The effects of environmental oxygen on CD4+ T lymphocyte activation and responses

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Abstract

The organs in which lymphocytes function are low in oxygen (<5% oxygen) and even lower oxygen levels may be more prevalent in inflammatory tissues. In this thesis the effects of environmental oxygen on human CD4+ memory T lymphocyte function *in vitro* have been investigated. The level of oxygen in normal air (21%) which historically has been used for most in vitro experiments with immune cells was found result in suboptimal responses of this cell type, especially with regards to proliferation. At physiologically more appropriate oxygen levels of 8.5%, optimal proliferation was observed which coincided with an increase in Th2-associated markers. At 3% oxygen, the average level found in the inflamed joint in rheumatoid arthritis, a more sustained pro-inflammatory response was observed. In 1% oxygen, cytokine production was not maintained over time paralleling observations of CD4+ T lymphocyte behaviour in both the tumour and chronic inflammatory environment. This comparison was further supported by the increased expression of the activation marker CD69 and the depression of CD4+ T lymphocyte proliferation. A model of reperfusion injury also highlighted the effect that varying oxygen levels can have on CD4+ memory T lymphocytes. Proximal T cell receptor signalling was found to be altered after equilibration at different oxygen levels, and preliminary experiments investigating the potential role that redox plays in regulating CD4+ memory T lymphocyte functions were performed. It is concluded that environmental oxygen levels significantly influence CD4+ memory T lymphocyte responses, have implications for their function in inflammatory sites *in vivo*, and need to be considered when designing or interpreting *in vitro* experiments.

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Contents

Abbreviations

APC antigen presenting cell

ARNT aryl hydrocarbon receptor nuclear translocator

BSA bovine serum albumin

CFSE carboxyfluorescein diacetate succinimidyl ester

CIA Collagen-Induced arthritis

CMV cytomegalovirus

CRACs calcium release activated channel

Csk C-terminal Src kinase

CTLA-4 Cytotoxic T-lymphocyte Antigen-4

CTLA-4 Cytotoxic T-lymphocyte Antigen-4

DC dendritic cell

DFX deferoxamine mesylate

DMSO dimethyl sulfoxide

EAE experimental autoimmune encephalomyelitis

EDTA ethylenediaminetetraacetic acid

FCS fetal calf serum

FIH-1 Factor Inhibiting HIF-1

FS forward scatter

GVHD graft-versus-host-disease

H_2O_2 hydrogen peroxide

HIF hypoxia inducible factor

HIV human immunodeficiency virus

HREs hypoxia-response elements

IDO indoleamine 2,3-dioxygenase

IEX-1 Immediate early response gene X-1

IPEX Immunodysregulation polyendocrinopathy enteropathy X-linked syndrome

IS immunological synapse

ITAM Immunoreceptor Tyrosine-based Activation Motif

iTregs inducible Tregs

LAT Linker of Activated T cells

Lck Lymphocyte-Specific Protein Tyrosine Kinase

LYP lymphoid protein tyrosine phosphatase

MFI mean fluorescence intensity

MHC Major histocompatibility complex

MnSOD mitochondrial superoxide dismutase

MS Multiple Sclerosis

mTOR mammalian target of rapamycin

NAC *N*-acetyl-*L*-cysteine

NSAIDs Nonsteroidal anti-inflammatory drugs

nTreg Natural regulatory T cells

OSAS Obstructive sleep apnoea syndrome

PBMC peripheral blood mononuclear cell

PBS phosphate buffered saline

PHA Phytohaemagglutinin

PHD prolyl hydroxylase domain enzyme

PI proliferative index

RA Rheumatoid Arthritis

RF Rheumatoid factor

ROS reactive oxygen species

S1P Sphingosine-1-phosphate

SCID Severe Combined Immunodeficiency

SH4 Src Homology 4

SHP-1 Src homology region 2 domain-containing phosphatase-1

SLO secondary lymphoid organ

SNP single nucleotide polymorphism

SS side scatter

STAT Signal Transducers and Activators of Transcription

T_{CM} central memory T lymphocytes

TCR T cell receptor

T_{EM} effector memory T lymphocytes

T_{RM} tissue-resident memory T lymphocytes

VEGF Vascular endothelial growth factor

VHL von Hippel Lindau tumour suppressor gene

Y394 Tyrosine 394

Y505 Tyrosine 505

ZAP70 zeta-chain-associated protein kinase 70

Figures and Tables

1. INTRODUCTION

The focus of this thesis is the effect of varying oxygen levels on CD4+ T lymphocyte behaviour and function. The motivation behind this research is the low oxygen levels found in inflammatory environments, particularly those observed in the chronic, autoimmune-associated inflammatory environment of the joints of rheumatoid arthritis (RA) patients. In this introduction, RA is discussed with a focus on CD4+ T lymphocytes. Then the effect of different oxygen levels on inflammatory cell behaviour and function are described.

1.1 Autoimmunity and the rheumatoid joint

1.1.1 An introduction to autoimmunity

The immune system in vertebrates has evolved to protect these multi-organ life-forms from the pathogens present in their environment. This system is clearly effective as evidenced by the fact that we still exist today. In the process of protecting an organism from pathogenic attack the immune system can cause localised, and sometimes systemic, tissue damage. However, sustaining such collateral damage is a small price to pay to ensure that a life-threatening infection is cleared. There are instances when the immune system is persistently activated within a tissue, and this can result in autoimmune disease. In these situations inflammation persists without the usual resolution of the immune response and this process is often driven by the adaptive immune response targeting self-antigens [1-3].

1.1.2 Rheumatoid arthritis

RA is an autoimmune disease involving the synovial membrane of joints affecting around 1% of the world's population [4, 5]. The normal synovial membrane is just 2-3

cells thick and consists of resident fibroblast and macrophage-like synoviocytes [6, 7]. These cells form a protective layer around the synovial space, providing synovial fluid to lubricate the joint [8, 9]. In RA, synovial membrane hyperplasia occurs alongside an influx of inflammatory cells, causing swelling, soreness and dysfunction of the joint [4, 10]. The proliferating RA synovium - many cells thick - forms a pannus tissue which invades and destroys cartilage at the joint interface and causes bone destruction [11, 12].

Although the reasons for disease onset are not fully understood, there are known risk factors. Concordant twin studies revealed that genetics can contribute around 60% of the risk of developing RA [13] and 98 genetic loci have been associated with the development of the disease [14]. The ‘shared epitope’ is a term used for certain alleles of *HLA-DRβ1* gene associated with the greatest genetic risk of developing RA which contain differences in an amino acid sequence in the third hypervariable region of the Major histocompatibility complex (MHC) Class II molecules. Since MHC class II proteins are expressed by antigen presenting cells (APCs) that present antigen to T lymphocytes, it is speculated that the associated alleles may be involved in altering responder T lymphocyte behaviour, potentially promoting autoreactive T lymphocyte activation and survival [4, 15-17]. Additional to genetic risk, environment is also known to play a part in disease onset. For example smoking for over 20 years is associated with increased risk of developing RA [1, 18], potentially via the generation of autoantibody production in the lung [19, 20].

It is not known whether proliferation of resident cells or infiltration of inflammatory cells drives the disease and the expansion of the synovium, although a mixture of the two is possible and potentially likely. When human fibroblasts from the RA joint are

placed into a severe combined immunodeficiency (SCID) mouse, an arthritic phenotype is generated with infiltrating murine cells [8, 21, 22]. Notably in these experiments, pathology is limited to the joint the fibroblasts were initially transferred into, suggesting they are not involved in the 'spread' of the disease to unaffected symmetric joints [22]. Synoviocyte proliferation can occur before inflammatory onset, suggesting that synovial hyperplasia pre-dates inflammatory cell infiltration and involvement [8, 23]. RA synovial fibroblasts may therefore be almost transformed in nature, and the immune involvement in the disease may be separate or secondary [21, 24].

1.1.3 Inflammatory cells of the rheumatoid joint

Despite the role of stromal cells in RA pathology, inflammatory cells - both of the innate and adaptive immune systems - migrate into the joint tissue and show signs of activation [15, 21, 25-30]. Inflammatory cells are able to activate fibroblasts by the secretion of cytokines such as TNFα and IL-1β, and by the release of microparticles during proliferation [21, 28, 31-33] and may therefore promote the synovial lining hyperplasia observed in RA. Migrating monocytes differentiate into macrophages, dendritic cells (DCs) and osteoclasts, further adding to the tissue-resident populations already present [6, 28, 34]. Macrophages show a stimulated, pro-inflammatory phenotype in the rheumatoid joint, producing pro-inflammatory cytokines such as TNF-α and IL-1. Furthermore, neutrophils (also known as polymorphonuclear leukocytes) are the most abundant cell observed in the RA synovial fluid and contribute to pathology by the production of factors that degrade tissue [26, 35, 36]. These factors include reactive oxygen species (ROS) that are

normally produced during the neutrophil respiratory burst, of which associated damage can be observed in the RA joint [36, 37].

Adaptive immunity plays an important role in RA with both autoreactive B and T lymphocytes involved in pathology. Occasionally adaptive immune cells reveal a high level of organisation; in around one third of patients, germinal centre-like structures are formed with B lymphocytes, T lymphocytes and follicular DCs [21, 27, 38, 39]. Lymphocytes in the RA joint show signs of recent activation and express CD69 [40]. Despite this, overall lymphocyte proliferation and turnover is thought to be low [27, 41]. B lymphocytes are important in the pathology of RA for several reasons. Firstly, they can act as APCs to autoreactive T lymphocytes [27, 42]. Secondly, their production of RANKL contributes to the formation of osteoclasts and bone destruction [29]. Thirdly, and most importantly, B lymphocyte autoantibody production is observed in a large number of patients [43, 44]. Rheumatoid factor (RF) - antibody targeted against the constant region of the IgG molecule - is present in over 80% of RA patients and was the first autoantibody associated with the disease. RF has been detected in patient samples' obtained years before disease onset suggesting it plays a role in disease initiation [1, 15, 44, 45]. Anti-citrullinated protein antibodies are also highly prevalent in RA patients, and their presence are a potential indicator of expected disease progression in early RA patients [3, 15, 27]. These antibodies can be generated locally in the synovial tissue [3, 43].

1.1.3.1 Basic biology of the CD4+ T lymphocyte

CD4+ T lymphocytes are also observed in the RA joint, and are potentially important in pathology [29, 46]. Before CD4+ T lymphocytes in RA are discussed, their basic biology is described.

1.1.3.1.1The importance of CD4+ T lymphocytes

T lymphocytes are necessary for a normally functioning immune system and severe disease results when T lymphocytes are physiologically lacking or present in low numbers [47, 48]. Genetic mutations associated with lymphocyte survival and function result in severe immunodeficiency. Patients with these mutations are prone to infectious diseases such as cytomegalovirus (CMV), and need to be rigorously treated early on in life [48]. Acquired immunodeficiency syndrome is an example of an acquired T lymphocyte deficiency and is most commonly caused by infection by human immunodeficiency virus (HIV) which causes depletion in peripheral CD4+ T lymphocyte numbers. When CD4+ T lymphocyte numbers drop below 200/mm^2 patients become susceptible to opportunistic infections such as bacterial pneumonia and candidiasis [47, 49]. T lymphocytes are therefore clearly important to maintaining human health, and are often described as having a ‘central role’ in immunity [50].

