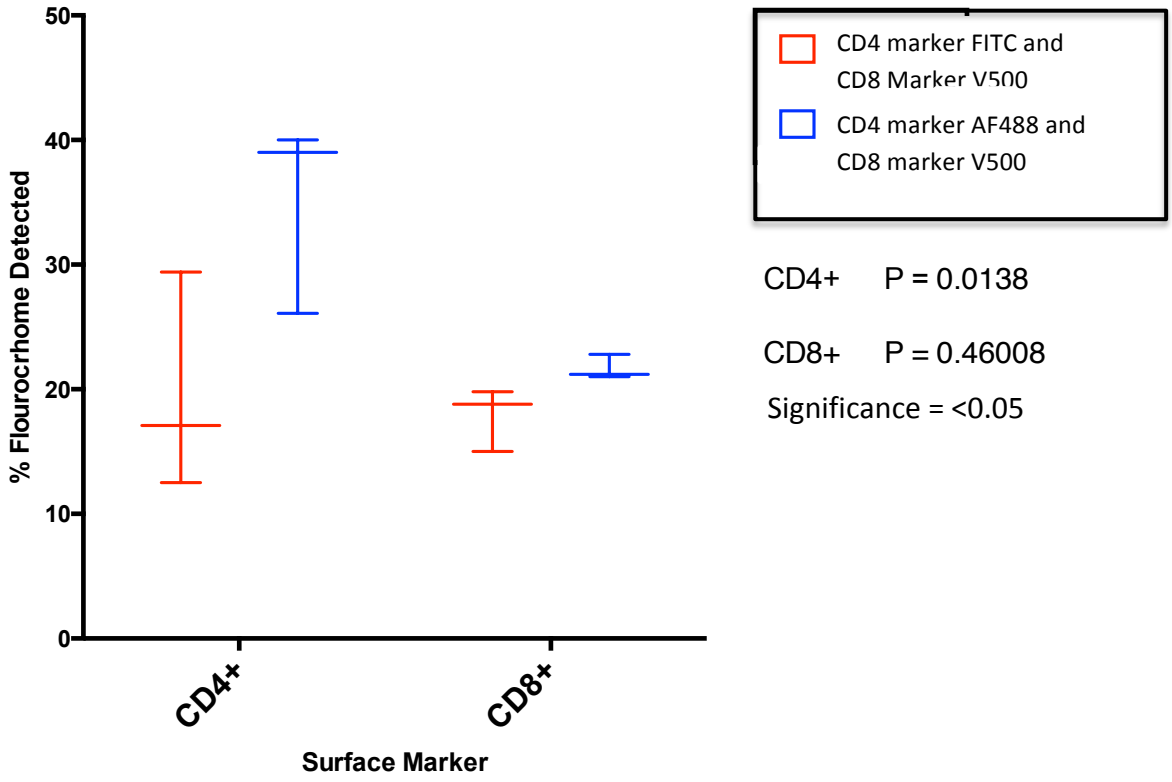


Figure 3.17 Comparisons of surface staining with either FITC CD4 flourochome or AF488 Fluorochrome with V500 CD8 fluorochrome on detection of surface markers CD4 and CD8 following activation and permeabilisation

Different fluorochromes were used to identify the optimal method for detecting $CD4^+$ surface marker in normal donors following 16 hour activation and a standard permeabilisation technique. A brighter fluorochrome was hypothesised to be better at detecting $CD4^+$ populations following the down-regulation during both the activation process and permeabilisation process. The fluorochrome chosen was AF488, a brighter conjugate fluorochrome than FITC but still using the same laser and filter on the flow cytometer. The experiment was repeated 3 times with 3 different healthy donors (n=3). The graph shows a significant difference p = 0.0138 in the ability to detect CD4+ population using fluorochrome AF488 as marker for CD4 and the fluorochrome FITC as marker for CD4.

3.6 Panel 3 Experiment

Although substituting AF488 fluorochrome for FITC in the flow cytometry panel number 2 improved detection of the $CD4^+$ population it was felt that this could be further improved by the addition of the surface marker $CD3^+$. Therefore a new panel was designed including an additional fluorochrome (table 3.1).

Through further research it became apparent that identifying Th17 T helper cells could also be aided by the addition of a further fluorochrome with an antibody to RORγt. Evidence suggested that this is a more sensitive marker for Th17 and could aid in improving accurate identification of this T helper cell phenotype (131, 132). It was decided that whilst redesigning a new panel to incorporate $CD3^+$ the fluorochrome for RORγt would also be added to the panel design.

To reduce the risk of overlap of spectra and help reduce the risk of false positives it was decided to use a live and dead discriminator on a completely different laser to the rest of the fluorochromes thus minimising spill-over from a very bright fluorochrome. Despite the addition of these new fluorochromes all of the fluorochromes were still on different filters and over as wide a rang of lasers as possible.

An experiment was designed to check the ability of this new flow-cytometry panel to detect key cytokines in the aim of identifying Th17 and Th22 Helper Cells (figures 3.18 and 3.19). Normal donor PBMCs were used from frozen samples and activated over 16 hours following thawing. These were then surface stained for CD3, CD4 and CD8 before permeabilisation followed by intracellular staining for cytokines. The samples were then analysed using flow-cytometer. Beaded controls, negative controls and FMOs were used to setup compensations on flow cytometer.

Table 3.1

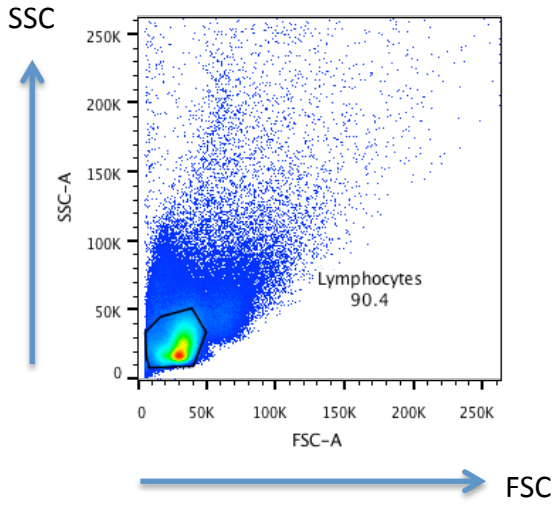
	CD4	IL17α	IL-22	CD8	INFγ	IL2	IL-10	TNFα	CD3	RORγt	Live/Dead
PANEL 3	Alexa Flouro 488	BV605	PE	V500	PerCP/CY5.5	PE/CY7	BV421	APC-CY7	PE-CY5	APC	Zombie - UV
	Blue **F**	Violet **B**	Blue **E**	Violet **E**	Blue **B**	Blue **A**	Violet **F**	Red **A**	Blue **C**	Red **C**	UV

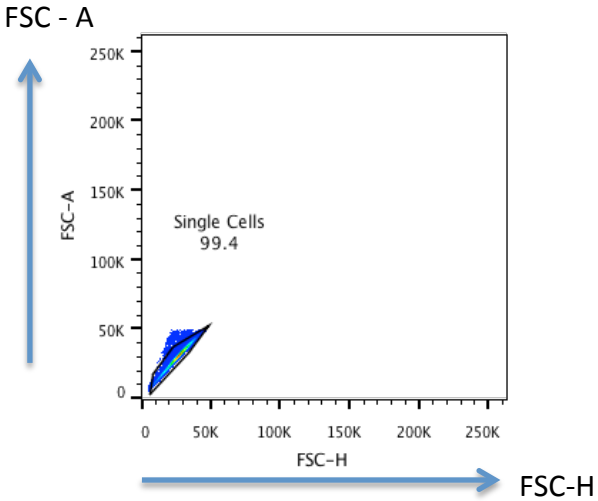
Table 3.1 Flow cytometry Panel Design Number 3

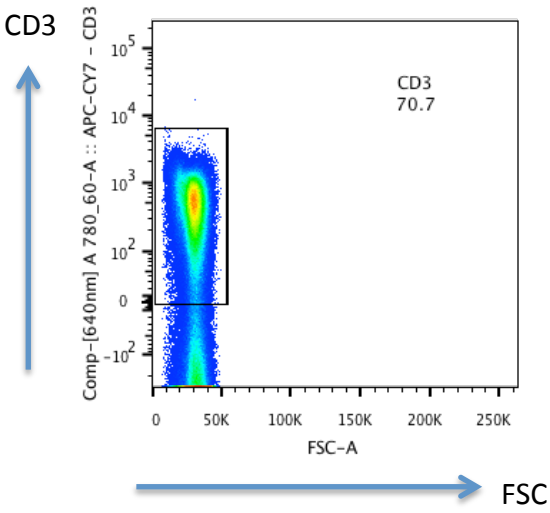
The table shows all the fluorochromes and the cytokine/surface markers chosen to optimise the detection and identification of $CD4^+$ T helper cell populations as well as specific phenotypes Th17 and Th22 based upon the previous experiments.

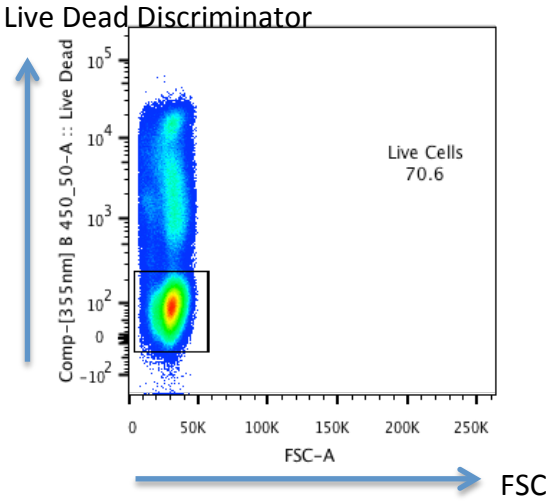
Figure 3.18

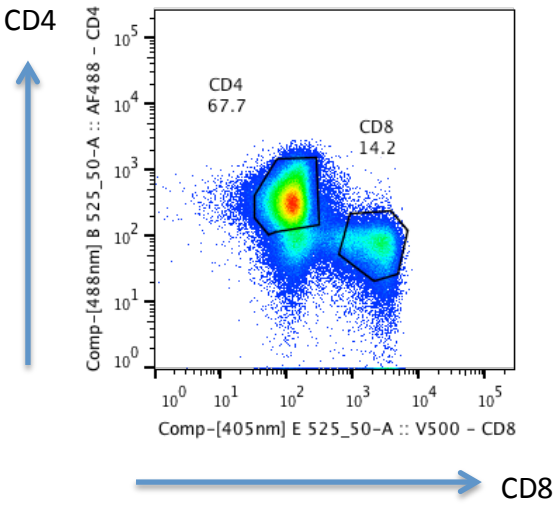
A) Gating technique to identify $CD4^+$ population in PBMCs



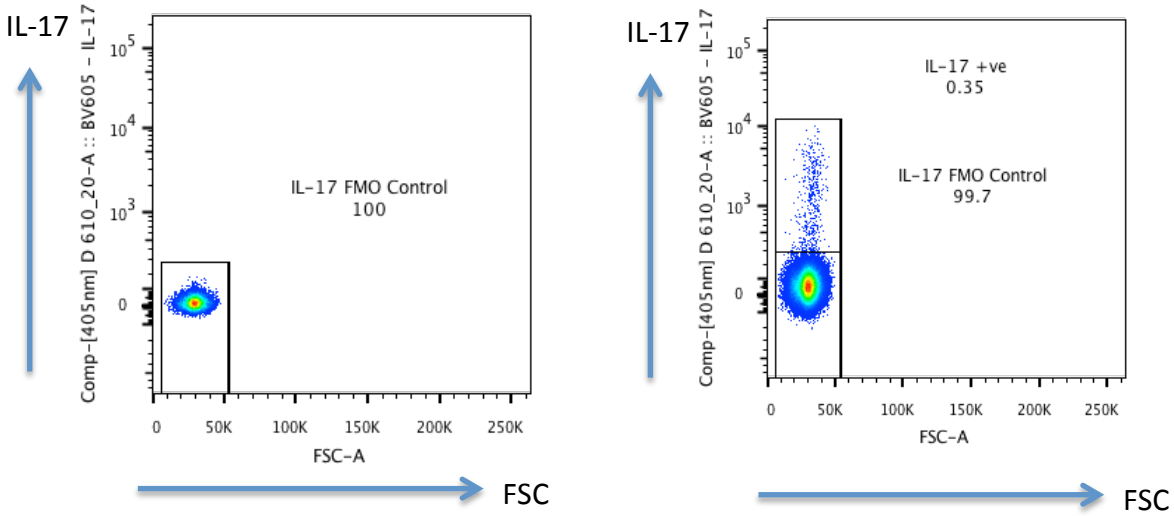




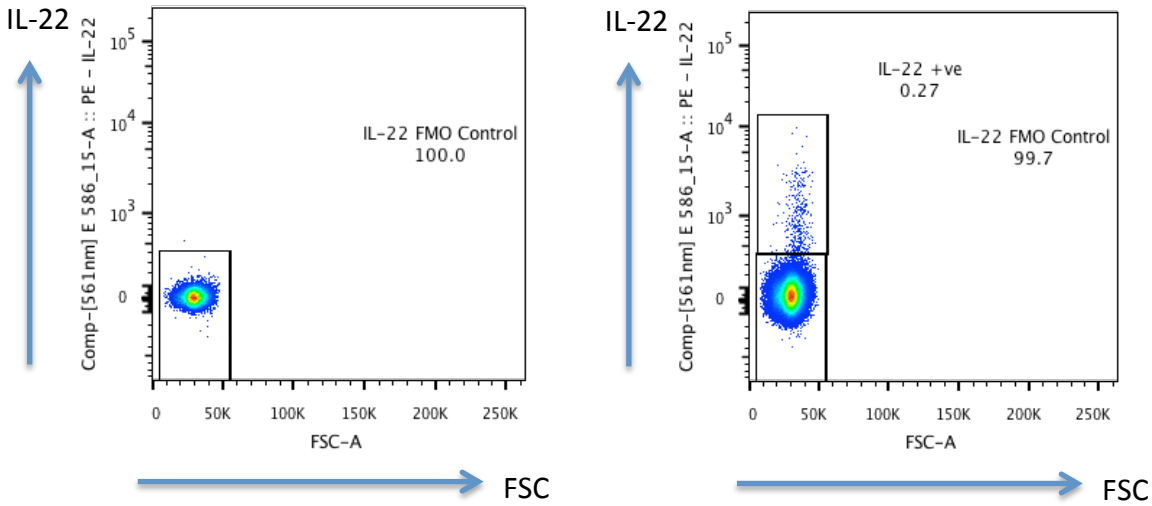




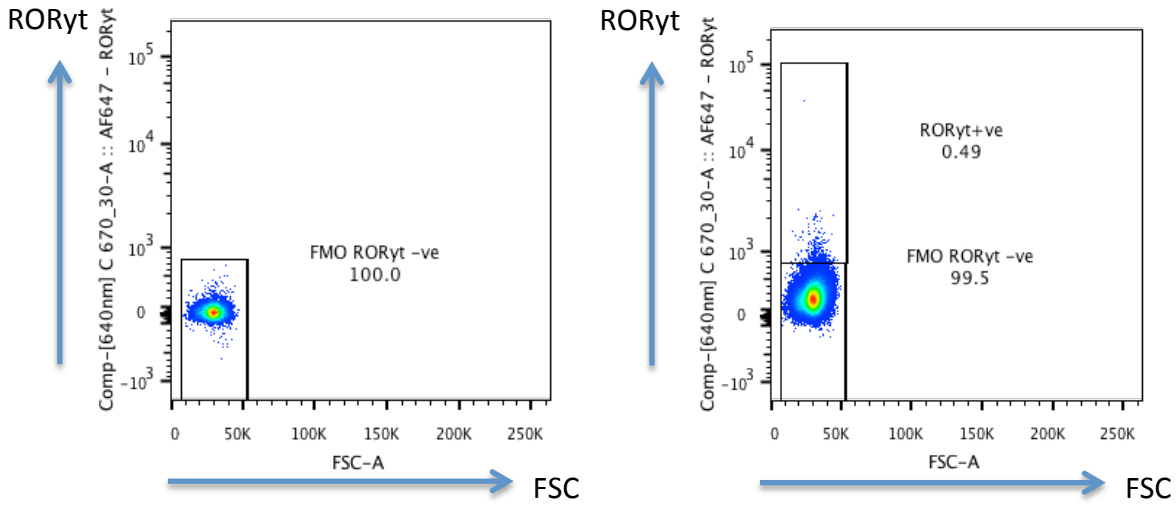
b) IL-17 producing population identified using FMO control