1.1.3.1.2 The CD4+ T lymphocyte life cycle

CD4+ T lymphocyte development

T lymphocyte precursors develop alongside all other immune cell precursors in the bone marrow but migrate to the thymus to complete maturation to single-positive CD4+ or CD8+ expressing cells [51-53]. The T lymphocytes that emerge from the thymus are termed ‘naïve’, but will retain some autoreactivity due to the process of thymic selection which only allows cells with some self-recognition to survive [52, 53].

CD4+ T lymphocyte response to antigenic exposure

CD4+ T lymphocytes’ main roles are to manage the immune system and to provide immunological memory. Naïve CD4+ T lymphocytes cycle between blood and

secondary lymphoid organs (SLOs) in a resting state to increase their chances of finding their cognate antigen, spending around 6-24 hours in each lymph node before moving on [54, 55]. They are maintained in SLOs by exposure to IL-7 [56-58]. Therefore a heterogeneous pool of naïve CD4+ T lymphocytes (with regards to antigen specificity) resides within the lymphoid system [57]. Once activated, naïve CD4+ T lymphocytes will proliferate and differentiate into different 'T helper' subsets within the lymph node, and after 3-11 days will migrate via the lymphatic and circulatory system to the infected site as effector CD4+ T lymphocytes [59, 60]. Effector T lymphocytes produce a wide variety of cytokines that guide the ongoing inflammatory process [47]. The different T helper subsets are described in more detail below.

Generation of CD4+ effector and CD4+ memory T lymphocytes

Once an infection is cleared the majority (90-95%) of effector T lymphocytes involved will die by the process of apoptosis [55, 58, 60-62]. However, a small subset persist as a population of memory cells that are able to act if re-exposure to the same pathogen occurs [63, 64]. Knowledge about these retained CD4+ T lymphocyte populations is increasing with recent descriptions of a pool of memory T lymphocytes that only reside in tissue (tissue-resident memory T lymphocytes, or 'T_{RM}') and cannot be isolated from blood samples as previously anticipated for memory T lymphocytes [55, 58, 61, 65]. In fact, the majority of memory T lymphocytes are found migrating through tissue, but, due to the ease of access, blood-borne memory T lymphocytes are the most widely investigated [55, 58]

Of the types of memory T lymphocyte that develop from an adaptive immune response, effector memory (T_{EM}) and central memory (T_{CM}) CD4+ T lymphocytes are the most well characterised [63, 64]. T_{EM} are able to act much more rapidly than both T_{CM} and naïve T lymphocytes and hence can clear pathogens quickly. T_{EM} directly enter infected tissues from blood and quickly produce cytokines such as IFNγ and IL-4 in response to re-stimulation [55, 58, 60, 62-64]. In contrast, T_{CM} circulate between blood and lymph tissues in a resting state and only become reactivated when antigen is again delivered to the lymph node. Unlike T_{EM}, they express CCR7 and high levels of CD62L, which allows them to enter the lymph node via high-endothelial venules [61, 64]. Once re-stimulated by antigen, T_{CM} readily proliferate and immediately produce IL-2 that is able to support the expansion and differentiation of any local naïve and regulatory T lymphocytes expressing the IL-2 receptor, CD25 [58, 60, 62]. They then differentiate into effector memory T lymphocytes, leave the lymph node and enter inflamed tissue as a secondary attack [55, 64].

It is not fully understood how two separate CD4+ T lymphocyte memory populations arise from the same inflammatory response. T_{CM} potentially arise after low-strength TCR-engagement [54, 61-63] although similar differentiation pathways may exist for the generation of both central and effector memory [58]. An elegant murine study showed that the differential generation of T_{CM} and T_{EM} may be due to the timings of the immune response. This was achieved by inhibiting lymphocyte entry into the lymph node at a specified time point during antigenic exposure. Naïve T lymphocytes present within a lymph node when antigen first entered became effector cells, whereas naïve T lymphocytes that migrated in after antigen differentiated into CCR7+ T_{CM}[54]. The authors suggested that the naïve T lymphocytes migrating in later may

be exposed to a lower dose of antigen which may contribute to their final differentiation [54]. Therefore, it is possible that the differentiation program of T lymphocytes from a naïve phenotype to a memory may be dependent on antigen dose, as shown in figure 1.1. Effector memory cells may either become tissue-resident memory T lymphocytes, or may die after re-exposure to antigen [58, 60].

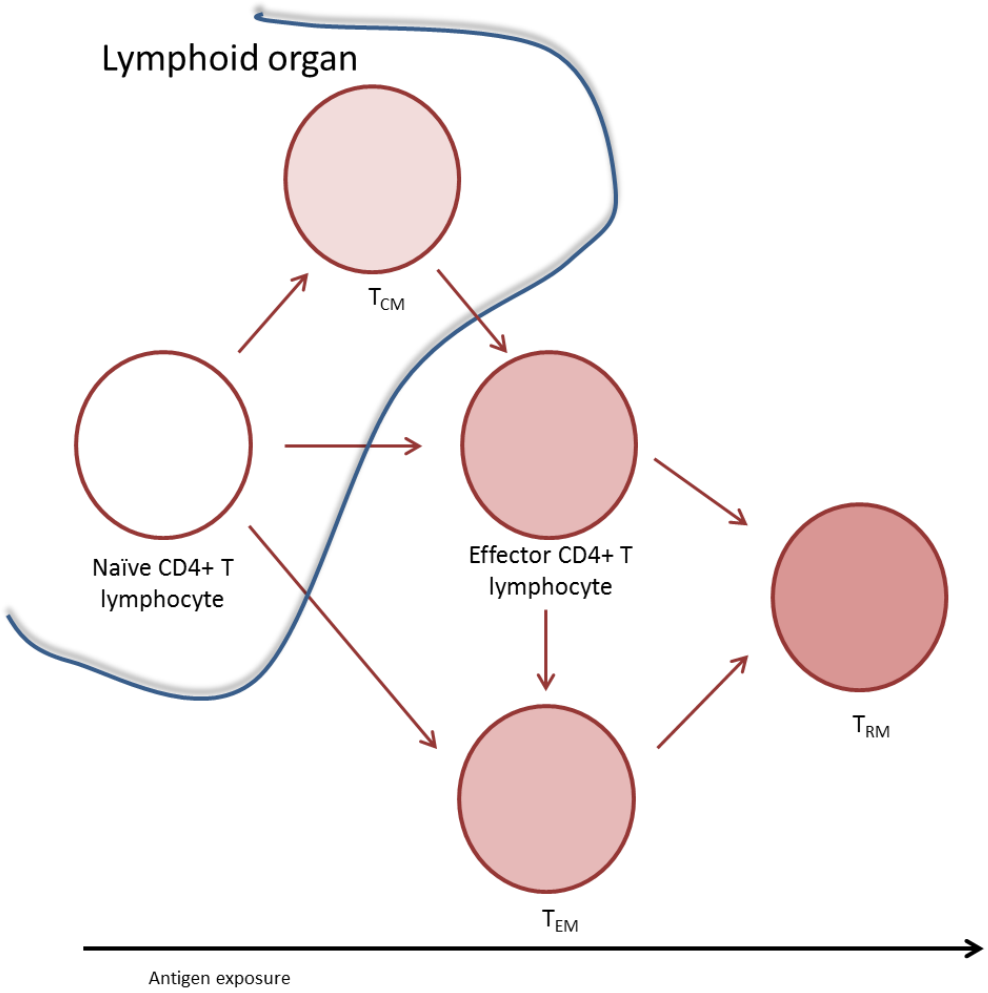

Figure 1.1 The generation of memory T lymphocytes from naïve precursors. *When exposed to antigen, naïve CD4+ T lymphocytes generate several populations of effector and memory cells. These include central memory T lymphocytes (T_{CM}), that circulate between the blood and lymphoid organs; effector T lymphocytes, that immediately enter inflamed tissue and produce cytokines; and effector memory T lymphocytes (T_{EM}), that circulate through blood and tissue. When T_{CM} are re-stimulated, they proliferate in lymph nodes and develop effector functions. Effector T lymphocytes and T_{EM} enter tissue and are able to form a population of tissue-resident memory T lymphocytes, T_{RM}. The degree of antigen exposure a naïve T lymphocyte experiences has been suggested to determine later memory development. Adapted from [58]*

1.1.3.2 Activating the CD4+ T lymphocyte

1.1.3.2.1 T lymphocyte activation – the main players

Antigen-dependent activation of T lymphocytes occurs via antigen presentation to the T cell receptor (TCR). TCR molecules are expressed on the surface of every CD4+ T lymphocyte and are members of the immunoglobulin superfamily [66]. The TCR exist as a heterodimer that associates with CD3 protein subunits to form a TCR:CD3 complex. The CD3 subunits potentiate downstream signalling after TCR engagement with an antigen. The relationship between the CD3 subunits and the TCR heterodimers is still not completely understood, although potential binding sites for the CD3 molecules have been identified on the TCR subunits [66, 67].

When CD4+ T lymphocytes are activated by APCs bearing peptide-loaded MHC II molecules, an ‘immunological synapse’ (IS) forms between the two cells [68]. On the T lymphocyte surface, an IS consists of concentric rings of molecules involved in T lymphocyte activation [68, 69]. CD3:TCR complexes are observed near the centre of the IS, but molecules with larger extracellular domains, such as CD45, are observed at the extremities [68, 70]. Signalling is thought to terminate in the very centre of the IS, with CD3:TCR complexes either being internalised [71], or secreted [72]. A brief migration of CD45 from the outermost rings of the IS to the centre is associated with this signal extermination [70, 73]. Additional molecular interactions contribute to the stability of the IS, such as the interaction between CD28 on T lymphocytes with CD80 and CD86 on APCs. CD28 is itself co-stimulatory and provides further intracellular signals into the T lymphocytes [68, 69, 74].

There are several schools of thought about how antigen binding to the TCR-CD3 complex initiates signalling [67]. The CD3 cytoplasmic tails are bound to the

membrane in resting cells, and therefore antigen binding is required to cause the dissociation of basic residues in the CD3 tails to allow them to interact with cytoplasmic proteins [66, 75, 76]. This may be achieved by a change in the lipid environment around the TCR:CD3 complex [77] or by mechanical pressure forcing their removal from the lipid bilayer [66, 78]. The newly-exposed CD3 cytoplasmic tails are then phosphorylated at key Immunoreceptor Tyrosine-based Activation Motif (ITAM) that allow zeta-chain-associated protein kinase 70 (ZAP70) to bind and phosphorylate Linker of Activated T cells (LAT). LAT then forms a complex with other signalling molecules that send diverse signals into the cell, initiating activation and effector functions [67, 79, 80]. Different levels of 'readiness' of the TCR signalling complex can result in differential thresholds in antigen exposure being required for successful signalling to activate a T lymphocyte. A primed TCR signalling complex, with kinases at the ready for ITAM phosphorylation, will have a lower signalling threshold and therefore T lymphocytes will be more readily activated [81, 82]. However, T lymphocyte apoptosis occurs in response to too great an intracellular signal, such as that seen in the deletion of autoreactive T lymphocytes in the thymus [52, 83].