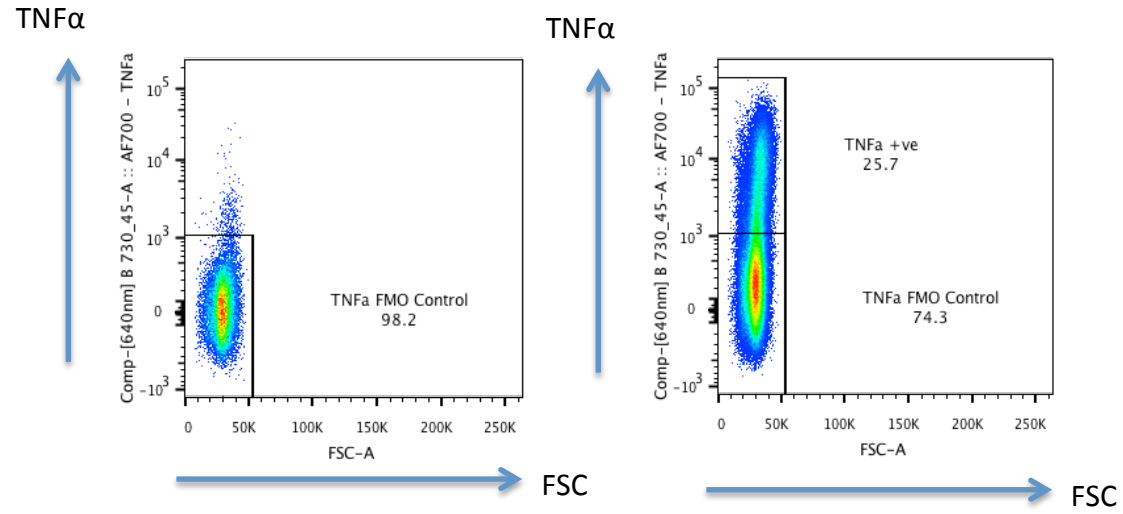
b) IL-22 producing population identified using FMO control



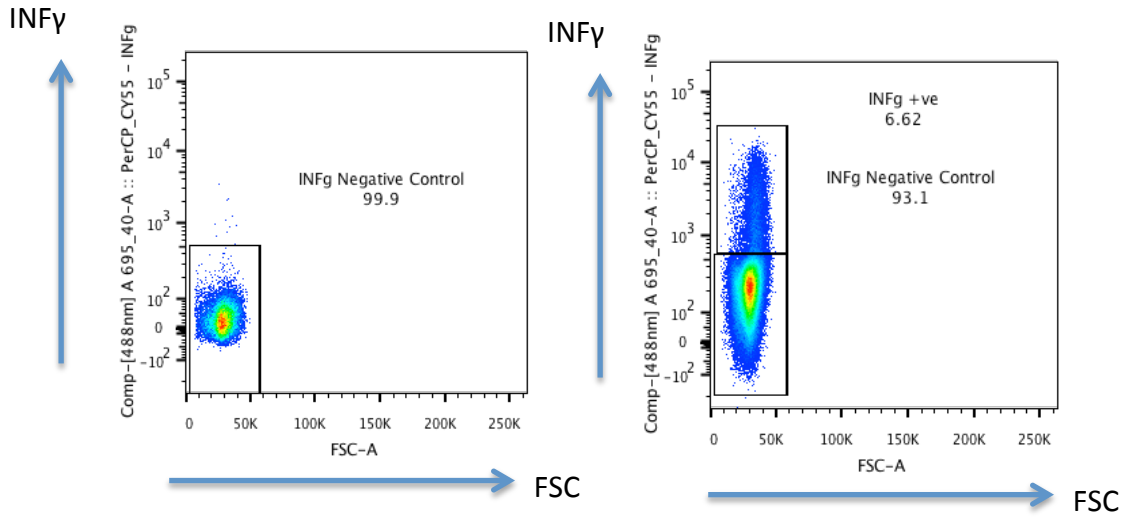
c) RORyt producing population identified using FMO control



d) TNFα producing population identified using FMO control



e) INFγ producing population identified using FMO control



f) IL-10 producing population identified using FMO control

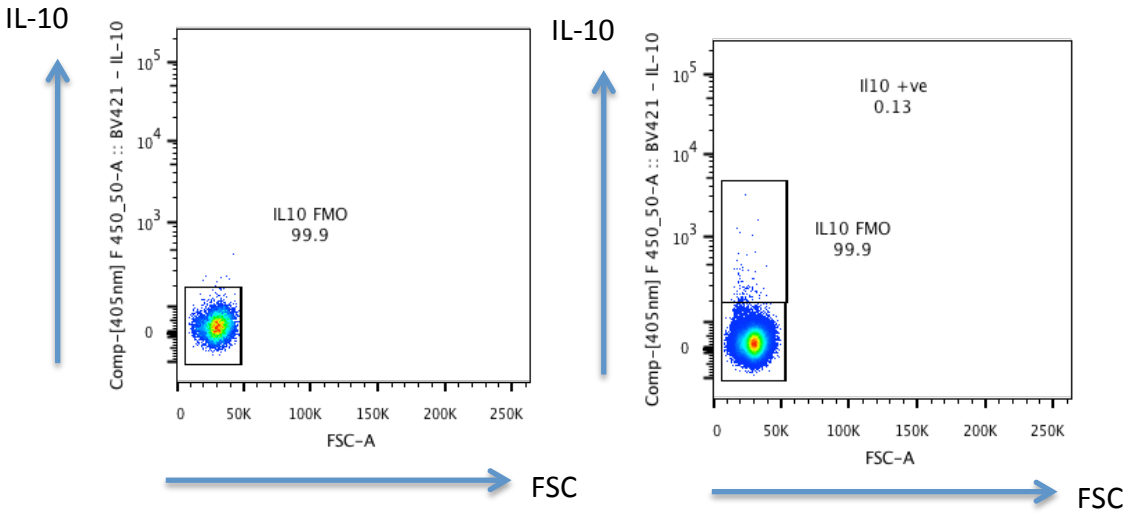


Figure 3.18 Results of final panel design for detection of cytokines IL-10, IL-17, IL-22, INFγ, TNFα, RORγt and surface markers CD4 and CD8 to identify subtype populations Th22 and Th17 phenotypes

Healthy donor PBMCs were frozen following a standard freeze process after lymphocyte seperation then thawed 1 week later. Activation was for a period of 16 hours using a standard technique. Populations of $CD4^+$ and $CD8^+$ were analysed following gating on lymphocyte groups as defined previously. A standard permeabalisation and staining technique was used based upon the panel design in figure 3.17. This experiment was repeated 3 times on further healthy donor samples (n=3).

The graphs in (a) show the gating protocal used to indetify accurately the $CD4^+$ population from PBMCs. Lymphocytes were gated on based on FSC and SSC followed by single cell identifcation from FSC-a and FSC-h. This excludes clumped cells which can effect the results. Live cells were gated on from the live dead discriminator. $CD3^+$ populations were subsequently gated to then allow idetificaiton of $CD4^+$ and $CD8^+$ populations.

Graphs (b-f) show the cytokine populations of the $CD4^+$ population for each of the cytokines listed in the panel in figure 3.17.

Figure 3.19

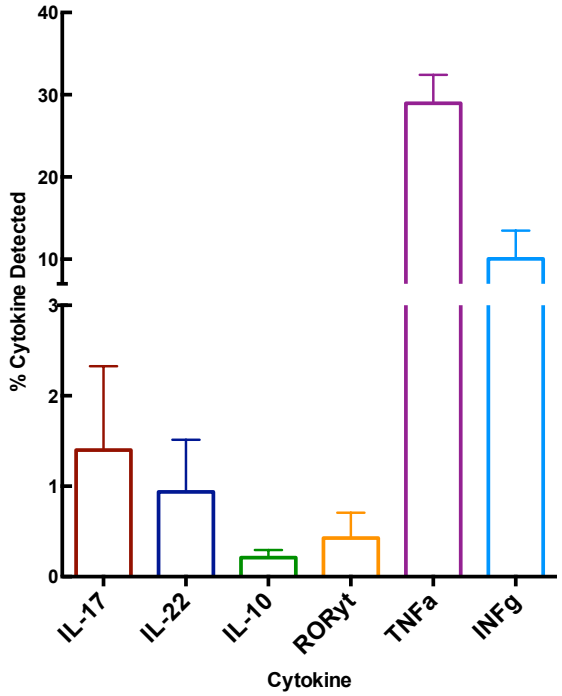


Figure 3.19 Average percentage of cytokines IL-10, IL-17, IL-22, TNFα, INFγ and RORγt produced following activation of frozen PBMCs for 16 hours using flow cytometry panel number 3

The mean number of key cytokines is shown with standard deviation. Activation, surface and intracellular staining and permeabilisation were performed using standard techniques. 3 healthy donors samples were used and the experiment repeated once for each donor sample.

3.6.1 Results of flow cytometry panel 3

Analysis of healthy donor PBMCs using the panel design 3 found that the ability to potentially identify TH17 and Th22 cells was significantly improved with reduce d risk of false positives. The use of AF488 for $CD4^+$ detection allowed far more accurate gating of this population, which was previously harder to detect in previous panel designs. Also adding in the surface marker $CD3^+$ did not impact the ability to detect the cytokines IL-10, IL-17, IL-22, INFγ, TNFα, and RORγt, however, it added to the accuracy in identifying these populations as T helper cells in the PBMCs rather than potential false positives such as other $CD4^+$ expressing populations (e.g.macrophages). Using a separate live and dead discriminator on a different laser also helped to more accurately eliminate dead lymphocytes, which have non-specific binding when dead and therefore can result in false positives. Using FSC-A and FSC-H to gate on single cells also helps to reduce potential sources of error and dead cells also tend to clump together and so can be excluded from this gate. Figure 3.19a highlight this gating technique to accurately identify the T helper cell population. To then subtype this T Helper cell population gating on specific cytokines was performed shown in figure 3.19b-f. In most cases the FMO was used as the control which is a more accurate control method as it takes into account any potential spillover from other fluorochromes and helps to eliminate this potential source of error. On one occasion, the negative control had to be used due to the poor quality of the FMO sample. However this did not significantly impact on the results.

Detection of the key cytokines IL-10, IL-17, IL-22, INFγ, TNFα and RORγt was significantly better with the fluorochromes in panel design3 compared with previous panel designs 1 and 2 and detection of the populations was very unlikely due to chance. Detection rates were also in keeping with expected population percentages in current literature.

Accurate detection of surface markers CD3 and CD4 is important in being able to confidently identify the T helper populations and subsequently the subtype populations Th17 and Th22 based on their cytokine profile. It was felt that this panel design 3 was optimal in detection and identification of these key subtypes and would be used in all future experiments.

Chapter 4 Comparisons of T Helper Cell Phenotypes between Normal Donors, Gastric Cancer Patients and Oesophageal Cancer Patients

4.1 Introduction

Previous studies have shown that in PBMCs of patients with upper gastrointestinal cancers there appears to be a significant increase in circulating Th17 and Th22 populations as well as in tumour infiltrating lymphocytes (113, 114). The role that these cells play in cancer development and prognosis still remains controversial, particularly in gastric cancer and oesophageal cancer where there is still very limited research. Several papers conflicted in whether these populations were beneficial inhibiting tumour growth or promote tumour progression (113, 114, 133).

A possible explanation of this difference in outcomes from these studies could be explained by the plasticity of Th17 populations and that a more specific definition of the Th17 population is necessary. Another area of potential conflict is in their definition of Th17 and Th22 populations which seems to vary between studies. Also the techniques used to detect their defined populations were limited and variable.

The aims of this chapter are using the optimised methodological technique outlined in the results of experiments in chapter 3 to more clearly define the subtype populations of T helper cells focusing on IL-17^{+} producing and IL-22^{+} producing subtypes, in healthy donors and upper GI cancer patients (oesophageal and gastric adenocarcinoma). Once these populations are defined a clearer view of their clinical relevance can be made. Therefore the chapter also aims to compare these populations with tumour stage for both gastric cancer and oesophageal cancer.

4.2 Patient and Healthy Donor Characteristics

A total of 28 patient samples were collected and analysed. The characteristics of the patients can be seen in table 4.1. The majority of patients were primary adenocarcinoma of the oesophagus with 25% presenting with primary gastric adenocarcinoma. The range of ages varied between the two groups but the median average was similar at 70 years old for oesophageal cancer and 70.5 years old for gastric cancer. There was a higher male prevalence for both cancer groups.

8 Healthy donors were also analysed. The median age was lower than compared to the patient groups at 45 year. The male to female ratio was even between groups and in keeping with incidence seen in the general population.

Table 4.1

	Oesophageal Cancer	Gastric Cancer	Normal Donors
Number	21	7	8
Age (median, range) years	70 (49– 79)	70.5 (62 – 79)	43 (26 – 50)
Sex (male/female)	15/6	6/1	5/3
Tumour Stage (T)			
I-II	3	3	0
IIIa	7	1	0
IIIb/c	4	1	0
IV	7	2	0
Histology			
Mod Differentiated	5	2	0
Poorly Differentiated	16	5	0

Table 4.1 Sample Patient Characteristics and Healthy Donor Characteristics

Summary of patient characteristics for both oesophageal adenocarcinoma and gastric adenocarcinoma compared against the healthy donor population.

4.3 Intracellular flow cytometry of Healthy donor, oesophageal cancer patient and gastric cancer patient PBMCs

Flow cytometry was performed using the previous 11 colour panel optimised through previous experiments. The samples were frozen PBMCs from patients as normal donors acquired with full ethical approved consent. The samples were defrosted as per previously noted protocol prior to immediate activation. Activation was with PMA, Ionomycin and Brefaldin A. The samples were activated overnight for 16 hours prior to analysis. Following this standard intracellular staining protocol was performed with FCR block and live/dead staining prior to surface staining with $CD4^+$, $CD3^+$ and $CD8^+$ antibodies. After surface staining was performed permeabilisation was performed using standard Biolegend FoxP3 permeablisation kit following which intraceullar cytokine staining was performed (figure). The samples were then analysed using the BD Fortessa flow cytometer. Data was collected with BD software FACSDiva, then results were analysed using Flow Jo V.10 as previously used. Data was subsequently collated and then analysed with Graphpad Prism 6.0 for statistical analysis. Patient and normal donor characteristics are seen in table 4.1.

4.4 Experiment to assess the correlation between T helper Cell Phenotypes and CD4+ subtypes defined by cytokine production in patients with gastric cancer and oesophageal cancer

The aims of this experiment was to look at the relationship between several distinct populations of T Helper cell phenotypes defined by the surface marker $CD4^+$. Th17 and Th22 whilst defined as distinct T cell phenotypes with distinct lineages increasing evidence has suggested that the two phenotypes may be linked with several studies showing that in a range of inflammatory conditions a positive correlation between the two phenotypes has been found(114, 134, 135). Previous evidence has suggested that their roles are very distinct and differentiation follows a different pathway. Th17 characterised by the cytokine Il-17, and IL-22 with differentiation being stimulated by IL-23, IL-6 and TGF-β and Th22 typically characterised by the production of cytokines IL-22 and TNFα but not IL-17 or INFγ with differentiation being stimulated predominately by IL-6 and TNFα (134, 136, 137).

As previously mentioned increasing evidence has supported the theory that Th17 may not have a definitive endpoint as an effector cell moreover it is likely that depending upon the tumour microvenvironment the role of Th17 cells may become plastic in nature either having a more regulatory function producing IL-10 or a effector function producing increased INFγ (138, 139).

Therefore this experiment also looked at the correlation between the Th17/Treg ($CD4^+IL\text{-}17^+IL\text{-}10^+INF\gamma^-$) T helper cells, characterised by the production of IL-17 and IL-10, and Th22 T helper cells and also TH17/Th1 ($CD4^+IL\text{-}17^+INF\gamma^+IL\text{-}10^-$) T helper cells, characterised by the production of IL-17 and INFγ, and Th22 T helper cells.

4.4.1 Results of comparisons between circulating T Helper cells and CD4+ subtype populations in healthy donor PBMCs, oesophageal cancer patients PBMCs and gastric cancer patients PBMCs

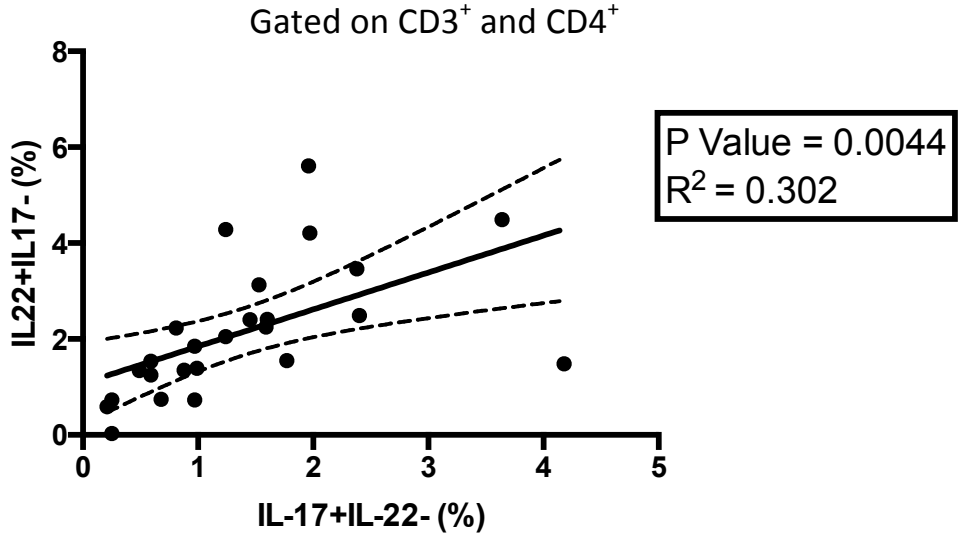
The results in figure 4.1 show an interesting difference in T helper cell subtype between healthy donors, Oesophageal cancer and gastric cancer. In both gastric cancers and oesophageal cancers there appears to be an increased number of $IL\text{-}22^+$ producing populations and Th22 populations compared to healthy donors but on statistical analysis this is not a significant difference. Also a difference is seen between $IL\text{-}17^+$, $IL\text{-}17^+IL\text{-}22^+$, Th17, and Th17/Th1 populations in gastric cancer and oesophageal cancer patients compared to healthy donor populations with a greater difference seen in gastric cancer patients for the Th17 and $IL\text{-}17^+$ populations. However again on statistical analysis this is not significant.

What can be clearly seen on graphs a, c, d, and f are 2 outliers highlighted in red. Interestingly the values seen were consistently from the same 2 individuals who when the samples were taken were known to have upper respiratory tract infections. Evidence has shown that Th17 and $IL\text{-}17^+$ producing cells are present within the respiratory tract and play an important role in host defence against RSV (Respiratory Syncytial Virus) infections (140-142). This may explain the results seen and the impact that this has on the significance of the data. Certainly when the outliers are removed from the analysis this appears to affect the significance of the results particularly for the $IL\text{-}17^+$ and Th17 subtypes (table 4.2). Another factor, which may be impacting on the significance of the data, is the n value particularly for the healthy donor population that is resulting in a type 2 error and could be improved by a higher-powered study.

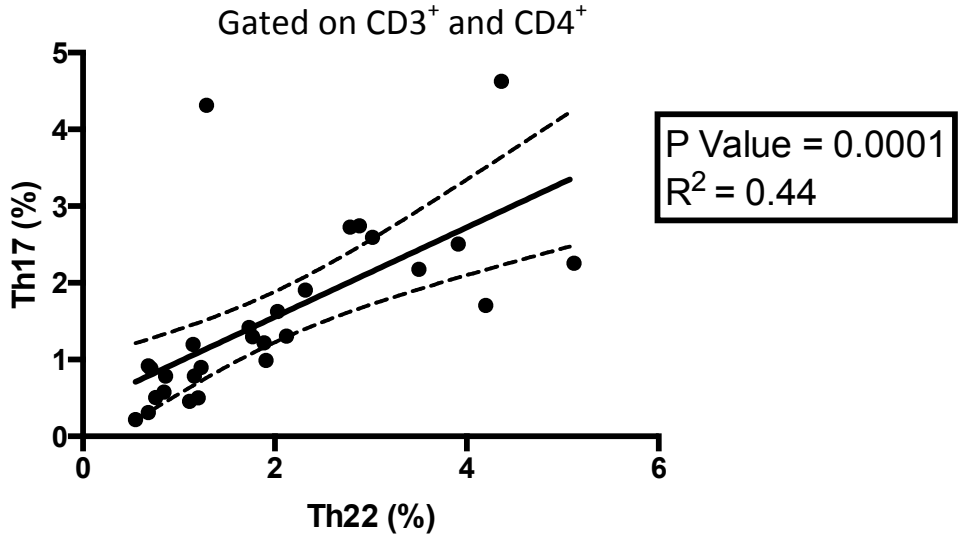
Despite these issue the data is in keeping with what has been observed in previous research and supports the theory that Th17 and Th22 cells may play a role in tumour progression of both oesophageal cancer and gastric cancer. Particularly when analysing the subtype of Th17, Th17/Th1 there also appears to be an increase in this population in both gastric cancer and oesophageal cancer however the subtype Th17/Treg appears to be possibly reduced in both cancer groups compared to healthy donors but again this was not significant therefore clinical inference cannot be made.

Figure 4.1

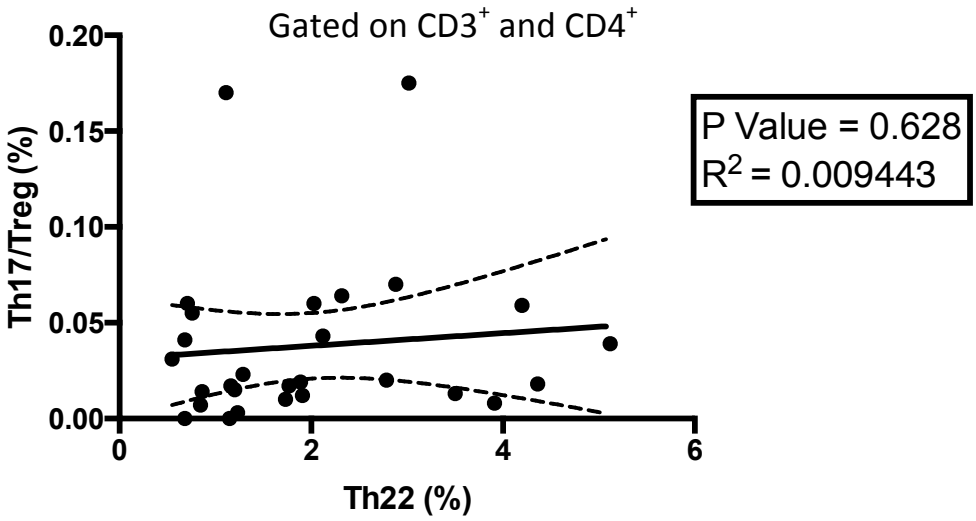
a)



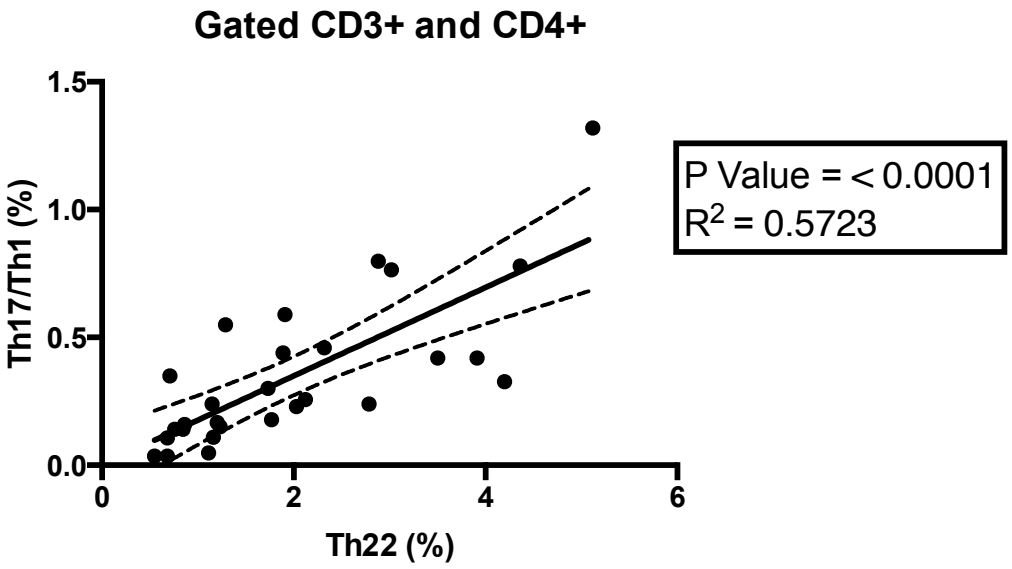
b)



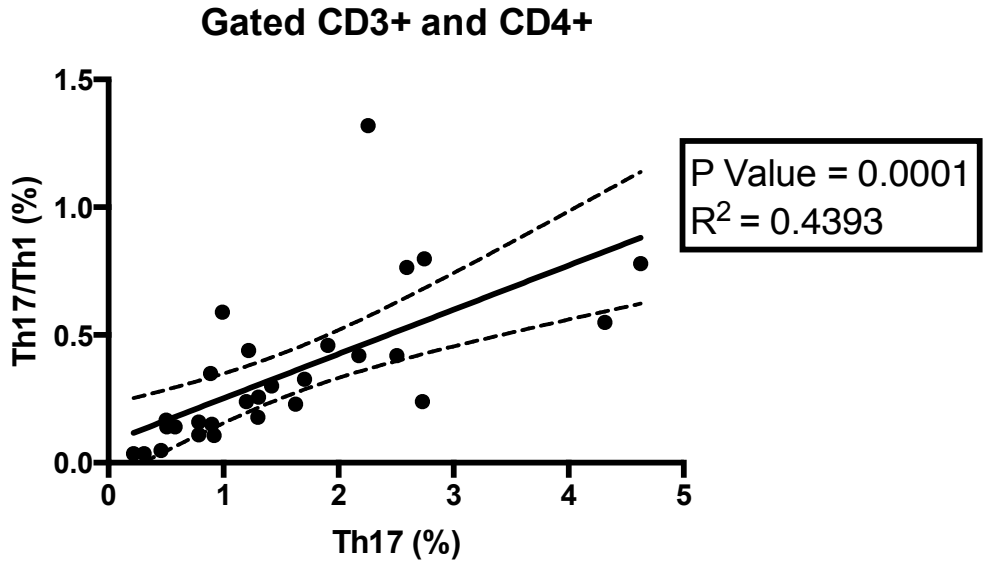
c)



d)


Gated CD3+ and CD4+
Th17/Th1 (%)
Th22 (%)
P Value = < 0.0001
R2 = 0.5723

e)


Gated CD3+ and CD4+
Th17/Th1 (%)
Th17 (%)
P Value = 0.0001
R2 = 0.4393

F.