Lck – a protein involved in proximal T lymphocyte signalling

Lymphocyte-Specific Protein Tyrosine Kinase (Lck) is a key kinase that phosphorylates the CD3-tail ITAMs, permitting ZAP70 association with the TCR/ CD3 complex. Lck can additionally phosphorylate and activate ZAP70 and other downstream signalling molecules [79, 80]. Lck is therefore extremely important in proximal T lymphocyte signalling and requires tight regulation. This regulation comes in the form of two key tyrosine residues – Y394 in the catalytic domain and Y505 in a

Src-homology 2 (SH2) domain [84-86]. Phosphorylation at Y505 is inhibitory as it causes the Lck molecule to fold into itself, obstructing the catalytic domain. Phosphorylation at Y394 is activatory and dominant over phosphorylation at Y505 [75, 84]. Therefore, doubly-phosphorylated Lck molecules are still able to phosphorylate downstream targets. Y394 is auto-phosphorylated and is dephosphorylated by phosphatases including lymphoid protein tyrosine phosphatase (LYP), CD45 and Src homology region 2 domain-containing phosphatase-1 (SHP-1) [73, 84, 85, 87, 88]. C-terminal Src kinase (Csk) phosphorylates the inhibitory Y505 residue [79, 85, 89] but to counteract this inhibition a cytoplasmic domain in the CD45 molecule can dephosphorylate this residue [85, 90].

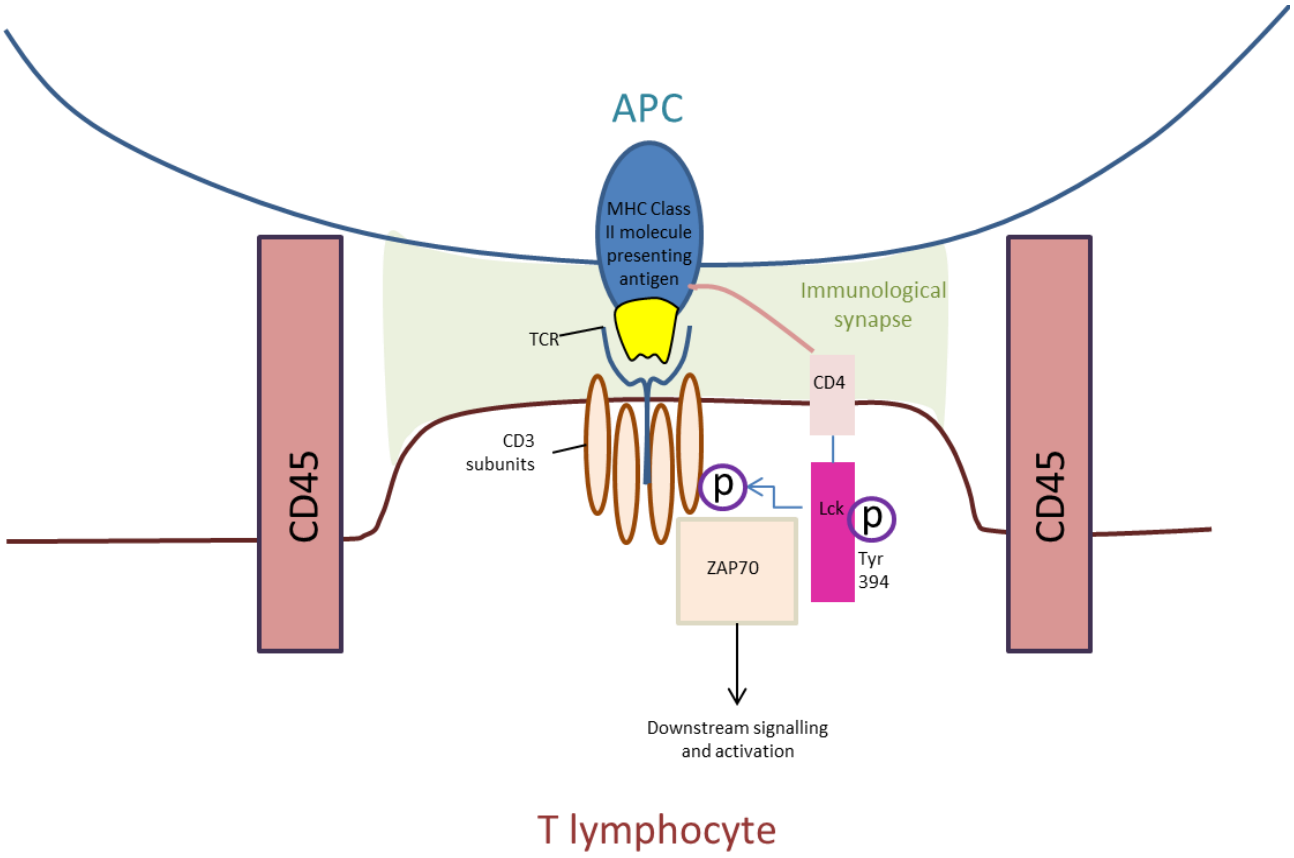

Figure 1.2. Activating the CD4+ T lymphocyte. *An immunological synapse (IS) forms between an antigen presenting cell (APC) and a CD4+ T lymphocyte when an antigen fragment is presented to a T cell receptor (TCR) by a major histocompatibility complex (MHC) Class II molecule. CD45 is excluded from the IS due to its large size. Binding causes a conformational change in CD3 subunits associated with the TCR, and activated Lymphocyte-Specific Protein Tyrosine Kinase (Lck) (phosphorylated at tyrosine 394) - often in association with CD4 molecules - are able to phosphorylate key residues in the CD3 subunits to allow for zeta-chain-associated protein kinase 70 (ZAP70) binding and initiation of downstream signalling. CD4 associated with the MHC Class II molecules acts to stabilise its interaction with the TCR.*

Presence of activated Lck in resting cells

The presence of activated Lck was recently described in resting human CD4+ and CD8+ T lymphocytes and in the T lymphocyte Jurkat cell line [75]. In naïve T lymphocytes, up to 37% intracellular Lck was found to be phosphorylated on the activatory tyrosine residue, Y394 [75]. This suggested that within a cell there was always a population of active Lck. This gave rise to a potential problem – if there is constitutively active Lck in T lymphocytes, how do unstimulated cells maintain a resting state without TCR signalling?

The answer to this conundrum probably lies within the discovery of different domains of the plasma membrane [82, 85]. Stephen *et al* visualised the separation of Lck from the TCR in maturing thymocytes by transition electron microscopy and gold particle staining. In 'untuned' thymocytes that had not experienced medullary MHC, Lck and the TCR could be seen to inhabit the same membrane domains, whereas in wild type thymocytes that had undergone thymic selection, Lck and TCR clearly inhabited separate domains within the membrane [82]. Therefore, it was concluded that when thymocytes undergo thymic selection, they reduce their chances of disorganised TCR signalling by separating the TCR from Lck, potentially reducing spontaneous autoreactivity within the periphery [82].

The separate zones that these molecules inhabit within the membrane are most probably due to membrane rafts, although regulation of protein transport and actin organisation may also play a role [85, 91]. Membrane rafts are areas of the plasma membrane rich in saturated lipids and provide an additional layer of regulation for TCR downstream signalling. There have been some discrepancies in their description due to the different experimental approaches used to investigate them,

including the use of different detergents [85]. Lck is thought to interact with membrane rafts via post-translational modifications of its Src Homology 4 (SH4) domain [85]. CD45 is also exposed to - and can dephosphorylate - Lck in resting cells, but when cells are activated it is isolated from Lck due to the movement of the latter into lipid rafts [73].

1.1.3.3 Processes and functions of CD4+ T lymphocytes

There are several defined subsets of CD4+ effector T cell with distinct cytokine-producing capabilities. These subsets are thought to be generated from the stimulation of naïve T lymphocytes in the lymph node [47]. The main subsets discovered to date include Th1, Th2, Th17 and regulatory T cells. However, there are also more recently described subsets including Th9, Th22 and T follicular helper cells. Distinct subsets are formed by differential priming by APCs involving exposure to cytokines and co-stimulatory molecules [47, 50, 92-94]. The cytokines produced by Th1 and Th2 cells also inhibit the differentiation of the alternate subsets [50, 95, 96], promoting the formation of discrete populations. The main subsets of CD4+ T lymphocytes are described in the sections below.

1.1.3.3.1 Th1 CD4+ effector T lymphocytes

Th1 cells, alongside Th2 cells, were one of the first T helper subsets to be officially recognised [97]. Th1 cells are associated with a more pro-inflammatory T helper cell phenotype, characterised by production of IFNγ, TNFα, lymphotoxin-α and high quantities of IL-2 [47, 50, 92, 97]. In Th1-polarised cells, the *ifnγ* locus is

hyperacetylated [98, 99] allowing for easy transcription of the gene. Th1 cells can also be identified by the expression of other markers such as CXCR3, a surface chemokine receptor [47, 50, 63, 100, 101].

Th1 cells are particularly effective in the reduction in load of intracellular pathogens such as mycobacterium and viruses [50, 92, 99]. This is due to their ability to activate phagocytic cells such as macrophages via IFNγ expression [102, 103]. Th1-like diseases are often described as ‘granulomatous’ due to the high number of macrophages present in the inflamed tissue [50]. Th1-produced IL-2 promotes CD8 memory cell formation, which will enable rapid destruction of virally infected cells if re-exposure should occur [92]. Several autoimmune diseases are also associated with a Th1 mediated inflammatory response, such as Type 1 diabetes, where IFNγ is thought to contribute to pathology [104, 105].

The production of IL-12 by APCs results in the polarisation of naïve T lymphocytes to Th1 effector cells [50, 92, 106, 107]. Additionally, IFNγ signalling from DCs and NK cells, and from autocrine mechanisms, can promote Th1 differentiation. Signalling of IL-12 and IFNγ results in the upregulation of the transcription factors Signal Transducers and Activators of Transcription (STAT)-4 and STAT1, respectively [92, 107]. STAT1 induces the expression of T-bet, deemed the ‘master-regulator’ of Th1 polarisation [47, 50, 92, 99, 108]. T-bet is important in the generation of the Th1 phenotype, and binds the *ifnγ* locus [99, 109, 110].

1.1.3.3.2 Th2 CD4+ effector T lymphocytes

Th2 are characterised by the expression of cytokines IL-4, IL-5, IL-9, IL-10, IL-13, IL-25 and low quantities of IL-2. Similar to the *ifnγ* gene in Th1 cells, the *il4-il13* gene

locus within Th2-polarised cells is hyperacetylated allowing for easy transcription [97, 98, 109, 111, 112]. Th2 cells are adapted to respond to extracellular parasitic infections such as helminths [47, 50, 92]. A Th2-mediated inflammatory response effectively seals off the area around large extra-cellular pathogens, containing the parasite by promoting the proliferation and activation of stromal cells [113]. The cytokines IL-4 and IL-13 are important in this role as they promote the tissue-regenerative activities of macrophages, and Th2-mediated immune responses are therefore associated with wound healing and fibrosis [113, 114]. Pathogenic Th2 cell activity is associated with allergy, for example Th2 cells are the predominant CD4+ T lymphocyte population in the asthmatic airway [115, 116]. Th2 cells are also involved in promoting the humoral immune response, as Th2-produced IL-4 is highly associated with B lymphocyte antibody production and IgE class-switching which is involved in the opsonisation of extracellular parasites [59, 92]. IL-4, IL-2 and IL-6 cytokine signalling promotes the generation of Th2 cells from naïve precursors [92], although the source of IL-4 for this differentiation has not been fully elucidated [50]. Differentiated Th2 cells are distinguishable by their expression of the transcription factors STAT5 and GATA-3 [92, 109, 117], and surface markers CCR4 and CrTH2 [63, 100, 101, 118] although CCR4 can also be detected on the surface of Th17 cells [119].

1.1.3.3.3 Th17 CD4+ effector T lymphocytes

Th17 cells are generated in response to extracellular bacterial and fungal infection [50, 92] and are characterised by their production of IL-17A, IL-17F, IL-21 and IL-22 [47, 50, 92]. Th17 cells are also capable of producing TNFα [120, 121] and therefore

have previously been confused with Th1 cells, partly resulting in their delayed description and classification [122]. CCR6 is a marker upregulated on Th17 cells and is therefore useful in their detection [32, 121], although it is also expressed on Th17 cells converting to a more Th1-like phenotype [123]. Many tissues contain IL-17 receptors [92, 93] but one of Th17 cells' main effect is to stimulate neutrophil pro-inflammatory activities [50, 92, 124] by promoting their survival [25] and proliferation in bone marrow [125]. Th17 are generated in response to low levels of TGF-β and IL-6 [50, 126], although the requirement of TGFβ *in vivo* has been questioned [127]. IL-21 further promotes Th17 differentiation, and DC-produced IL-23 maintains the Th17 phenotype [92, 126]. RORγt (also known as RORc) has been identified as a master regulator of Th17 cells. Another transcription factor, it promotes the expression of IL-17, and is absolutely required for Th17 differentiation [92, 122, 128, 129].

Since their discovery, Th17 cells have been found to be involved in autoimmune diseases previously attributed to Th1 cells, such as multiple sclerosis (MS) [93, 121, 122, 130, 131]. There are still some disagreements as to the designation of Th1 or Th17-mediated pathology in certain diseases [105, 131] and it is possible a combination of both subsets, or phenotypic plasticity, contributes to some conditions [127]. In a model of autoimmune uveitis, inflammation was induced and sustained by either Th1 or Th17 cells, and each subset was not reliant on the presence of the other. Instead, IL-23 was found to be absolutely required for pathology [132]. IL-23, as already mentioned, is known to be important in the maintenance of the Th17 phenotype, but it may have other roles within immunity [132]. Varying animal models involving different CD4+ subsets associated with similar pathologies also confuse matters. The plasticity of Th17 cells is discussed in more detail below.

1.1.3.3.4 Regulatory T lymphocytes

Regulatory T lymphocytes are distinct from the other T lymphocyte subsets in that their main role is to suppress inflammation. There are several types of regulatory T lymphocyte classified mainly by their origin. Natural regulatory T lymphocyte (nTreg) are generated in the thymus and are therefore a first line of defence against over-aggressive inflammatory responses. nTregs are CD4+ Foxp3+ CD25+ CD127lo expressing cells [133-135] and have been shown to suppress CD4+ effector T lymphocyte behaviour *in vivo* by reducing their activation [136].

CD4+ lymphocytes with regulatory functions can also be generated in the periphery from naïve T lymphocyte precursors, and are designated as inducible Tregs (iTregs) [47, 133, 137]. However, there is some uncertainty as to the nature of iTreg due to the fact that all T lymphocytes upregulate regulatory markers and are suppressive in nature post-stimulation [138]. Despite this added complexity, two main types of iTregs have been described in both humans and mice: Foxp3+ Th3 and Foxp3- Tr1 [139, 140] . There is some debate as to the extent of iTregs' immunosuppressive nature in human disease, although they do appear to be immunosuppressive *in vitro* and in mouse *in vivo* models [47, 133]. A recent paper has highlighted a potentially important role in iTreg suppression of overt Th2-mediated pathology at mucosal sites in both the gut and lung [137], a role that may delineate them from nTregs.

Recent data suggests that Tr1 do function in human immunity. Lack of them is associated with inflammatory conditions such as Crohn's disease [133], and IL-10

(which is produced by Tr1 cells) has been suggested to be important in regulating inflammation in the gut, lung and skin [137, 141]. Patients who develop drug-independent tolerance from mixed chimerism after allogeneic haematopoietic stem cell transplant have higher numbers of circulating Tr1 [139, 140]. Additionally, Immunodysregulation polyendocrinopathy enteropathy X-linked syndrome (IPEX) patients (who have mutations in Foxp3 and are therefore unable to generate nTregs) are able to generate Tr1 in the periphery, although these regulatory lymphocytes are not enough to stop overt inflammation [139].

1.1.3.3.5 The plasticity of CD4+ T lymphocytes

There seems to be some disparity between the generation of T helper cell subsets in mouse and human, with mouse CD4+ T lymphocytes forming more distinct and discrete subsets in *in vitro* experiments [142] whereas human CD4+ T helper lymphocytes appear to have increased plasticity between the different subsets, with characteristics associated with more than one subset present in a single cell [63, 98, 101, 143]. This may be due to procedural differences between murine and human experiments, and increased murine plasticity of similarities have been proposed more recently [127]. Furthermore, humans experience a wide range of pathogens in their environment, whereas mice used experimentally for research have a more restricted exposure to pathogens. Nonetheless, it is therefore important to bear cell origin in mind when comparing data from mouse and human studies.

The greatest plasticity amongst the subsets appears to lie with Th17 cells which have been observed to co- express cytokines associated with alternative subsets,

including IFNγ [93, 121, 144-146] and IL-10 [128]. Indeed, some Th17 cells appear to become IFNγ-single expressing cells [147]. Unlike Th1 and Th2 cells, Th17 cells have been reported as transient and unstable in their phenotype [93, 127, 132], but conversely have also been described as long-lived effector memory cells [120]. It must be stated that in the publication that made this latter claim the stimulation method used (including the duration) was not stated [120]. Therefore the phenotype observed may have been due to the fact that stimulating CD4+ T lymphocytes inherently leads to loss of CD45RA expression [148]. Genetic imprinting in Th17 cells appears to be less stable than in Th1 or Th2, again pointing to a less terminally differentiated phenotype [144].

Ex vivo, Th17-like cells from both blood and tissues have been observed to express both IFNγ and IL-17, suggesting these double positive cells have physiological relevance [145, 149]. Hence there is now a considerable amount of evidence that a Th17 to Th1 conversion occurs physiologically [93, 95, 145, 149], and this may even be the normal course of differentiation for Th17 cells. T-bet expression has been identified in pathologic Th17 cells [93, 128, 147], and it is therefore possible that Th17 conversion to a more Th1-like phenotype is associated with greater pro-inflammatory effector functions. IFNγ+ IL-17+ double positive cells have been observed physiologically in autoimmune conditions such as MS [93, 145]. IFNγ+IL-17A+ cells were also detected in the synovial fluid of juvenile idiopathic arthritis patients and shared TCR clonotype with Th17 cells isolated from the same joint, suggesting they had originated from IL-17 single positive cells [145].

Th17 plasticity is thought to be regulated epigenetically. Th17 cells in Th1-favouring conditions contain ‘open’ *ifnγ* loci and limit transcriptional access to their *il-17* loci

[144]. Interestingly, Th17 cells re-stimulated in Th17 –favouring conditions still upregulate IFNγ transcription, further supporting the notion that a Th17 to Th1 switch is normal for Th17 biology [144]. Murine Th17 cells have also been shown to convert to a Th2 phenotype under the right polarising conditions [95, 149]. An increasing number of publications have also highlighted the stem-cell like nature of Th17 cells compared to more differentiated Th1 and Th2 cells [93, 128] and propose that Th17 cells are less determined in their phenotype, thus facilitating their more effective response to environmental cues.

Markers associated with Th17 and Treg phenotypes have also been shown co-exist within the same cell [95, 146, 150, 151]. Both Foxp3+ RORγt+ and Foxp3+ T-bet+ T lymphocytes have been identified [146, 152] and a recent study has suggested that the conversion of Treg to Th17 cells occurs in human RA patients [151]. However, the lack of specific Treg-cell markers can make such analysis fraught and more work needs to be done to validate the assumptions made from these studies. As Foxp3 can be expressed transiently in all activated T lymphocytes [146, 153] it is possible that the Foxp3+ cells detected in some experiments are not true Tregs.

Th1 and Th2 cells are thought to be more set in their phenotype but there is evidence that phenotypic switches can occur [95, 146, 154]. A murine *in vivo* study found that previously Th1 and Th2-differentiated cells could switch phenotype when challenged with an antigen that normally promoted the opposite subset to develop [154]. Interestingly, Th2 memory cells revealed more plasticity than Th1 memory cells. However, central memory cells revealed the greatest plasticity in this study. Central memory cells hypoacetylate their cytokine genes resulting in a natural plasticity compared to effector memory cells [98]. As this *in vivo* model used APCs loaded

with antigen, it is possible that these APCs migrated to lymph nodes and activated central memory cells [98]. Furthermore, an *in vitro* murine study found that after a certain number of divisions in specific polarising conditions, CD4+ T lymphocytes were reported to become fixed in their phenotype, even when exposed to reciprocal polarising conditions [142].

Th1 and Th2 differentiated lymphocytes are not able to produce IL-17 when re-stimulated in Th17 differentiating conditions, and epigenetically completely inhibit transcription of RORγt, signifying that the phenotypic switch of these subsets may be limited [93, 149, 150]. As Th1 and Th2 may be more terminally differentiated than Th17 cells, the 'de-differentiation' this may entail may be the reason for the lack of plasticity to a Th17 phenotype.