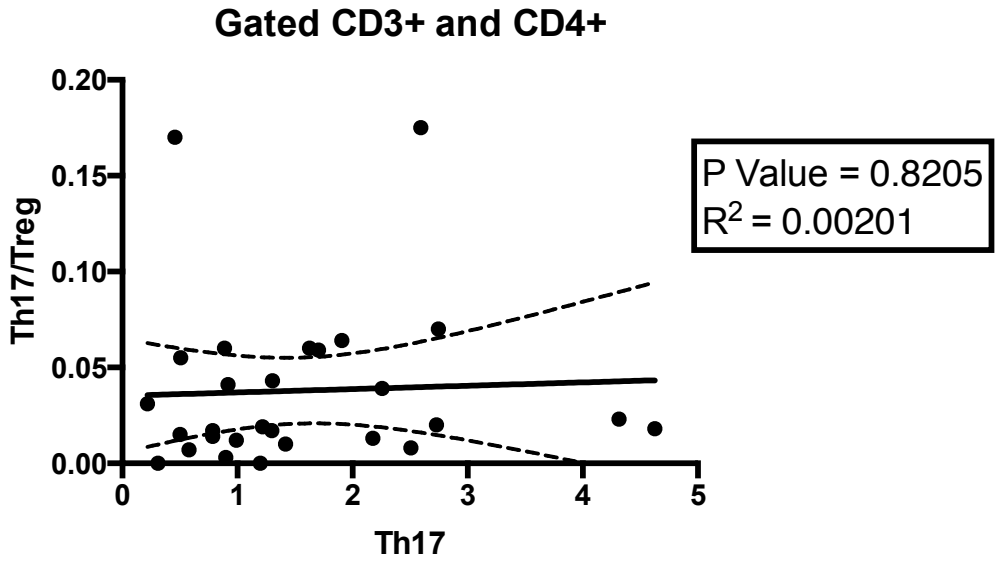

Gated CD3+ and CD4+
Th17/Treg
Th17
P Value = 0.8205
R2 = 0.00201

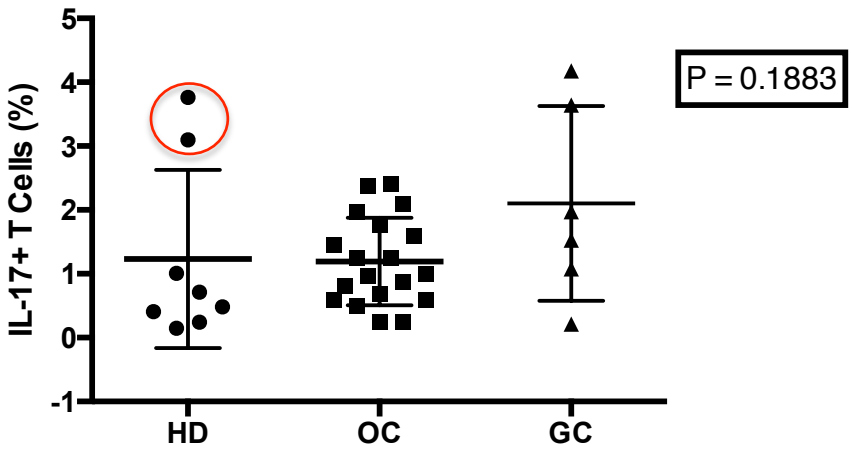
Figure 4.1 Correlations between T helper Cell Phenotypes and CD4+ subtypes defined by their cytokine production in upper GI cancer patients (n=28)

The correlations between the frequencies of $CD3^+CD4^+IL\text{-}17^+IL\text{-}22^-$ cells and $CD3^+CD4^+IL\text{-}22^+IL\text{-}17^-$ cells (a), Th17 ($CD3^+CD4^+IL\text{-}17^+IL\text{-}10^-INF\gamma^-$) and Th22 ($CD3^+CD4^+IL\text{-}22^+IL\text{-}17^-TNF\alpha^+$) (b), Th17/Treg ($CD3^+CD4^+IL\text{-}17^+IL\text{-}10^+INF\gamma^-$) and Th22 ($CD3^+CD4^+IL\text{-}22^+IL\text{-}17^-TNF\alpha^+$) (c), Th17/Th1 ($CD3^+CD4+IL\text{-}17^+IL\text{-}10^-INF\gamma^+$) and Th22 ($CD3^+CD4^+IL\text{-}22^+IL\text{-}17^-TNF\alpha^+$) (d), Th17/Th1 ($CD3^+CD4+IL\text{-}17^+IL\text{-}10^-INF\gamma^+$) and Th17 ($CD3^+CD4^+IL\text{-}17^+IL\text{-}10^-INF\gamma^-$) (e), Th17/Treg ($CD3^+CD4^+IL\text{-}17^+IL\text{-}10^+INF\gamma^-$) and Th17 (f) were analysed. Correlation coefficients were computed to assess the relationships between these T-cell subsets. Significance of correlation between two T-cell sub- sets was indicated by P value, $P<0.05$ was considered significant. Statistical analysis was performed with Graphpd FlowJo V6 using the statistical test linear regression.

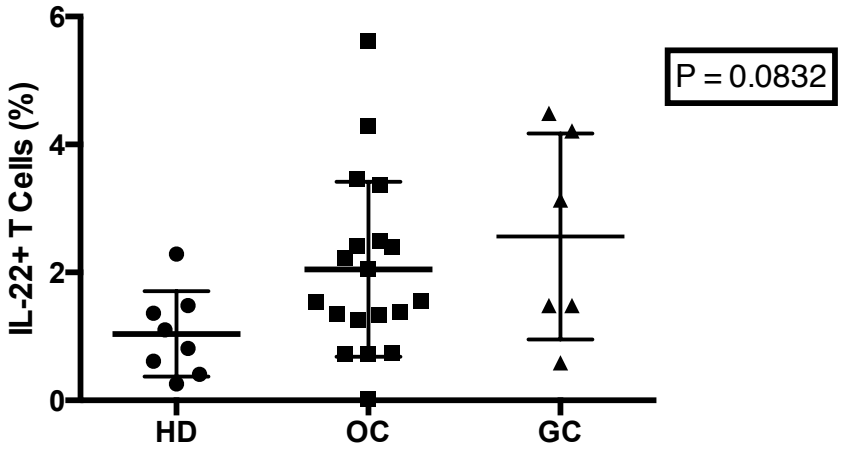
Graphs a, b, d, and e show a positive correlation between the percentages of $IL\text{-}22^+IL\text{-}17$- and IL-22-$IL\text{-}17^+$ expressing cells (p=0.004), Th17 and Th22 cells (p=0.0001), Th17/Th1 and Th22 cells (p=<0.0001), Th17/Th1 and Th17 cells (p=0.0001). No correlation was seen between Th17/Treg cells and Th22 cells (p=0.628), and Th17/Treg and Th17 cells (p=0.8205)

Figure 4.2

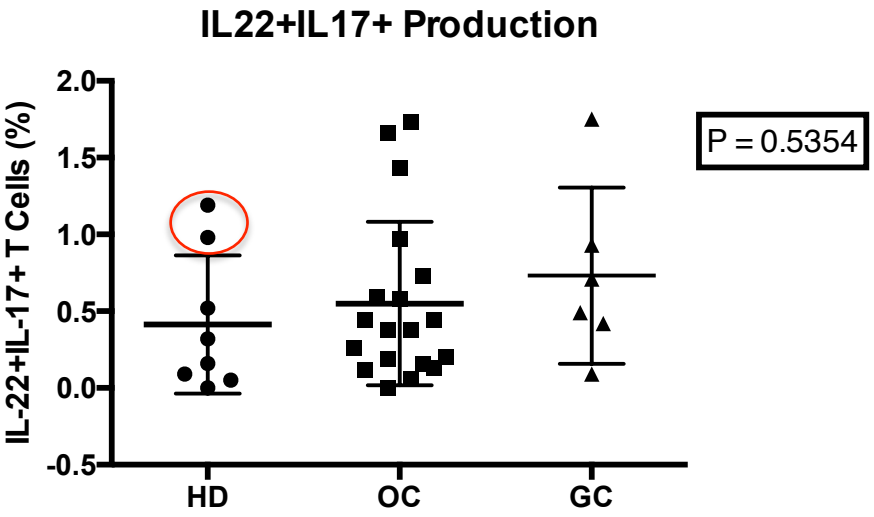
a)



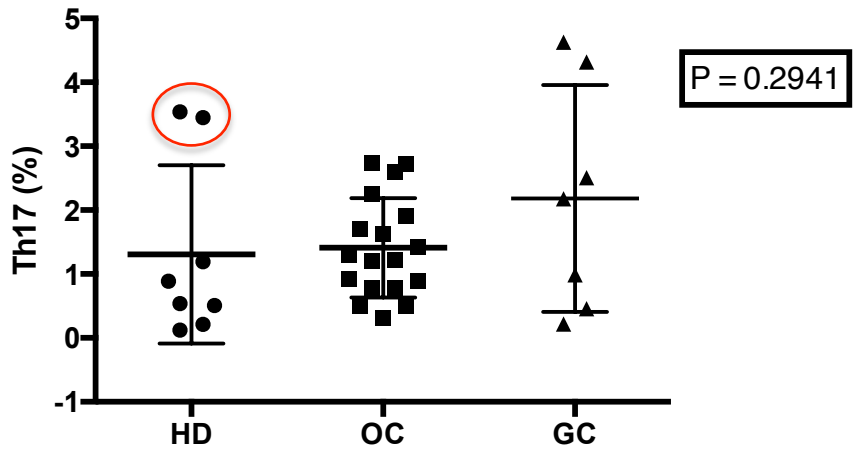
b)



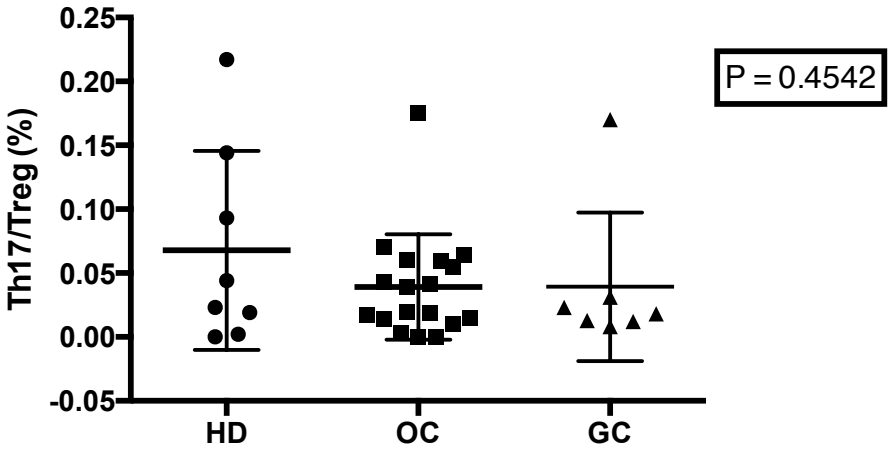
C)



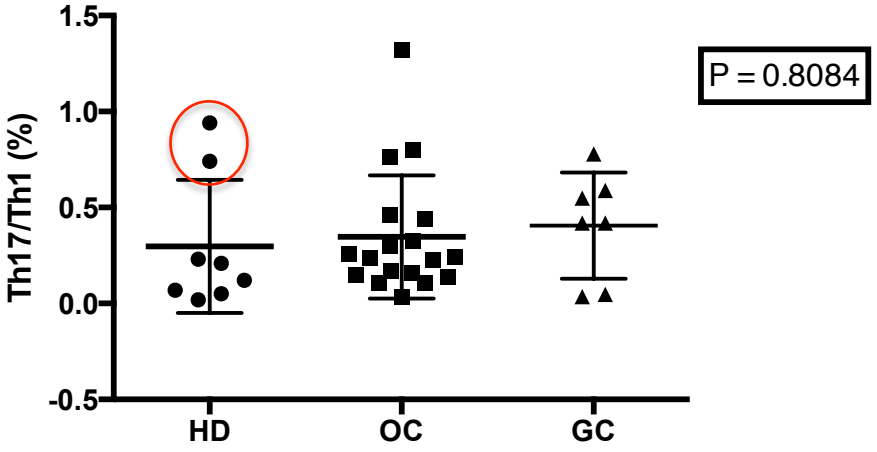
D)


Th17 (%)
5
4
3
2
1
0
-1
HD
OC
GC
P = 0.2941

E.


Th17/Treg (%)
0.25
0.20
0.15
0.10
0.05
0.00
-0.05
HD
OC
GC
P = 0.4542

f)


Th17/Th1 (%)
1.5
1.0
0.5
0.0
-0.5
HD
OC
GC
P = 0.8084

g)

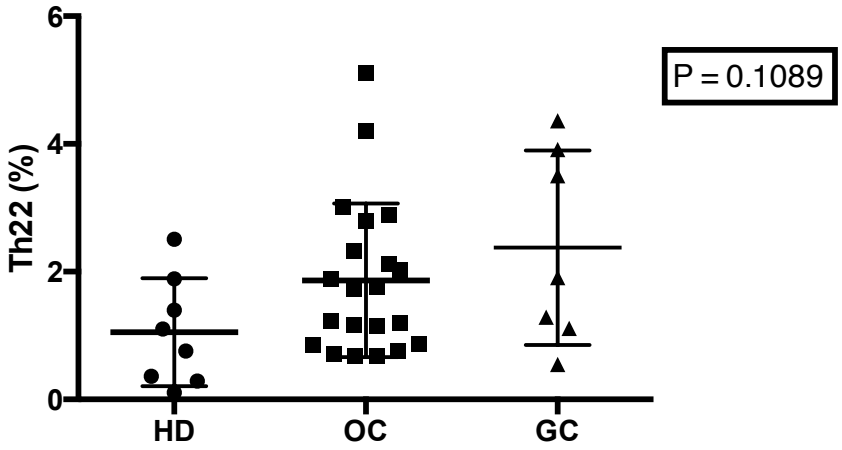


Figure 4.2 Comparison of circulating T Helper cells and CD4+ sub populations in healthy donor PBMCs (n=8), oesophageal cancer patients PBMCs (n=21) and gastric cancer patients PBMCs (n=7)

Graphs a – d show an observable difference in the percentage of IL-17^{+}, IL-22^{+} expressing cells, Th17 and Th22 cells between healthy donor patients and oesophageal/gastric cancer patients. However the results are not significant (statistical significance p=<0.05) although on observation there appears to be a potential group of outliers highlighted in red circles in graphs a,c,d, and f which could be skewing the results. The highlighted group are the same 2 patients in each of the 4 graphs. Statistical difference was calculated between the means by the statistical test ANOVA, using FlowJo software V6.