1.1.3.4 CD4+ T lymphocyte function in the RA joint

This project is primarily concerned with CD4+ T lymphocytes and the role they play in low-oxygen environments such as the RA joint; the autoimmune disease used as a model for this research. The role of CD4+ T lymphocytes in RA is not completely understood but evidence does suggest they play an important role. As already alluded to, genetic mutations associated with RA are related to CD4+ T lymphocyte function [14, 35, 45, 155, 156] including the genes of *ptpn22, cytotoxic T-lymphocyte antigen-4 (ctla-4),* and *ccr6* [1, 14, 15, 157, 158]. *ptpn22* encodes the phosphatase, LYP [159-162], and the polymorphic variant associated with RA is thought to be a gain-of-function [159, 161, 162]. CTLA-4 is a surface molecules involved in Treg function that reduces CD28 co-stimulation on effector T lymphocytes [74, 163]. Interestingly, Treg cells were found to have the genes of many of the polymorphisms

associated with RA in an epigenetically ‘open’ conformation, suggesting that these cells may be the most susceptible to genetic mutations [14].

Experimental data has also suggested that CD4+ T lymphocytes are important in the pathology of RA. Injection of human synovial T lymphocytes from a subset of RA patients into the joints of SCID mice initiated synovial hyperplasia. Interestingly, the T lymphocytes that caused positive transfer of disease were from patients with disease duration of 6 years or less, suggesting that distinct cell types may be involved in pathology at different times during the course of the disease [164, 165]. Additionally, an upregulation in the number of both CD4+ and CD8+ T lymphocytes expressing the activation marker CD69 in lymph nodes has been observed in both early RA patients, and in non-diseased individuals carrying autoantibodies [166]. The CD4+ T lymphocytes present in the RA joint show a memory phenotype [46, 156, 167-170] and constitute around 20-30% of the overall inflammatory cell infiltrate in the RA joint, [35, 46], although some papers report even higher percentages and describe T lymphocyte-rich zones [40, 164, 171]. Varying numbers of lymphocytes may be found due to the different stages of disease, and higher numbers of CD4+ T lymphocytes may be more associated with established disease [171].

Despite evidence suggesting CD4+ T lymphocytes do play a role in the pathology of RA, some data indicates that their behaviour is odd. For example, synovial fluid T lymphocytes have reduced proliferation [172-174]. Furthermore, despite T lymphocyte-related cytokines being detected in early arthritis [33], it seems that in more established RA T lymphocyte cytokine production is depressed [175, 176]. Further to this, decreasing numbers of CD4+ T lymphocytes have been associated with increased pain in RA, suggesting that CD4+ T lymphocytes may play a

protective role in the disease [30]. Smeets *et al* compared CD4+ T lymphocytes taken from RA patient biopsies to cells of removed tonsils. CD4+ T lymphocytes from both early and late RA patient biopsies showed a reduction in expression of IFNγ, the proliferation marker Ki67 and the activation marker CD25 [176]. However, all the RA patients in this study were on Nonsteroidal anti-inflammatory drugs (NSAIDs), whereas it was not clarified whether tonsillitis patients were on anti-inflammatory drugs. Additionally, lymphoid tissue such as tonsils may be a hot-bed for recent lymphocyte activation and expansion and thus it is possible that the comparison between peripheral inflammatory and lymphoid tissue is not completely viable, as T lymphocytes will be in different stages of development and maturation. Effector cells found in peripheral tissue routinely need to be re-stimulated for investigation *ex vivo* [60, 132], suggesting that there is a small window of opportunity to detect effector functions *in vivo* in tissue.

In general, Th2 cells are not thought to be associated with established RA as IL-4 is only detected at low levels in samples [177], and van Hamburg *et al* found that overexpression of GATA-3 resulted in decreased arthritis [178]. However, early arthritis patients' samples revealed increased levels of both IL-13 and IL-4 in patients who went on to develop RA, suggesting that Th2 and humoral immune mechanisms may play a role in the early stages of the disease [33]. Oddly, RA patients do show an increase in the numbers of CCR4+ CD4+ T lymphocytes in peripheral blood [179]. As explained, CCR4 is a chemokine receptor associated with Th2 cells but it can also be expressed on Th17 cells [119]. Ratios in the periphery may also be a consequence of increased depletion of particular subsets of T lymphocytes as they preferentially migrate in to inflamed tissue.

It was initially suggested that RA is a Th1-like disease after an increase in the amount of IFNγ was detected in the rheumatoid synovium of established RA patients' samples compared to osteoarthritic joints [176]. However, double staining revealed that only 1% of CD3+ T lymphocytes were expressing IFNγ in rheumatoid joints, and other cell types, perhaps macrophages, were expressing the majority of IFNγ [180]. Additional evidence further questions a Th1-polarisation in RA, as one study found no IFNγ in the synovial fluid of early arthritis patients who went on to develop RA, and little was detected in the synovial fluid of established arthritis patients [33]. Some murine models of inflammatory arthritis find IFNγ deficiency, either by genetic deletion of systemic removal, can lead to exacerbated disease [35, 45, 181], although local administration can enhance inflammation [35, 182]. Additionally, synovial membrane IFNγ mRNA levels negatively correlated with disease progression in the 2006 DAMAGE study, suggesting again that IFNγ is protective or non-pathological [183]. However, a lack of IFNγ detection may be due to its increased uptake, and this would need to be addressed in future studies.

Recently it has become apparent that it is not just Th1-associated mechanisms involved with the disease as Th17 cells are now known to play an important role in the pathology of RA [31, 122, 155, 184]. IL-17A has been detected in greater amounts compared to healthy and disease controls in RA synovial fluid in both early and late RA patients, and in the synovial tissue of established RA [31, 33, 184-187]. IL-17 is important in the development of murine models of arthritis, including the ZAP70 KO arthritis models, Collagen Induced Arthritis (CIA) and a model of spontaneous arthritis [130, 155]. IL-17 producing cells in the rheumatoid joint are associated with CCR6+ CD45RO+ T lymphocytes, which are able to migrate in to the

RA joint in response to increased CCL20 levels [32]. Increased numbers of IL-17 producing Th17 cells have also been observed in the peripheral blood of reactive arthritis patients, ankylosing spondylitis and RA patients, suggesting a systemic alteration in the immune system [119, 131, 188], although they do not necessarily correlate with disease activity [105].

Evidence thus far has therefore led some to conclude that Th17 cells play an important role in RA pathology. However, histology of rheumatoid synovial membrane suggests that the percentage of IL17+ T lymphocytes is similar to the number of IFNγ+ T lymphocytes – only around 1%-8% [185, 189, 190] and it is possible that neutrophils and mast cells produce the most IL-17 in this tissue [189, 190]. Additionally, in the DAMAGE study, all patients' synovial membrane samples expressed mRNA for TNFα and IL-10, but only 28% of patients' samples expressed IL-17 mRNA [183].

RA patients have increased numbers of circulating Tregs in their periphery and within their synovial fluid compared to healthy controls [168]. However, there are some disagreements as to whether synovial Tregs are functionally suppressive. Some *in vitro* assays have reported successful suppression of effector T lymphocyte proliferation [168, 191] whereas others have observed a reduction in suppressive capabilities compared to matched peripheral blood Tregs [151]. Conflict has also arisen over whether RA patients Tregs are able to suppress effector T lymphocyte cytokine production. Peripheral blood $CD25^+$ Tregs from RA patients showed reduced ability in suppressing TNFα and IFNγ by CD4+ CD25- T lymphocyte effector cells, even though their ability to suppress proliferation was intact. In these experiments, anti-TNFα treatment restored the suppressive capabilities of peripheral Tregs three

months after treatment [192]. Synovial Tregs cells express more CTLA-4 than their equivalent peripheral blood regulatory counterparts but, as mentioned, contact with APCs is required for CTLA-4 to function. Suppression physiologically in the RA joint may therefore be partly dependent upon cellular organisation and locality [163, 168, 191].

Although cytokine production by T lymphocytes in RA may be diminished, the role of cell contact may still play an important role. Fixed T lymphocyte clones were able to induce the production of IL-1β from cells of the THP-1 macrophage cell line [193]. T lymphocyte contact with macrophages also results in the production of TNFα, IL-6, MMPs and other pro-inflammatory mediators [40, 155, 194]. In *ex vivo* RA synovial samples, isolated RA CD4+ T lymphocytes were only able to induce TNFα production from peripheral blood monocytes when contact was uninhibited [40, 194]. These RA CD4+ T lymphocytes mimicked cytokine-stimulated peripheral blood CD4+ T lymphocytes in their response to PI3K inhibitors, suggesting that in the joint they had been stimulated by cytokines such as IL-6, IL-2, IL-15 and TNFα [40]. The activation marker CD69 may be important in these cell-contact dependent mechanisms, as blocking CD69 inhibits TNFα production from synovial macrophages in these models [155, 164, 194, 195]. However, T lymphocytes do not inhabit similar regions as TNFα producing macrophages in the RA joint and therefore the extent of cell contact that occurs physiologically may be minimal [175].

1.2. Hypoxia at inflammatory sites

A link between hypoxia and inflammation is becoming increasingly apparent. Altitude sickness, in which blood oxygen levels are decreased, is associated with a systemic

increase in pro-inflammatory cytokines [196, 197]. Furthermore sleep apnoea, a condition in which blood oxygen levels drop during sleep due to airway blockage, is associated with an increase in circulating pro-inflammatory cytokines and a decrease in regulatory-associated markers [198]. Thus, systemic hypoxia appears to be linked with pro-inflammatory mechanisms.

Microelectrodes have been utilised to measure oxygen levels of tissues experimentally, and averages of around 3% oxygen have been recorded in RA joints [199-202]. This is lower than those that have been found for osteoarthritic (5.6% oxygen) and healthy joints that had experienced trauma (8.2% oxygen) [199]. In RA, joint oxygen levels negatively correlate with macroscopic synovitis [200], mutations in mitochondrial DNA [202], lipid peroxidation [201] and lactate production [203] highlighting the glycolytic switch that occurs when oxygen is more limited. Hypoxia is also known to prevail in other autoimmune conditions such as systemic sclerosis, and also in the tumour microenvironment [204-206]. One reason for this includes poor oxygen delivery. Blood vessel occlusion caused by infiltrating phagocytes and the increasing distance between blood vessels caused by oedema result in decreased oxygen perfusion of tissue. Additionally, infiltrating cells place extra demands on the local oxygen supply, causing its localised depletion [200, 201, 207, 208]. Many chronic inflammatory conditions are associated with neovascularisation, and this is often thought to be in response to hypoxia found in the inflammatory environment [9, 209]. This neovascularisation can often be disorganised and inadequate, resulting in badly formed vessels that can be detrimental to the environment and actually reduce oxygen delivery to the tissue, further contributing to hypoxia [9, 35, 196, 210] .This is probably the case in RA, where blood vessels are immature and unstable, and are

associated with low oxygen levels. It is therefore possible that the hypoxic environment in the joint may cause constant remodelling of blood vessels and contribute to pathology [210]. In effect, angiogenesis never seems to catch up with oxygen turnover, and hypoxia therefore prevails [210]. However, it is not known whether low environment oxygen levels contribute to the initiation of the disease.

1.2.1 Reperfusion injury after hypoxia

Reperfusion injury is a phenomenon where a previously sustained hypoxic site is again exposed to higher oxygen levels. Reperfusion injury is associated with ischaemic inflammatory conditions such as graft rejection, stroke and myocardial infarction [211-213], and if not caused by an inflammatory response, is normally accompanied by one. In a reperfused tissue endothelial cells activate incoming inflammatory cells by upregulating the expression of adhesion molecules and releasing inflammatory mediators [211-214]. Reperfused tissues are associated with oxidative damage of cellular structures and signalling molecules and can result in tissue damage [215, 216]. Both endothelial cells and activated neutrophils contribute to oxidative stress associated with reperfusion injury due to the release of ROS [211, 214, 216]. These ROS originate from several sources including the enzyme xanthine oxidoreductase and mitochondria [211, 214, 215, 217]. Xanthine oxidoreductase is an enzyme normally involved in oxidising hypoxanthine to xanthine, but during ischaemia it is converted to a form that preferentially reduces oxygen to superoxide. When oxygen becomes more readily available during reperfusion the presence of this converted form of the enzyme increases cellular ROS production [211, 215, 218].

However, its involvement in reperfusion-injury associated pathology has been questioned due to its production of vaso-protective nitric oxide [218].

With regards to chronic inflammatory diseases, reperfusion injury is thought to play a role in pathology in liver disease [219] and autoimmune diseases such as RA [220-222]. The healthy joint is thought to maintain a similar intra-articular pressure even when pressure is placed on it. In RA, however, intra-articular pressures fluctuate in response to pressure and exercise. These changes in pressure can alter blood flow through the joint, and therefore give rise to periods of ischaemia and reperfusion [199, 220, 222]. Additional evidence of reperfusion injury in the rheumatoid joint comes from the oxidative damage of joints structures, and the detection of xanthine oxidoreductase [221, 223].

1.2.2 Hypoxia's effect on gene regulation: Introducing hypoxia inducible factor (HIF)

1.2.2.1 HIF

One of the best understood responses to cellular hypoxia is the stabilisation of the transcription factor HIF. HIF is a heterodimeric transcription factor consisting of α and β subunits. The β subunit (also known as aryl hydrocarbon receptor nuclear translocator or 'ARNT') is constitutively found in the nucleus. The α subunits are constitutively transcribed but their protein stability is variable depending on environmental oxygen levels and other stimuli [204, 224]. Three HIF- α subunits have been described but HIF-1α is the most well-understood. Normally they are found in the cytoplasm and are degraded rapidly in the presence of oxygen. However, when stabilised, they translocate to the nucleus, where they dimerise with β subunits, bind

hypoxia-response elements (HREs) in the DNA and initiate transcription of genes involved in metabolism (including pyruvate dehydrogenase kinase), angiogenesis (such as Vascular Endothelial Growth Factor (VEGF)), cell cycle and many other aspects of cellular biology [224-227].

1.2.2.2 Regulation of HIF

As shown in figure 1.3, two hydroxylated proline residues on HIF-α subunits allows the von Hippel Lindau tumour suppressor gene (VHL) complex to bind, targeting them for proteasomal degradation via ubiquitinylation. Hydroxylation at these key residues is due to the action of prolyl hydroxylase domain enzymes (PHDs) that use dioxygen as a substrate and act as direct sensors of environmental oxygen. Fe(II) is additionally required as a co-factor for successful PHD-directed proline hydroxylation, providing a means for *in vitro* manipulation of this system [224, 225, 228]. HIF-α subunits are also further regulated by Factor Inhibiting HIF-1 (FIH-1), another oxygen-sensitive regulatory enzyme that hydroxylates a key asparagine residue on HIF-α molecules. The asparagine residue hydroxylated by FIH-1 is normally involved in the binding of CBP/p300, a key co-factor for HIF-α transcriptional activity [225, 226]. As FIH-1 is active at lower oxygen levels than PHDs due to its higher affinity for oxygen [229], it exerts further delicate regulation of HIF activity.

1.2.2.3 Stabilisation of HIF

When oxygen is deficient, PHDs and FIH-1 are no longer able to hydroxylate the proline residues on HIF-α subunits. This stabilises cytoplasmic HIF-α subunits,

allowing them to translocate to the nucleus. The oxygen level that HIF-α stabilisation occurs at varies depending on cell type and HIF-α isotype. In a study where oxygen was increasingly depleted, HIF-1α protein detection increased between 5-2% oxygen in all cell lines tested [230]. HIF-1α is also only stabilised in stimulated T lymphocytes at 5% oxygen, but is more strongly stabilised at 3% oxygen and below [204]. Variability in the oxygen level at which HIF-1α is stabilised may be due to intrinsic differences in cellular metabolism and thus different oxygen turnover rates within the cytoplasm [225, 230]. Therefore, in this thesis, oxygen levels of below 5% are deemed ‘hypoxic’ which is in agreement with much of the literature [204, 230-240]. 5% oxygen itself is probably a reasonably normal and healthy oxygen level physiologically, and found in smaller blood vessels and some tissues [204, 235, 236, 241-244]. This definition does mean that several physiologically ‘normal’ oxygen levels are therefore hypoxic, such as those seen in the thymus [245, 246] and eye [247]**.**

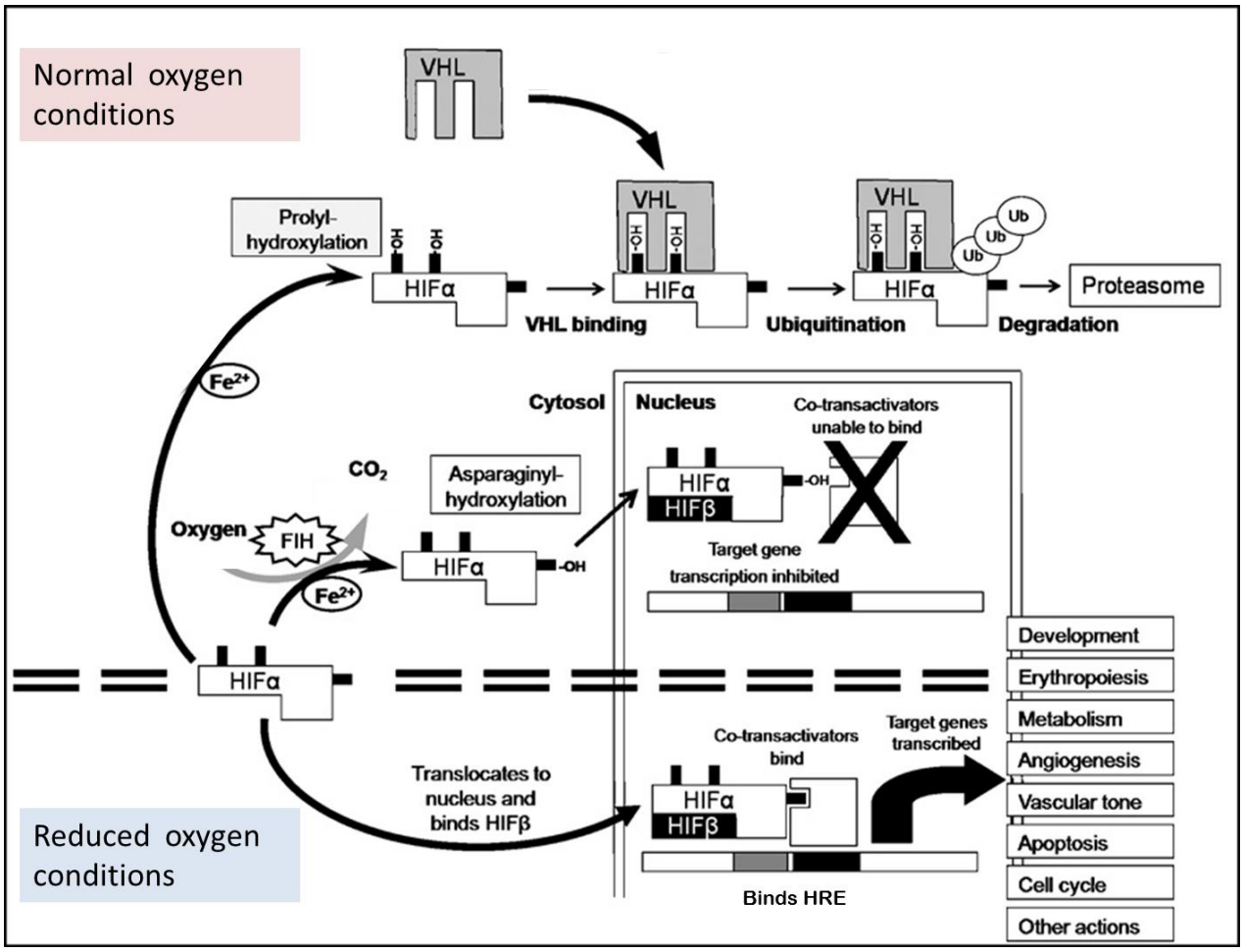

Figure 1.3 The stabilisation of HIF-α subunits under reduced oxygen conditions*. In normal oxygen conditions, prolyl hydroxylases domain enzymes (PHDs) hydroxlate two prolyl residues in the HIF-α subunits, targeting them for proteasomal degradation via ubiquitinylation via von Hippel Lindau tumour suppressor gene (VHL) complexes. Furthermore, Factor Inhibiting HIF (FIH) hydroxylates an asparagine residue important for transcriptional co-factor binding, converting oxygen to carbon dioxide in the process. When oxygen is reduced, both the PHDs and FIH-1 are inhibited in their action due to the lack of oxygen, and hence HIF-1α can translocate to the nucleus, dimerise with HIF-β and its transcriptional co-factors, and initiate transcription of genes associated with multiple cellular processes. Adapted from [224].*

1.2.2.4 Expression of HIF in inflammation

HIF-1α expression has been detected in several inflammatory sites. In RA, both HIF-1α and 2α expression has been detected [208, 248-252]. Specifically, HIF-1α expression has been observed in RA macrophages [250, 252] synoviocytes [251, 252], T lymphocytes [204, 252] and plasma cells [252] indicating that all of these cell types may experience hypoxia in the RA joint. HIF expression is also detected in other inflammatory tissues associated with autoimmune diseases including MS [253] and inflammatory bowel disease [234].

1.2.3 Mitochondrial responses to hypoxia and reperfusion injury

Low environmental oxygen levels can result in the increase in ROS production from the mitochondria [254-256]. Counterintuitively, this requires dioxygen, and therefore has the potential to perpetuate intracellular hypoxia [254, 255]. ROS production from the electron transport chain in reasonable oxygen levels most commonly occurs at complex I. During hypoxia, however, complex III has been credited with most ROS production [254, 256], producing superoxide which is rapidly converted to hydrogen peroxide (H_2O_2) by mitochondrial superoxide dismutase (MnSOD) which then leaks out of the mitochondria. It is now understood that this ROS signal is important in cellular responses to hypoxia. For example, mitochondrial ROS can stabilise NFκB [257], and is probably supported by the hypoxic-induced downregulation of anti-oxidants such as thioredoxin [258, 259]. There is also evidence that mitochondrial ROS can stabilise HIF-α by inhibiting PHD activity [255, 256], although this has been challenged [258].