Table 4.2

	HD population **including** Outliers P-Value	HD population **excluding** outliers P-Value
IL-17^{+}	0.1883	0.0112
IL-22^{+}IL-17^{+}	0.5354	0.1687
Th17	0.2941	0.0320
Th17/Th1	0.8084	0.1627

Table 4.2 Comparison of P values calculated using ANOVA statistical test

The table shows the difference between the calculated P-values of the different subtype populations when the outliers are removed from the healthy donor population. When outliers are excluded there is statistical significant difference (p = <0.05) in the percentages of IL-17^{+} cells (p= 0.0112) and Th17 cells (p=0.032) observed in healthy donor PBMCs and both Gastric and Oesophageal cancer patient PBMCs.

4.5 Experiment to compare circulating T helper cell phenotypes in PBMCs between stages of oesophageal cancer and also stages of gastric cancer

The aim of this experiment was to identify if the observed increased subtype populations of T Helper cells identified previously in patients with gastric and oesophageal cancers is associated with clinical presentation. Lui et al in their study of gastric cancer found that there was a significant difference in their defined Th22 populations and tumour stage and that as tumour stage increased there was a positive correlation with Th22 percentages (114). They also found that same for Th17 defined populations but not Th1 populations. Similar results were seen by Chen et al in oesophageal cancer with their defined Th17 population and tumour stage (133). However the limitations of both of these studies included their definition of the Th17 population. The cytokine profile of both studies was vague and could include other IL-17^{+} producing populations. This experiment aimed to more clearly identified the subtype populations of T helper cells by a more specific cytokine profile and correlate these populations to tumour stage.

The results analysed were taken from the same previous intracellular flow cytoemtry procedure performed on the previous patient samples. The results were collated in Graphpad Prism 6.0 and analysed using Students T Test to assess for clinical significance.

4.5.1 Results from experiment to compare circulating T helper cell phenotypes in PBMCs between stages of oesophageal cancer and also stages of gastric cancer

The results shown in figures 4.3, 4.3 and 4.5 highlight some interesting differences between oesophageal cancer and gastric cancer when comparing different T helper subtype populations and tumour stage. On review of the results of the patients with oesophageal cancer the results are in keeping with other previous studies. Figure 4.3 a), c), d), f) and g) show an observable difference between the percentages of IL-22+, IL-22+, IL-17+ expressing cells, Th22, Th17/Treg and Th17/Th1 cell percentages in PBMCs and tumour grade suggesting a positive correlation with worsening tumour grade. However the results were not significant. There was no difference observed for IL-17 expressing cells and Th17 cells, and tumour grade. A clear difference was seen in populations of T helper cells producing IL-22^{+}. In figure 4.3 a), b) and d) the subtype populations IL-22^{+}, IL-17^{+}IL-22^{+} and Th22 can clearly be seen to be increasing in population numbers as tumour stage increases. However the results do not show a significant difference.

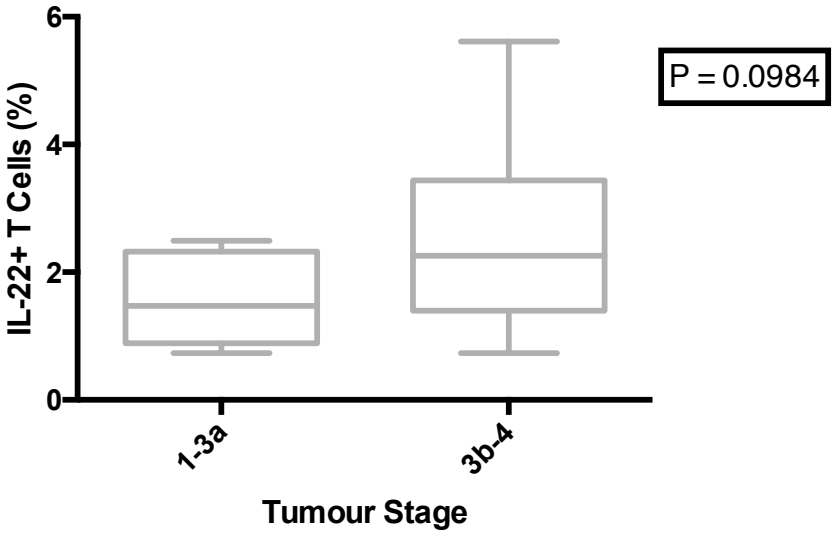
Interestingly there appears to be no correlation between IL-17^{+} and Th17 subtype populations and tumour grade although the more specific subtype populations Th17/Th1 and Th17/Treg do appear to show a clear positive correlation between population numbers and tumour stage but again the p values of p=0.15 and p=0.12 respectively are not significant. This variation in the subtype populations particularly of Th17 could explain the variability in the literature of the benefit or harm of these populations and that the plastic nature of Th17 may results in different functions, either regulatory or effector, depending upon the microenvironment of the tumour.

Previous evidence suggested that in gastric cancer there was a positive correlation between tumour stage and both Th17 and Th22 populations however the results from this experiment show the converse. Figure 4.4 a), b) and e) show a significant difference in the percentage of IL-22+, IL-17+ expressing cells and Th17 cells between early stage gastric cancers (1-3a) and later stage gastric cancers (3b-4) with a similar trend seen for IL-17+IL-22+ expressing helper cells and Th22 cells suggesting a negative correlation between percentages of these cells and tumour grade. Figures 4.4 a), b) and e) all show that as tumour stage increases there is a significant reduction in IL-22^{+}, IL-17^{+} and Th17 populations ($p = <0.05$). Also a clear reduction is seen in IL-22^{+}IL-17^{+}, Th22, and Th17/Th1 subtype populations as tumour stage increases, although not significant. However there was a positive correlation between Th17/Treg populations and tumour stage but again this was not significant (p=0.154).

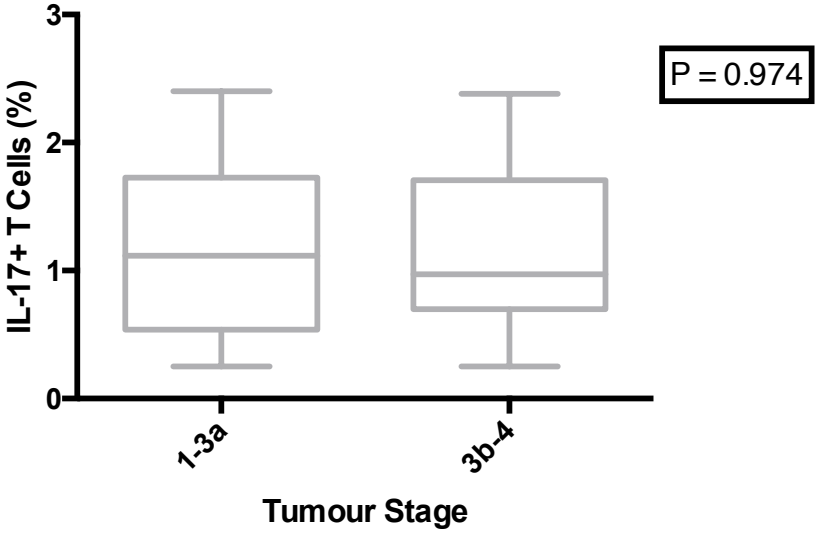
Figure 4.5 (a to d) highlight the difference of the immunological profile of these two cancers. Although they follow a similar pathophysiological process the role that the immune system plays appears to be very different. Particularly Th22 cells may play a very different role in oesophageal cancer compared to gastric cancer (figure 4.5a). Also Th17 cells may play a far more significant role in gastric cancer than oesophageal cancer (figure 4.5b).

Figure 4.3

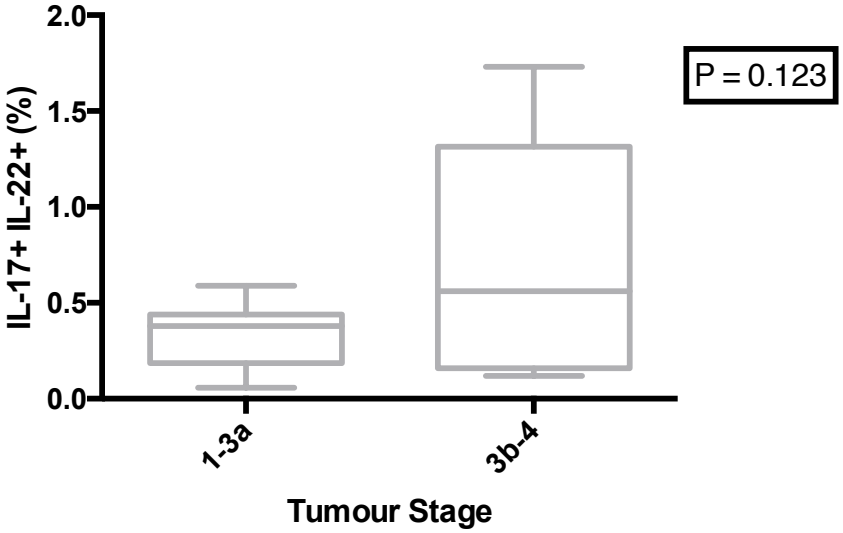
a)



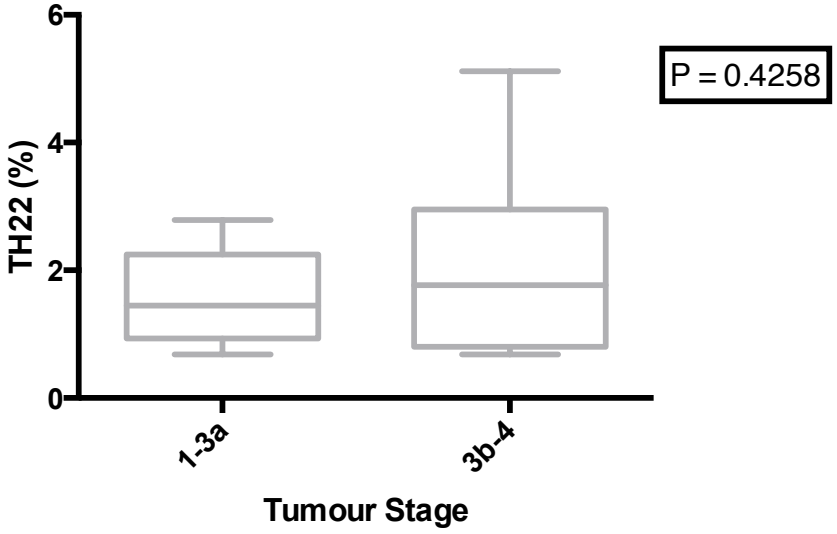
b)



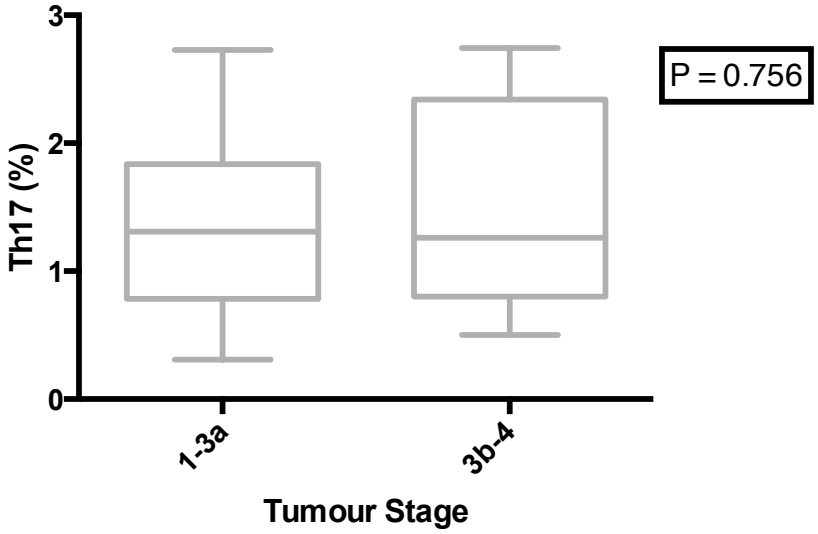
c)



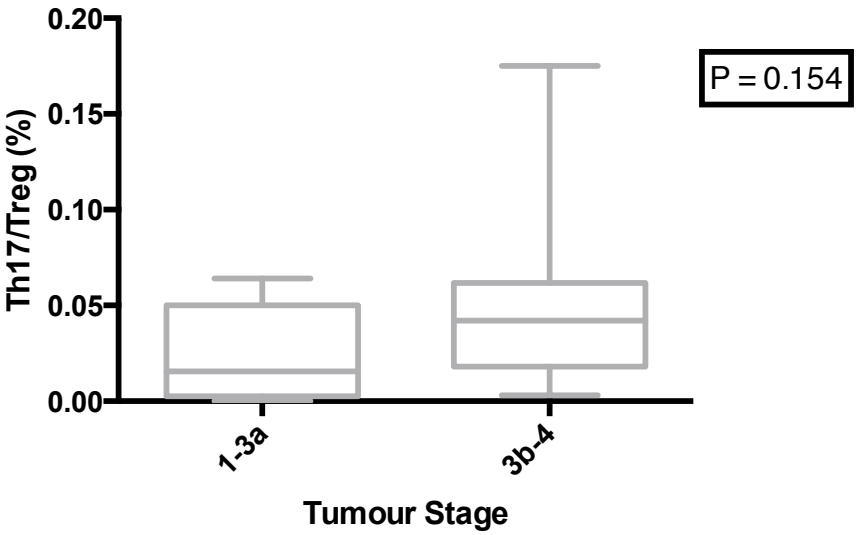
d)


P = 0.4258
TH22 (%)
6
4
2
0
1-3a
3b-4
Tumour Stage

e)


P = 0.756
Th17 (%)
3
2
1
0
1-3a
3b-4
Tumour Stage

f)


P = 0.154
Th17/Treg (%)
0.20
0.15
0.10
0.05
0.00
1-3a
3b-4
Tumour Stage

g)

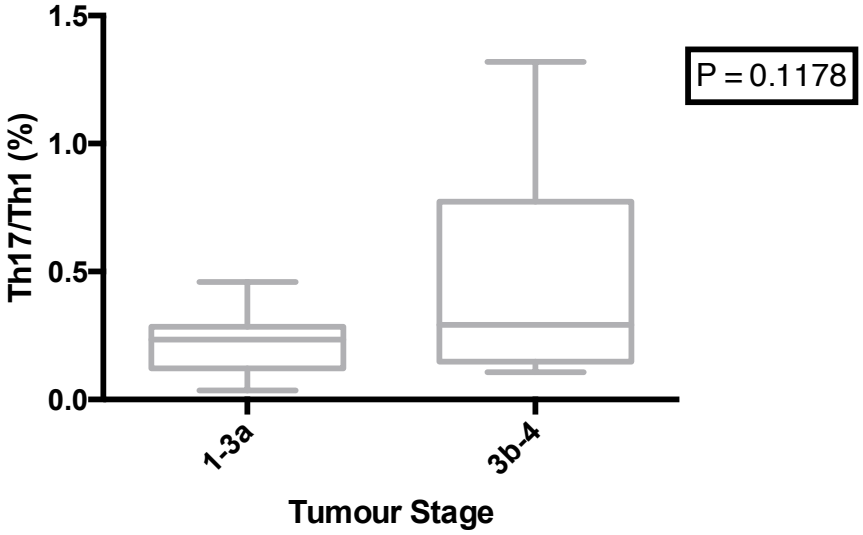
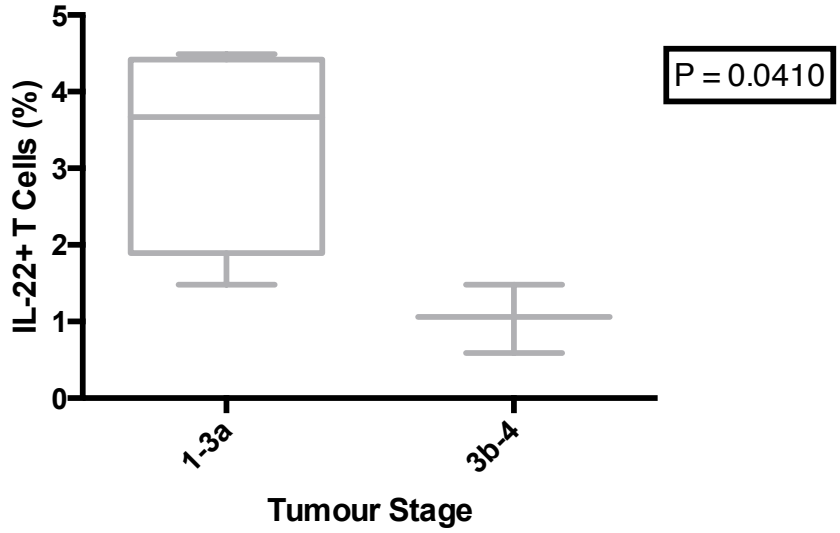


Figure 4.3 Comparisons of circulating T helper cell phenotypes and CD4+ subtypes based on cytokine production between stages 1-3a and 3b-4 of <u>Oesophageal</u> cancer patients (n=21)

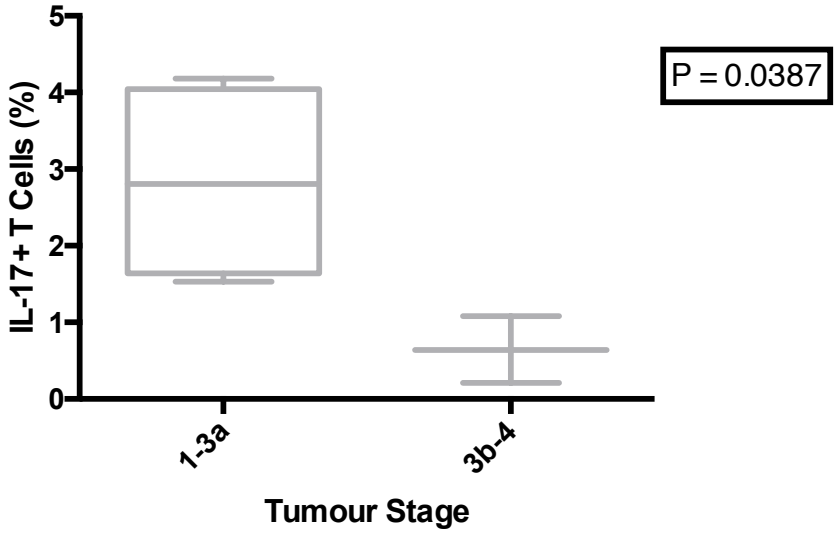
Graphs a – g show the comparison of different T helper cells subtype populations and tumour stage for Oesophageal cancer patients only. Tumour stage was grouped in Stage 1 to 3a and then 3b to 4 using the TNM classification system (tumour, node, metastasis). Different subtype populations of T helper cells were the compared against the grouped tumour stages. The subtype populations were defined by the following cytokine profiles a) IL-17^{+} (CD3^{+}CD4^{+}IL-17^{+}IL-22^{-}), b) IL-22^{+} (CD3^{+}CD4^{+}IL-22^{+}IL-17^{-}), c) IL-17^{+}IL-22^{+} (CD3^{+}CD4^{+}IL-17^{+}IL-22^{+}), d) Th22 (CD3^{+}CD4^{+}IL-22^{+}IL-17^{-} TNFα^{+}), e) Th17 (CD3^{+}CD4^{+}IL-17^{+}IL-10^{-}INFγ^{-}), f) Th17/Treg (CD3^{+}CD4^{+}IL-17^{+}IL-10^{+}INFγ^{-}), g) Th17/Th1 (CD3^{+}CD4^{+}IL-17^{+}IL-10^{-}INFγ^{+}). Significance of correlation between two T-cell sub- sets was indicated by the P value, P<0.05 was considered significant. Statistical difference was calculated by the statistical test ANOVA, using FlowJo software V6.

Figure 4.4

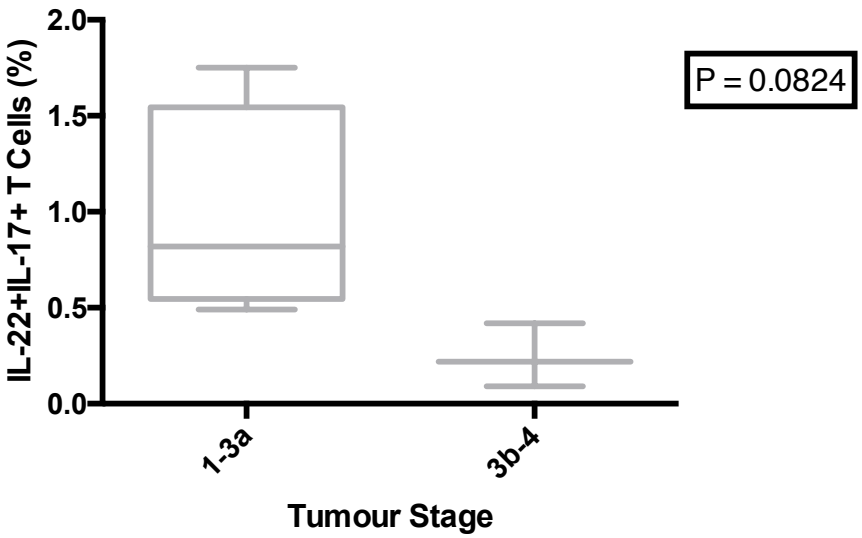
a)



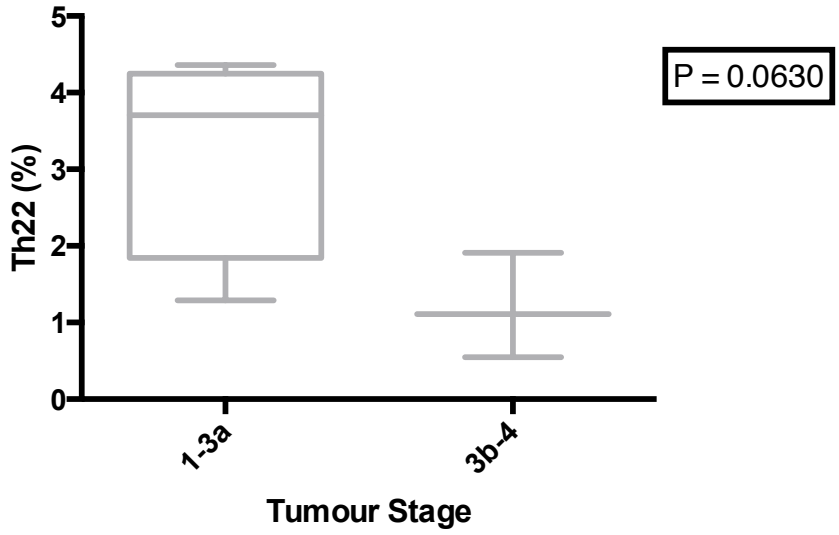
b)



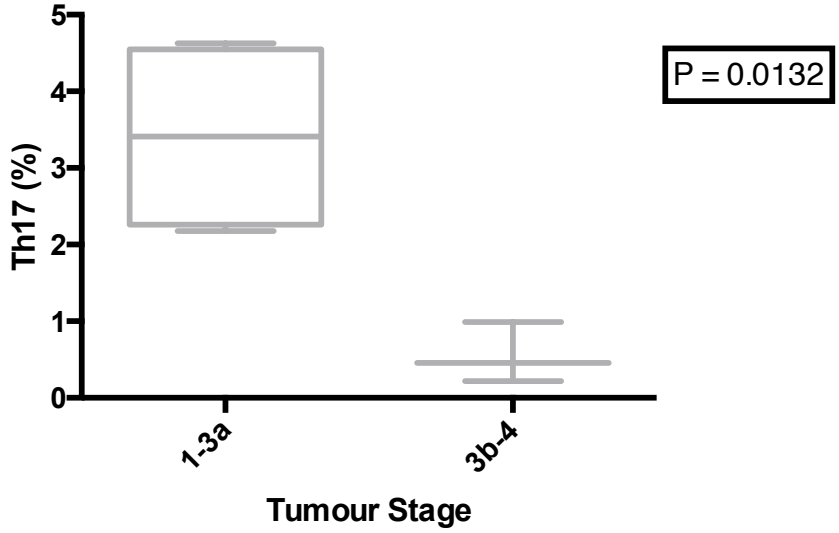
c)



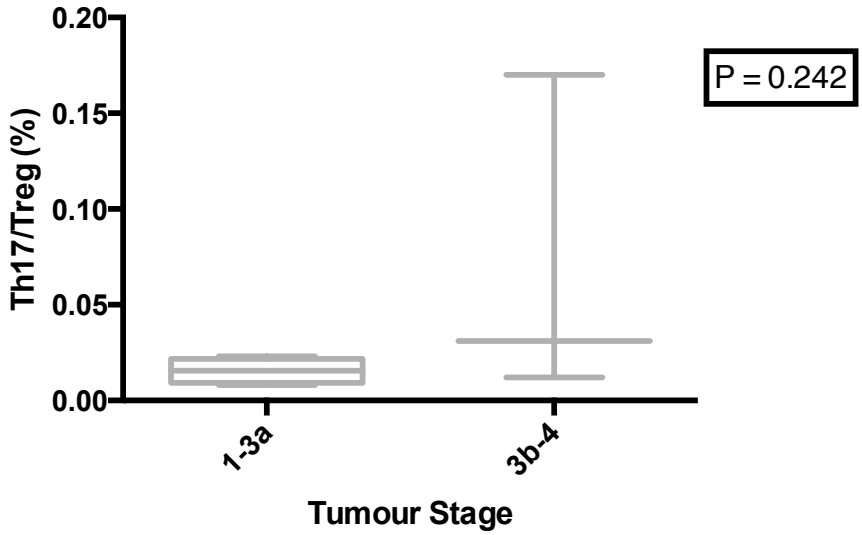
d)


P = 0.0630
Th22 (%)
5
4
3
2
1
0
1-3a
3b-4
Tumour Stage

e)


P = 0.0132
Th17 (%)
5
4
3
2
1
0
1-3a
3b-4
Tumour Stage

f)


P = 0.242
Th17/Treg (%)
0.20
0.15
0.10
0.05
0.00
1-3a
3b-4
Tumour Stage

g)

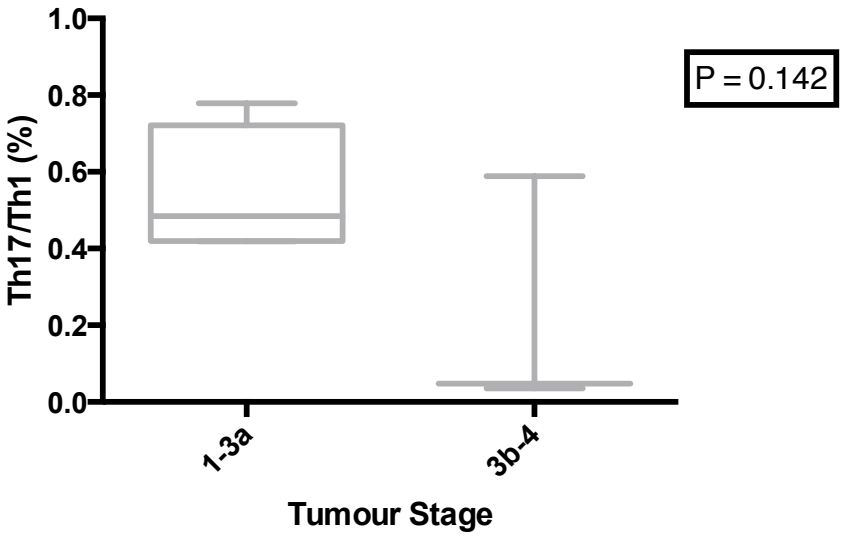
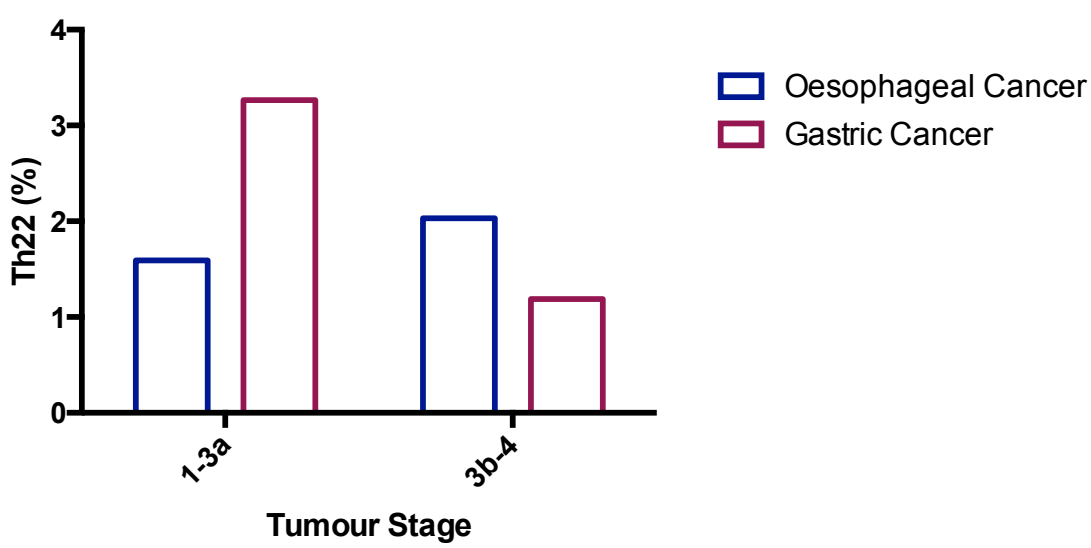