Hypoxia also alters mitochondrial biology during reperfusion [211, 260, 261]. Post-transcription alterations in mitochondrial membrane proteins occur during oxygen deprivation [215, 260], and mitochondrial anti-oxidants become less available [211, 258, 259]. This therefore limits the capability of mitochondria to deal with subsequent re-exposure to oxygen and can result in mitochondrial ROS production. The membrane potential of the mitochondria is also depressed during hypoxia, reducing the mitochondria's ability to generate ATP [211, 215, 261]. When oxygen is re-introduced the membrane will instead hyperpolarise; a state in which ROS production is more favoured [261]. The combination of activated inflammatory cells and ROS can result in considerable tissue damage [212].

1.2.4 Hypoxia and its effect on the innate immune system

Innate cells of the immune system are often the first cells to respond to a pathogen or inflammatory stimuli. The environments into which these cells migrate can be physiologically harsh, being both low in oxygen and depleted of other key nutrients. However, evidence suggests that innate immune cells are well-adapted to the hypoxic environment. For example, macrophages and neutrophils utilise glycolysis even when oxygen is abundant [262], and neutrophils have delayed apoptosis in hypoxic conditions [25, 263]. Macrophages and neutrophils also both show increased phagocytic activity at low oxygen levels, and HIF-1α expression appears to be imperative for their proper pathogenic clearance [227, 242, 262, 264-266]. HIF-1α also regulates macrophage cytokine production, as TNF-α production is depleted in HIF-deficient macrophages [227]. In contrast, hypoxia may depress neutrophil cytokine production [267]. Interestingly, animals with myeloid-specific HIF-1α

deficiency had lower clinical scores of arthritis, indicating that hypoxia and HIF-1α stabilisation may play a role in maintaining pathology via the activities of innate immune cells [227, 242]. Oxygen levels can also influence the behaviour of APCs. One study using kidney tubule clamping, a model of reperfusion injury, observed an increase in the maturation of resident DCs [268] and an increase in the expression of CD80 and CD86 has also been observed on DCs cultured at constant 1% oxygen and below [268, 269].

1.2.5 Low environmental oxygen levels and their effect on the adaptive immune system

Akin to innate immune cells, the cells of the adaptive immune system are not likely to experience high oxygen levels physiologically. Measurements with microelectrodes have shown the murine thymic oxygen partial pressure to be around 10mmHg (around 1.3% oxygen) [243, 245, 246]. Furthermore, measurements of the murine spleen revealed changeable oxygen levels, varying between 0.5-4.5% oxygen in one study [239], and 1.3-3.3% oxygen in another study [245]. Rabbit spleens have higher measured oxygen levels than those recorded in mouse [245], and it therefore cannot be ruled out that human lymphoid organ oxygen levels vary from what has been reported in the literature.

In both the thymus and spleen, proximity to blood vessels influences local hypoxia, with increasing distance from the blood vessel resulting in reduced oxygen levels [239, 246]. Increased metabolic activity in these tissues may also deplete hypoxia [245]. Lymph fluid is also hypoxic, with decreasing oxygen levels compared to corresponding tissue and blood vessels [270]. Lymphatic vessels contain varying

oxygen levels, with the greatest oxygen levels in large lymphatics due to increased proximity to blood vessels [270]. Lymphocytes will also experience a wide variety of oxygen levels in blood circulation, depending on whether they are in well-oxygen perfused alveolar capillaries, or less oxygenated venous capillaries [224]. With regards to chronic inflammatory environments observed in autoimmune diseases, the hypoxic environment will be acute to any T lymphocyte migrating in. However, they soon become part of the chronic architecture of the disease. Thus, oxygen levels will peak and trough throughout a CD4+ T lymphocyte's lifetime, but they often are exposed to oxygen levels below 5% oxygen, and never will experience 21% oxygen physiologically.

1.2.5.1 Experimental observations of the effect of hypoxia on T cells

The majority of *in vitro* work carried out with T lymphocytes is performed at 21% oxygen, the oxygen level of air at sea level. However, as already outlined, this experimental condition is inappropriate. In the literature, terminology with regards to hypoxia can be slightly misleading. Some papers will label 21% oxygen as 'normoxia'. It is 'normal' in respect to current experimental procedures, but is not a 'normal' oxygen level found physiologically. In fact, T lymphocytes will never experience this oxygen level physiologically, and it may be better to term 21% oxygen as 'hyperoxia'. Several studies have investigated the effect of low oxygen on T lymphocyte activity and behaviour, and have revealed varying reponses from T lymphocytes at different oxygen levels.

1.2.5.1.1 Varying oxygen levels: effect on CD4+ T cell activation and signalling

Effect of environmental oxygen levels on the T lymphocyte oxidative response

Culture of mixed CD4+ and CD8+ T lymphocytes at 21% oxygen reduces levels of glutathione, a major cellular antioxidant, compared to culture at 5% oxygen [244]. Concurrent with this observation, increased oxidative damage has been reported during 21% oxygen culture [271]. CD4+ T lymphocytes reveal different patterns of intracellular ROS production in response to 1% oxygen compared to 21% oxygen, with a delay in initial intracellular ROS in hypoxia, but a steady subsequent increase observed thenceforth [235]. 5% oxygen revealed similar patterns to culture at 21% oxygen, with only an initial peak in ROS observed (associated with the initial TCR signal) [235]. Hence lymphocytes cultured at different oxygen levels have altered behaviour as soon as signalling is initiated, and this may take the form of altered resilience to oxidative attack.

Effect of hypoxia on calcium influx

One of the first events to happen after T lymphocytes signalling is the mobilisation of calcium stores resulting in a calcium signal [68, 272]. The potential of the T lymphocyte outer membrane is maintained by differential expression and activation of calcium and potassium channels [273, 274]. Activation of T lymphocytes causes mobilisation of intracellular calcium stores, which subsequently results in the activation of plasma membrane calcium release activated channel (CRACs), giving rise to extracellular calcium influx and intracellular calcium oscillations. Membrane potential is maintained, and balanced, by the expression of potassium channels such as Kv1.3. Potassium channels are able to hyperpolarise the membrane by the

removal of cellular potassium, thus increasing the electrochemical gradient across the membrane and promoting the movement of calcium into the cell [273, 274].

Hypoxia (1% oxygen) inhibits Kv1.3 channel function in T lymphocytes [275] resulting in the depolarisation of the membrane, and reducing extracellular calcium influx and the calcium oscillations required for T cell activation. This alteration in membrane potential was observed as soon as T lymphocytes were placed into a hypoxic environment and resulted in a depression in calcium signalling in around half of T lymphocytes pre-stimulated by phytohaemagglutinin (PHA). Calcium influx was very variable depending on donor and cell subsets present within the population, revealing the complex nature of calcium signalling in different T lymphocyte subsets [273]. These observations are particularly interesting as T lymphocytes in the rheumatoid joint have reduced calcium signalling [41]. A longer duration of exposure to 1% oxygen also reduced Kv1.3 membrane expression [275].

Effect of HIF on activation and signalling

In T lymphocytes, TCR engagement – via mammalian target of rapamycin (mTOR)-dependent processes - is required for the hypoxic-induced stabilisation of HIF-1α protein, whereas resting cells do not appear to stabilise its protein expression [204, 224, 238, 276, 277]. VHL deficient T lymphocytes, (in which HIF-1α is constitutively stabilised), have reduced calcium signalling in response to TCR signalling caused by increased endoplasmic reticulum and mitochondrial uptake of cytoplasmic calcium [278]. It is therefore possible that the stabilisation of HIF-1α post-TCR signalling may function as a negative feedback regulatory mechanism, inhibiting further calcium signals [279].

1.2.5.1.2 Apoptosis

A considerable number of studies do not observe a difference in apoptosis or viability in peripheral blood mononuclear cell (PBMC) or CD4+ T lymphocytes stimulated in hypoxia compared to culture at higher oxygen levels [237, 280-283], even up to 7 days culture [283]. In fact some papers describe an increased viability at lower oxygen levels [204, 284, 285], and this may be potentially due to the stabilisation of HIF-1α [204, 224]. However, there are some reports of increasing apoptosis in low environmental oxygen conditions of both PBMC mixed cultures and pure T lymphocyte populations [240, 244] and HIF has been implicated as a cause [279, 286] contradicting other literature [204, 224]. PHA stimulation of CD4+ T lymphocytes at 1% oxygen resulted in increased apoptosis compared to 5 and 21% oxygen but resting cells were unaffected [235] . Naïve T lymphocytes may be more susceptible to apoptosis in low environmental oxygen [236, 287], whereas effector T lymphocytes appear to be much more protected and resilient [287]. As different experimental procedures exist between laboratories, it is possible that simple variance in cell density or the presence of anti-oxidants in medium may give rise to contradictions within the literature.

1.2.5.2 Proliferation

1.2.5.2.1 Proliferation in more than 5% oxygen

Physiologically healthy oxygen levels of 5% and above generally reveal a different pattern in proliferation compared to 21% oxygen cell culture, however, the literature is contradictory. Several groups have observed an increase in proliferation at 5% oxygen in PBMC cultures compared to 21% oxygen culture [284, 285, 288], whereas

alternative studies have found the opposite [244, 281] or find culture at 5% similar to 21% oxygen [235].

1.2.5.2.2. Proliferation in less than 5%oxygen

At oxygen levels less than 5%, a different pattern begins to emerge in the proliferative capacity of T lymphocytes revealing the intricate and subtle influence environmental oxygen levels can have on lymphocyte biology. Several reports indicate that proliferation is reduced in oxygen levels lower than 3% [235, 236, 240, 275, 280, 282, 287, 289, 290]. For example, PBMC stimulated with PHA for 40 hours at 2% oxygen had a lower DNA content than those stimulated at 21% oxygen [282]. Additionally, pure CD4+ T lymphocytes populations cultured in low environmental oxygen levels revealed a reduction in proliferation when stimulation with PHA [235, 289] or with anti-CD3/anti-CD28 antibodies [240] compared to those cultured in 21% oxygen oxygen. This reduction in proliferation maybe subset-specific, with a greater proliferative defect in effector CD4+ T lymphocyte subsets compared to cells with a more regulatory phenotype [291]. Furthermore, a study that generated T lymphocyte clones from healthy donors observed that long-term culture at 3% oxygen reduced replication compared to 21% oxygen culture [271]. However, observations of no difference in proliferation between low oxygen (2%) and 21% oxygen culture have also been reported [283].