Figure 4.4 Comparisons circulating T helper cell phenotypes and CD4+ subtypes based on cytokine production between stages 1-3a and 3b-4 of gastric cancer patients (n=7)

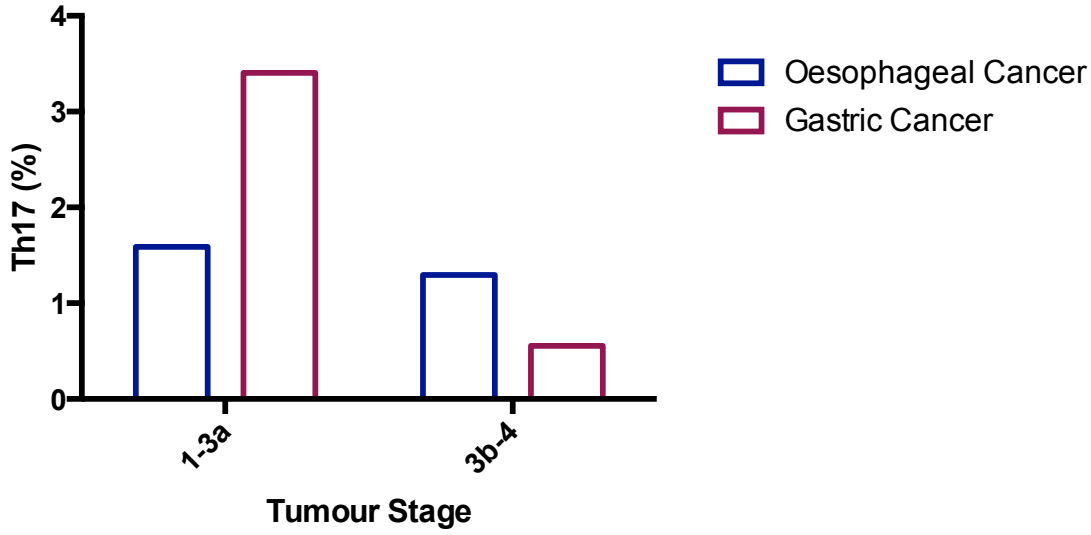
Graphs a – g show the comparison of different T helper cells subtype populations and tumour stage for Gastric cancer patients only. Tumour stage was grouped in Stage 1 to 3a and then 3b to 4 using the TNM classification system (tumour, node, metastasis). Different subtype populations of T helper cells were the compared against the grouped tumour stages. The subtype populations were defined by the following cytokine profiles a) IL-17^{+} (CD3^{+}CD4^{+}IL-17^{+}IL-22^{-}), b) IL-22^{+} (CD3^{+}CD4^{+}IL-22^{+}IL-17^{-}), c) IL-17^{+}IL-22^{+} (CD3^{+}CD4^{+}IL-17^{+}IL-22^{+}), d) Th22 (CD3^{+}CD4^{+}IL-22^{+}IL-17^{-} TNFα^{+}), e) Th17 (CD3^{+}CD4^{+}IL-17^{+}IL-10^{-}INFγ^{-}), f) Th17/Treg (CD3^{+}CD4^{+}IL-17^{+}IL-10^{+}INFγ^{-}), g) Th17/Th1 (CD3^{+}CD4^{+}IL-17^{+}IL-10^{-}INFγ^{+}). Significance of correlation between two T-cell sub- sets was indicated by the P value, P<0.05 was considered significant. Statistical difference was calculated by the statistical test ANOVA, using FlowJo software V6.

Figure 4.5

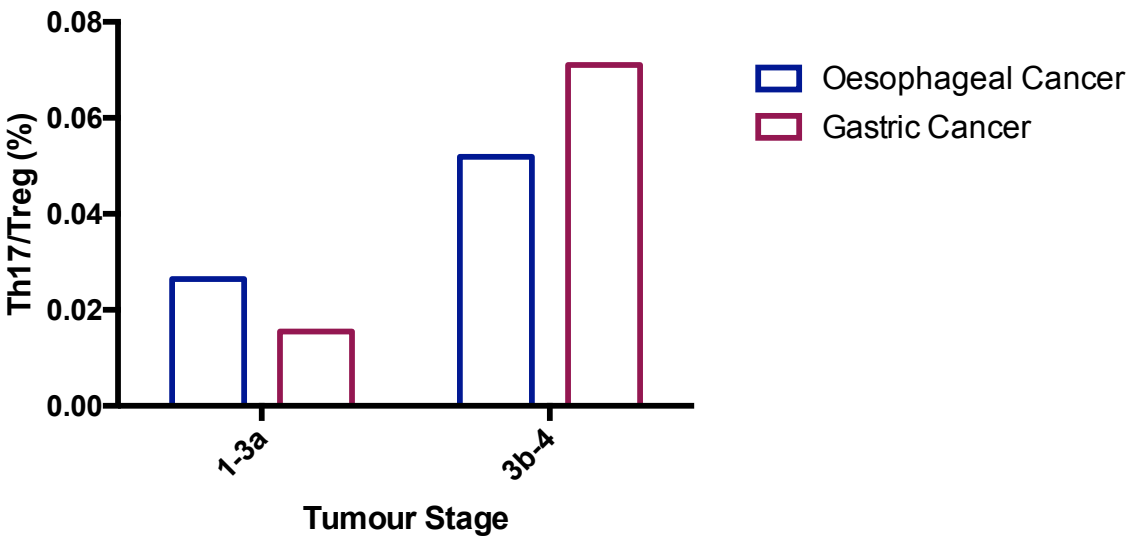
a)



b)



c)



d)

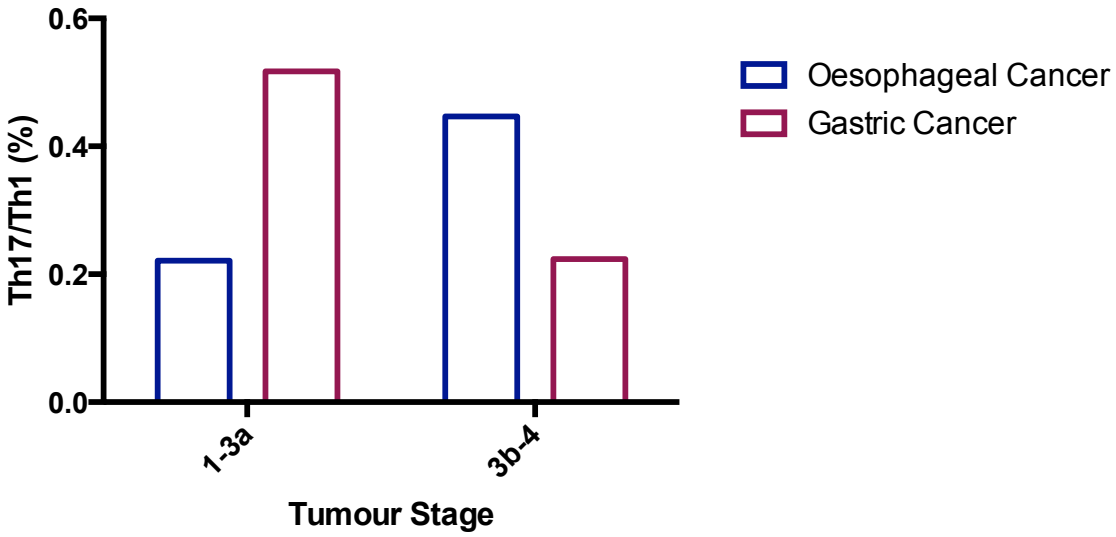


Figure 4.5 Graphs showing the Comparisons of Th17, Th17/Treg, Th17/Th1 and Th22 T helper cell phenotypes with tumour grade for both gastric (n=7) and oesophageal cancer (n=21)

Different subtype populations of T helper cells were the compared against the grouped tumour stages for both Gastric cancer and Oesophageal cancer. The subtype populations were defined by the following cytokine profiles a) IL-17^{+} (CD3^{+}CD4^{+}IL-17^{+}IL-22^{-}), b) IL-22^{+} (CD3^{+}CD4^{+}IL-22^{+}IL-17^{-}), c) IL-17^{+}IL-22^{+} (CD3^{+}CD4^{+}IL-17^{+}IL-22^{+}), d) Th22 (CD3^{+}CD4^{+}IL-22^{+}IL-17^{-}TNFα^{+}), e) Th17 (CD3^{+}CD4^{+}IL-17^{+}IL-10^{-}INFγ^{-}), f) Th17/Treg (CD3^{+}CD4^{+}IL-17^{+}IL-10^{+}INFγ^{-}), g) Th17/Th1 (CD3^{+}CD4^{+}IL-17^{+}IL-10^{-} INFγ^{+}). This thesis went on to further examine if this difference in Th22 and Th17 cells was associated with disease progression and could be a potential biomarker of disease progression. In patients with oesophageal cancer there was an observed difference in IL-22^{+} T helper cells and tumour stage with levels increasing in PBMCs with increasing disease stage (stage 1a-3a mean = 1.6%, stage 3b-4 mean = 2.5% (p=0.01)). Similar differences were observed in IL-17^{+}IL-22^{+} T helper cells (stage 1-3a mean = 0.33, stage 3b-4 mean = 0.7 (p = 0.1)), Th22 cells (stage 1-3a mean = 1.6, stage 3b-4 mean = 2.1 (p = 0.43)), Th17/Th1 (stage 1-3a mean = 0.22, stage 3b-4 mean = 0.45 (p = 0.12)), Th17/Treg (stage 1-3a mean = 0.22, stage 3b-4 mean = 0.45 (p = 0.12)).

Chapter 5 Discussion and Future Work

5.1 Aims

In recent years, the incidence of both oesophageal cancer and gastric cancer in the UK has significantly increased despite the reduction of risk factors such as smoking rates and improvement in preventative medicine with the introduction of proton pump inhibitors. The pathophysiological process and, in particular, the role of the immune system in upper gastrointestinal adenocarcinomas is still relatively poorly understood. There have been several hypotheses presented to explain the sequale of metaplasia-dysplasia-adenocarcinoma and citing the potential role of T helper cells in this process (12, 13). Evidence from other areas looking at the role of the immune system in cancer development have suggested a role for T cells in tumour defence through 'immunoediting' particular the role of T helper cells (82, 84).

The aims of this project were to improve detection and identification of certain phenotypes of T helper cells in PBMCs of both healthy donors and oesophageal and gastric cancer patients using flow cytometry. This would then be used to help identify which phenotypes may play in role in the development of these cancers.

5.2 Optimising Panel Design for Flow cytometry to identify Th17 and Th22 cells in PBMCs

In order to be able to identify the phenotype of T Helper cells Th17 and Th22 from PBMCs with confidence, it was first necessary to design a methodology to identify these cell subsets based on their surface markers and pattern of intracellular cytokine production. Flow cytometry was chosen as the optimal methodology since this enabled the detection of multiple surface markers and intracellular cytokines simultaneously. The challenges this methodology presented included the potential bias in using frozen PBMCs or fresh PBMCs for analysis, the activation required to detect the intracellular cytokines and the impact this had on surface marker expression, and the potential bias of using multiple fluorochromes to identify these phenotypes that could cause false positives through spill over into other channels of detection.

The first step was to assess the effect freezing had upon the PBMC populations and particularly the detection of the key cytokines IL-17^{+} and IL-22^{+}, which were central to the successful

identification of the Th22 and Th17 phenotypes. The ability to freeze samples was important due to the issues surrounding the irregular collection of patient samples and the advantage of batch assays to reduce inter-sample variation. Additionally, identifying the period of mitogenic T cell activation to enable the optimal identification of these cytokines was critical. However, the challenge with prolonged activation was significant cell death due to Brefeldin A cell toxicity. Brefeldin A was essential for intracellular cytokine detection as it inhibited intracellular protein transfer. Therefore, an experiment was designed to identify what the ideal balance would be for cell activation and the effects freezing would have on cytokine detection. As part of this experiment the development of the fluorochrome panel was important to be able to confidently and accurately identify the cytokines IL-17 and IL-22, as well as markers of activation INFγ and IL-2, and surface markers CD4 and CD8 and thus define the T helper phenotype Th17 and Th22. The design of the panel was challenging as it became important to choose a range of fluorochromes that covered different lasers on filters on the flow cytometer thus reducing potential sources of bias. The results highlighted some issues with the choice of fluorochromes. In trying to choose a wide range of lasers and filters it resulted in the selection of certain fluorochromes that were not particularly bright for the smallest potential population of cytokines. The detection of the IL-17 cytokines was lower than expected and it was hypothesised that the FITC fluorochrome chosen, which is known to be a dim fluorochrome, was not bright enough to enable the clear identification of this small population. The results from this experiment suggested that there was an impact on the detection of the cytokines IL-17 and IL-22; however, it was felt that the ability to batch freeze the samples for analysis outweighed the reduction in detection of IL-22. Also the 16 hour period of activation was optimal for the detection of both cytokines, which was significantly improved by using a live/dead discriminator along with a greater number of cells for analysis then the expected number of cell deaths from a longer duration of activation would not overall impact on the results.

A subsequent experiment optimised the flow cytometry panel further to improve the detection of both IL-17 and IL-22. In addition, two further cytokines were included into the panel to refine the characterisation of the Th22 and Th17 phenotypes by a broader cytokine pattern. Evidence suggests that Th17 may have an element of plasticity and thus its role in cancer progression may differ dependent upon the cytokine environment (57). Therefore it was felt that adding in the cytokines IL-10 and TNFα would help more clearly define the role of Th17. To improve IL-17 detection, the brighter fluorochrome BV605 was chosen. In the first panel design Fluorochrome BV421 was chosen for the surface marker CD4. As CD4 is expressed at high level, it was felt that a bright fluorochrome was not needed for this surface marker and FITC was chosen instead. The

final panel design for this experiment seen in figure 3.6 highlights the importance of the choice of fluorochromes to make sure a widest range of lasers and filters are chosen and thus reducing the risk of false positives. As two new fluorochromes were added to the panel, the experiment for optimal activation period to detect Il-10 and TNFα was also repeated with this new panel design as well as the effects of freezing on the detection of these. The results showed that the freezing process had a small but not significant an effect on the cytokine expression. The results also showed that 16-hour activation was an adequate period of activation to detect the key cytokines IL-17, IL-22, IL-10, TNFα and INFγ. However, in this experiment, changing the fluorochrome for CD4 from BV421 to FITC reduced the ability to accurately gate on this population. Figure 3.7 (d) clearly showed that the population of $CD4^+$ cells became very difficult to define and thus gate on accurately and be able to define this population as T helper cells. It appeared that a longer duration of activation that improved cytokine expression was associated with a reduction in CD4 expression. It was also hypothesised that the permeabilisation process required to detect intracellular cytokines may also effect the expression of CD4 on the surface of the cell.

A further experiment was designed to investigate the effect that both permeabilisation and activation had upon the expression of CD4 surface marker following 16 hours of activation. It was decided to use the same panel for the flow cytometry as in the previous experiment and 16 hours activation as the results from the previous experiment suggested this was an optimal duration of activation for cytokine detection. The experiment showed that both activation and permeabilisation reduced CD4 relative surface expression that adversely affected the ability to define the $CD4^+$ population. It was felt that to try and overcome this issue would be difficult as both permeabilisation and activation were necessary to detect the appropriate cytokines. It was decided to try to use a very bright fluorochrome, which may help identify this population more easily. Therefore, a further experiment was designed to compare the CD4 detection using the dimmer fluorochrome FITC and the brighter fluorochrome AF-488. AF-488 was chosen as the fluorochrome alternative as it used the same laser and filter as FITC and therefore have little impact on the rest of the panel used in the previous experiment. The results clearly showed that the ability to identify the $CD4^+$ population was significantly increased using AF-488 compared to FITC but the $CD8^+$ population remained relatively the same. It was decided that AF-488 would be used for CD4 surface marker detection in all further experiments.

Carrying on from these previous experiments, the next experiment was designed to optimise the detection of Th17 and Th22 phenotypes in PBMCs. The panel used the AF-488 as the fluorochrome marker for CD4, BV605 for IL-17 but also used a different fluorochrome for

live/dead cell detection. Due to the increasing number of fluorochromes being used in the panel, it was felt that for improved accuracy of identifying the $CD4^+$ cells that a fluorochrome should be used to detect CD3 surface marker. This would hopefully remove any false positives of non T-helper cells expressing $CD4^+$ being included within the $CD4^+$ gate which could skew the results. By gating on the $CD3^+$ population, then the $CD4^+$ population it would help in more accurately defining these cells as T helper cells and thus in defining Th17 and Th22 cells. Also to allow more accurate gating a different live/dead discriminator Zombie UV was used. This particular live/dead discriminator used a completely different laser to all other fluorochromes and it was felt this would reduce any false positives. A final additional fluorochrome was added for the detection of RORyt. Increasing evidence suggested that this transcription factor could be a marker for Th17 cells and thus aid in the accuracy of defining this population. The results from this experiment showed clear improvement in the detection of the $CD4^+$ population. Also the detection rates for all the cytokines examined for were as expected for healthy donors. This final ten-colour flow cytometry panel was then used in all subsequent experiments on patient and healthy donor PBMCs.

5.3 Comparison of T Cell phenotypes in Upper GI cancers with healthy donors

With an optimised flow panel design that could clearly identify Th17 and Th22 cells in PBMCs from healthy donors, this was then used to examine the relative frequency of these cells of upper GI cancer patients. Previous studies suggested a direct relationship between IL-22 and IL-17 producing T helper cells and also INFγ producing cells in gastric cancers (113). Zhuang et al described a significant positive correlation between these cytokine producing cells which became increasingly significant in lymphocytes isolated directly form the tumours. Liu et al also found a significant positive correlation between IL-17 producing helper cells and $IL\text{-}22^+$ producing cells; however, they did not find a significant correlation between INFγ producing T helper cells and either $IL\text{-}22^+$ producing cells or $IL\text{-}17^+$ producing T helper cells (114). The results this thesis found a similar significant positive correlation between IL-17+ T helper cells ($CD3^+CD4^+IL\text{-}17^+IL\text{-}22^-$) and IL-22+ T helper cells ($CD3^+CD4^+IL\text{-}17^-IL\text{-}22^+$) for gastric and oesophageal cancer patients ($p=0.0044$). However, this thesis also found a positive correlation for more specifically defined Th17 ($CD3^+CD4^+IL\text{-}17^+IL\text{-}10^-INF\gamma^-$) and Th22 ($CD3^+CD4^+IL\text{-}22^+IL\text{-}17^-TNF\alpha^+$) cells ($p=0.0001$, $r2 = 0.44$). This thesis also found a positive correlation between IL-17 producing cells which also

produced INFγ, that this thesis defined as Th17/Th1 ($CD3^+CD4$+$IL\text{-}17^+IL\text{-}10^-INF\gamma^+$), and both Th22 and Th17 cells. However, no correlation was found between those cells expressing the regulatory cytokine IL-10, which was defined as Th17/Treg ($CD3^+CD4^+IL\text{-}17^+IL\text{-}10^+INF\gamma^-$), and Th17 cells and Th22 cells. Lui et al suggested a possible explanation for this observed correlation based on their findings that serum cytokine IL-23 was found to be significantly higher in gastric cancer patients than healthy donors and that this may have a role in simultaneous Th17 and Th22 differentiation contributing towards the positive correlation observed (114) . Current evidence suggests that the role of IL-23 in Th17 and Th22 differentiation, along with other cytokines, may be due to inhibition of T-bet and FoxP3 transcription factor expression and induction of IL-22 production (143). This thesis supports the hypothesis of IL-23 being an important cytokine in upper GI cancers as evidence suggests that naıve $CD4^+$ T cells cultured in only IL-6 and TGF-b and without IL-23 secrete IL-17A but also produce high levels of IL-10 (56). McGeachy et al found that cells stimulated with IL-23 did not produce IL-10 but maintained production of IL-17 suggesting that IL-23 was important in the production of IL-17. They also found that as long as there was a maintained environment of IL-6 and TGF-b, IL-10 would be produced. This is supported by the results in this thesis, which found no correlation between IL-10 producing Th17 cells (Th17/Treg ($CD3^+CD4^+IL\text{-}17^+IL\text{-}10^+INF\gamma^-$)), and non IL-10 secreting Th22 ($CD3^+CD4^+IL\text{-}22^+IL\text{-}17^-TNF\alpha^+$) or Th17 ($CD3^+CD4^+IL\text{-}17^+IL\text{-}10^-INF\gamma^-$) cells. These results propose that a tumour cytokine microenvironment with high levels of the pro inflammatory cytokines IL-22 and IL-17 and less regulatory cytokines IL-10 may be the result of high levels of IL-23 and not TGF-B or IL-6.

After showing there was a relationship between Th22 and Th17 cells in upper GI cancers, this thesis went onto identify whether there was a significant difference between in the relative frequency of these cells between healthy donors, gastric cancer and oesophageal cancer patients. The results observed showed a small but not significant difference between the upper GI cancers and healthy donors. Both gastric and oesophageal cancers when examined separately were showed higher percentages of $IL\text{-}17^+$ T helper cells, $IL\text{-}22^+$ T helper cells, $IL\text{-}22^+IL\text{-}17^+$ T helper cells, Th17 ($CD3^+CD4^+IL\text{-}17^+IL\text{-}10^-INF\gamma^-$), Th22 ($CD3^+CD4^+IL\text{-}22^+IL\text{-}17^-TNF\alpha^+$) and Th17/Th1 ($CD3^+CD4^+IL\text{-}17^+IL\text{-}10^-INF\gamma^+$) but not Th17/Treg ($CD3^+CD4^+IL\text{-}17^+IL\text{-}10^+INF\gamma^-$) when compared with healthy donors. The lack of statistical significance was felt to be potentially down to 2 reasons;

1) On data analyses, there were 2 outliers identified in each of the comparisons in Figure 4.3 a,c,d and f. In each of these graphs the 2 values highlighted in red were the same 2 patients who at the time had upper respiratory tract infections. On exclusion of these,

healthy donors the results become statistically significant ($p < 0.05$) when comparing the population of IL-17^+ T helper cells and Th17 cells.

2) For both healthy donors and gastric cancers the sample sizes were both small (7 healthy donors and 8 gastric cancers). This small sample size may have contributed to a lack of statistical significance resulting in a false negative.

This observed difference was in keeping with previous results and also current evidence (144, 145). Lui et al found in a samples size of 32 gastric cancer patients and 19 healthy donors there was a significant difference for both IL-17^+ T helper cells and IL-22^+ T helper cells in PBMCs of healthy donors and gastric cancer patients (114). Chen et al also found a similar difference in the percentages of circulating IL-17^+ T helper cells (IL-17^+CD4^+) of oesophageal cancer patients compared with healthy donor controls (133). On examination of the tumour microenvironment in eight oesophageal cancer patients, they found IL-6, TGF-b and consistently elevated levels of IL-23 compared to tumour-free tissue. This may help to explain the findings of this thesis found which found higher levels of Th22, Th17, Th17/Th1 but not Th17/Treg in cancer patients compared to healthy donors suggesting this increase may be driven by higher levels of IL-23 in the tumour cytokine microenvironment.

This thesis went on to further examine if this difference in Th22 and Th17 cells was associated with disease progression and could be a potential biomarker of disease progression. In patients with oesophageal cancer, there was an observed difference in IL-22^+ T helper cells and tumour stage with levels increasing in PBMCs with increasing disease stage (stage 1a-3a mean = 1.6%, stage 3b-4 mean = 2.5% ($p=0.01$)). Similar differences were observed in IL-17^+IL-22+ T helper cells (stage 1-3a mean = 0.33, stage 3b-4 mean = 0.7 ($p = 0.1$)), Th22 cells (stage 1-3a mean = 1.6, stage 3b-4 mean = 2.1 ($p = 0.43$)), Th17/Th1 (stage 1-3a mean = 0.22, stage 3b-4 mean = 0.45 ($p = 0.12$)), and Th17/Treg (stage 1-3a mean = 0.22, stage 3b-4 mean = 0.45 ($p = 0.12$)). However, there was no difference comparing just Th17 cells or IL-17+ cells suggesting that these may play a lesser role in oesophageal cancer. Certainly there appears to be an association between disease stage and Th22 cells. Lack of difference in the Th17 and IL-17+ secreting cells may highlight the plastic nature of Th17 cells mentioned previously in this thesis and that the tumour microenvironment may change depending upon disease progression resulting in a different phenotype of Th17 such as Th17/Treg or Th17/Th1 having a more significant role. There is little current evidence on the role of Th17 or Th22 cells in oesophageal cancer to be able to compare our results with however a paper by Chen et al found a significant increase in Th17 cells associated with increasing disease stage ($p=<0.01$) but they included both squamous cell

oesophageal cancer patients and adenocarcinoma patients in their study which are known to have very different pathophysiological processes and thus may affect the results. There are currently no papers examining the role of Th22 in oesophageal cancer to enable a comparison with published work.

In gastric cancer patients, there appeared to be a significant reduction in TH22, Th17, IL-17^+ T helper cells, IL-22^+ T helper cells, and IL-22^+IL-17^+ T helper cells with worsening disease stage. Th17/Treg appeared to increase with increasing tumour stage and Th17/Th1 appeared to decrease with increasing tumour stage however, neither result achieved statistical significance. The apparent increase in Th17/Treg cells may in part explain the observed results as these have a more regulatory role secreting high levels of regulatory cytokines such as IL-10 thus the observed decline in most of the phenotypes in this study may suggest that these cells play a role in disease progression. Interestingly, previous studies have found the opposite to this thesis' results showing an increase in both Th17 and Th22 populations with disease stage (113, 114, 146). One potential explanation for this difference may be geographical location since a significant number of gastric cancer studies have been performed in Asian countries (China/Japan) where the incidence of gastric cancer is far higher and aetiology may result in a different pathophysiological disease process compared to patients developing gastric cancer in western countries. There are a number of theories as to this epidemiological difference but certainly *H.Pylori* may play a role as Asian countries are known to have a high prevalence of *H. pylori* infection in comparison to western countries (147). It has also been well established that *H.Pylori* is associated with an increase risk in gastric adenocarcinoma (148). Another potential area of is bias secondary to a small sample size of only 7 patients in this thesis compared to the Lui et al's sample size of 32 patients. Also, a potential source of bias could have come from the staging of the disease. Both oesophageal cancer and gastric cancers are initially staged using TNM system through predominately radiological staging however for early cancers for which patients then go on to have surgery many some may be restaged using pathological staging. The current literature does not mention in detail the staging process only that the TNM system was used.

The results of this thesis suggest that the T helper cell phenotypes Th22 and Th17 may play a significant role in the pathophysiological process of both gastric and oesophageal adenocarcinomas. However, it is clear that the role may be complex and, in particular, the plasticity of Th17 cells and their role may change depending upon the cytokine environment of the tumour. The results clearly show that both levels of Th22 and Th17 are raised in PBMCs of upper GI cancer patients compared with healthy donors, which is in keeping with the rest of the

literature. This presents the possibility that these cell phenotypes could be used as biomarkers to aid diagnosis as well potential predictors of prognosis. Another interesting point raised by this work is the involvement of IL-23 in the differentiation of both these phenotypes in upper GI cancers and that this may be a potential area for development of immunological therapies. What remains unclear is the role that these phenotypes play in the disease process. The evidence is still mixed as to whether these play a beneficial role in control of the disease or a contributory factor in disease progression. Either way, this thesis underlines the complexity of the tumour microenvironment and the detectable effects in PBMCs. The lack of immunological research in oesophageal cancer and in studies of gastric cancer on western patients highlights the challenges in researching these particular cancers. There is certainly scope for further research focusing on the immunological phenotypes in upper GI cancers and the roles that they may play. This thesis highlights some of the challenges in the methodology of being able to examine for these phenotypes and further research could be done a larger sample size of patients to improve the power of the study. Also, longer follow-up would help to identify potential biomarkers of prognosis paying particular attention to 5 year survivals, overall survivals as well as disease free survival. This thesis only examine the peripheral blood mononuclear cells and one aim of further research would be to examine the phenotypes Th17 and Th22 in both PBMCS and TILs of upper GI cancer patients focusing on these phenotypes but also the tumour microenvironment paying particular attention to the potential role of IL-23. The overall aims of future research would be to potentially identify biomarkers to improve accuracy of diagnosis and also to be used as markers of disease progression. However, ultimately future research could aid in the development of improved management and development of immunological therapies that would hopefully aid prognosis of two diseases that are not only increasing in prevalence but also associated with very poor outcomes.

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