1.2.5.2.3 Differences in the literature regarding effects of environmental oxygen on proliferation

Differences in the literature may have arisen due to the different experimental procedures used including the selection of particular CD4+ T lymphocyte subsets, purity of lymphocyte populations investigated, and stimulation methods examined. It is possible that differences in response to environmental oxygen may only be observed after stimulation through the TCR, as some report no differences in response to PMA/ ionomycin stimulation in hypoxia compared to membrane-receptor engagement [275]. However, this is not consistently observed. Indeed, one study found that whilst a difference in proliferation could be observed at different oxygen levels after Con A or anti-CD3/ anti-CD28 stimulation, no difference could be found after PHA stimulation [281]. This lack of difference after PHA stimulation was also observed in another study, whereas PMA/ ionomycin stimulation did reveal a difference [235], questioning the role of signalling through the TCR. Therefore, the picture is still incomplete as to how environmental oxygen levels influence T lymphocyte proliferation.

1.2.5.3 Cytokine production and Polarisation of CD4+ T lymphocytes

The effects of hypoxia on purified T lymphocyte cytokine production is also not completely clear. Some papers find an increase in cytokine production in hypoxia [205, 235, 237, 292], whereas others find a reduction [204, 236, 239, 243, 289, 293].

1.2.5.3.1 An increase in T lymphocyte cytokine production in response to low environmental oxygen

An increase in cytokine production at lower oxygen levels has been observed in response to several different stimulation methods. Roman *et al* found that stimulating murine CD4+ T lymphocytes for 48 hours with anti-CD3/ anti-CD28 resulted in increased detection of IL-2, IL-4 and IFNγ levels at 1% compared to 21% oxygen, although IFNγ mRNA was reduced. IFNγ and IL-4 secretion levels continued to increase over 72 hours. Preliminary investigations into the role of cytokine turnover and receptor expression showed that this did not play a large role in the difference in cytokine levels observed [237] suggesting the increase was due to heightened expression. In another study, PHA stimulation revealed an increase IL-2, IL-3 and IL-6 production at 1% oxygen compared to 21% oxygen [235]. In addition, a PHA-stimulated transcriptome analysis of T lymphocytes stimulated under different oxygen levels also found an increase in pro-inflammatory cytokine transcript levels in hypoxia; IL-6, IL-1β, IL-23A and TNFα transcripts were increased in human CD4+ T lymphocytes placed in 1% oxygen compared to 21% oxygen [205]. Pure CD4+ cultures placed in cumulative hypoxia by the use of a sealed container also increased production of IL-2, IL-6, IL-8, IL-10 and IL-1β in response to PMA/ ionomycin stimulation [292].

1.2.5.3.2 Reduced T lymphocyte cytokine production in low environmental oxygen

In other studies, a drop in CD4+ T lymphocyte cytokine production has been observed at lower oxygen levels [204, 236, 239, 243, 289, 293]. Murine naïve CD4+ T lymphocyte IFNγ and IL-17A intracellular staining was reduced at 1% oxygen

stimulation compared to both 5% and 21% oxygen [236]. A DNA microarray of peripheral human T lymphocytes found no change in the expression of some cytokines in response to stimulation in hypoxia but IFNγ, GM-CSF and TNFα transcripts were downregulated compared to stimulation at 21% oxygen [204].

1.2.5.3.3 The effect of HIF on cytokine production

The deletion of HIF-1α has provided some interesting results with regards to explaining the effects of hypoxia on cytokine production. HIF-1α deficient T lymphocytes produce more IFNγ, TNFα, IL-4, IL-13 and IL-2 [243, 293, 294]. However, the *ifnγ* and *il2* genes are known to not contain HREs [239], suggesting complex mechanisms may be involved in the regulation of these cytokines in response to differing environmental oxygen. In a murine model of colitis IL-10. IL-1β, IL-6, IL-17, IL-23, IFNγ, IL-12a and TNFα were all significantly upregulated in the colonic mucosa of T lymphocyte-specific HIF1α-deficient mice compared to wild type [234]. This suggests that HIF stabilisation may have a negative effect on cytokine production.

1.2.5.4 Polarisation

Observations of the effect of hypoxia on cytokine production begins to give some clues as to the effect hypoxia has on CD4+ T lymphocyte polarisation. However, current literature is somewhat contradictory. Some papers have found increases in cytokines associated with both Th1 and Th2 subsets [237, 282], suggesting that hypoxia may not influence polarisation, but enable better overall effector functions.

However, other papers suggest that hypoxia favours the formation of distinct CD4+ T lymphocyte subsets, although there is some disagreement as to which subsets are favoured. The strongest evidence for the role of hypoxia in the regulation of T lymphocyte phenotype is in differentiation to Th17 and Treg subtypes, as discussed below. The different metabolic requirements of the various T helper cell subsets needs to be considered when probing the literature. Th17, Th2 and Th1 upregulate glycolysis upon activation [276, 295], whereas Tregs appear to utilise fatty acid oxidation [295]. This will influence their oxygen requirements and utilisation, and therefore their response to hypoxia.

1.2.5.4.1 Th1 or Th2?

The effect of hypoxia on Th1 and Th2 cell differentiation and function is not well understood. As already mentioned, cytokines associated with both Th1 and Th2 phenotypes are upregulated in response to hypoxia in certain models [237, 282]. However, it is possible that Th1 differentiation is favoured more than Th2 in an hypoxic environment [237]. Studies looking at HIF-1α expression in lung epithelial cells found that when HIF-1α was deleted, an eosinophilic, Th2-like inflammatory response ensued in response to cobalt treatment [296]. Cobalt normally results in the stabilisation of HIF-1α and is therefore used as a hypoxic mimetic. When HIF-1α was intact, a more pro-inflammatory response occurred associated with an increase in TNFα, suggesting the cobalt-stabilisation of HIF-1α reduced Th2 responses [296]. Lymphocytes were not directly investigated in this study, but the results give an indication of what effect overall environmental hypoxia may have on T helper cell differentiation.

Th1-associated cytokine secretion has been found to be upregulated in response to hypoxia in some studies [204, 205, 237]. Despite this, both *IFNγ* and *T-bet* mRNA have been observed to be downregulated at 1% oxygen [237]. HIF-1α overexpression results in IFNγ inhibition [297], whereas HIF-1α deletion appears to upregulate IFNγ production [293, 294]. As HIF-1α deficiency has resulted in the upregulation of many cytokines' expression [234] there may be additional regulatory factors at work here yet to be discovered, especially that, as already stated, the *ifnγ* gene locus does not contain an HRE [239]. Interestingly Th2 cells were observed to utilise glycolysis more than Th1 and Th17 cells during differentiation, but in a HIF-1α independent fashion, unlike Th17 cells [276]. Further understanding about Th2 cells' response to hypoxia is somewhat lacking. Despite an increase in IL-4 being found in response to hypoxia in some studies [237, 280, 282], a reduction in GATA3 mRNA has also been reported [237].

1.2.5.4.2 Th17and Treg cells

Pro-inflammatory Th17s response to hypoxia

Th17 and Treg cells differentiation follows similar pathways, and in murine cells both differentiate in response to TGF-β stimulation (with Th17 differentiation also requiring IL-6). However, it appears Th17 differentiation may be more favoured in low oxygen-conditions. Obstructive sleep apnoea syndrome (OSAS) is a disorder where blood oxygen levels can considerably drop during sleep, essentially causing whole-body hypoxia. Sleep apnoea has been found to increase the ratio of Th17 cells compared to Treg cells circulating in OSAS patients' blood [198]. The stabilisation of HIF-1α is potentially important in the differentiation of Th17 cells from naïve precursors.

Increased HIF-1α expression has been observed in Th17 polarised T lymphocytes [120, 236, 276, 298], with Th17 cells upregulating HIF-1α mRNA more than their Th1, Th2 and Treg counterparts [276]. HIF-1α deficiency is associated with protection from the development of experimental autoimmune encephalomyelitis (EAE) which is commonly associated with Th17-mediated pathology [276, 277]. In *in vitro* experiments performed at 21% oxygen, HIF-1α deficiency was found to inhibit the expression of RORγt and IL-17 in stimulated murine CD4+ T lymphocytes polarised towards a Th17 phenotype. True to HIF-1α's function as an oxygen sensor, HIF-1α-intact cells doubled IL-17 production in hypoxia compared to their 21% equivalents, whereas limited IL-17 was detected in HIF-1α deficient T lymphocytes [276, 277]. Additionally, a model of reperfusion injury found that initial stimulation of differentiating Th17 cells at 3% and 5% oxygen resulted in increased, HIF-1α-dependent IL-17 production. However, at 1% oxygen with reperfusion injury, IL-17 production was reduced [236].

Stabilisation of HIF-1α in Th17 cells has been found to be via both STAT-3 induction [277] and mTOR signalling [276] and may provide an additional advantage to Th17 cells in an hypoxic inflammatory environment, promoting their survival and function. Indeed, HIF-1α expression in Th17 cells is associated with an upregulation of glycolysis [276] and the inhibition of apoptosis [120]. HIF-1α also appears to promote the Th17 phenotype through its master regulator, RORγt, as these two proteins co-operate (and co-immunoprecipitate) to promote IL-17 expression, and potentially cause the degradation of Foxp3 [277, 299], although this result has been questioned [291]. Furthermore, the RORγt promoter contains an HRE, making it HIF-1α-responsive [277, 299].

Other factors present in the hypoxic environment also seem to promote Th17 differentiation. For example, IL-6 is required for naïve T lymphocyte differentiation to a Th17 phenotype and IL-6 has been found to be upregulated in response to hypoxia [205, 282]. Additionally, mitochondrial ROS can promote IL-17 production. In immediate early response gene X-1 (IEX-1)-deficient mice mitochondrial ROS is increased. When T lymphocytes from these mice were differentiated to a Th17 phenotype, IL-17 production was upregulated, but anti-oxidants were able to abrogate this response [300].

Hypoxia favours a regulatory phenotype?

There is ongoing debate as to the role of HIF-1α in the development of Th17 and Treg cells [276, 277], with cells needing to make a 'checkpoint decision' about which way to differentiate. As discussed, strong evidence favours Th17 differentiation from a naïve T lymphocyte. As nTregs are formed in the thymus, and are often selected for in *in vitro* experiments due to their CD25 expression even when resting, it is difficult, and potentially ill-advised to compare them to Th17 cells differentiated from naïve T lymphocytes in the periphery. iTregs do not always express all the markers associated with nTregs, and therefore are not always compared correctly with Th17 cells. Despite this, there is some evidence to suggest that once cells are on the differentiation pathway to becoming, or already are fully differentiated to a Treg, hypoxia and stabilisation of HIF-1α may promote regulatory function. Indeed, hypoxic tumours preferentially recruit differentiated Tregs, suggesting that Tregs are primed to function in environments low in oxygen [301].

Hypoxia promotes *FOXP3* transcription in resting CD4+ T lymphocytes [234], potentially via HIF-1α stabilisation due to the *FOXP3* promoter containing a HRE [291, 302] and Foxp3 expression is upregulated in HIF-1-intact cells [234]. Suppressive capabilities of CD4+CD25+ T lymphocytes were increased after hypoxic exposure (although the experiment modelled reperfusion injury to some extent) and hypoxic CD4+CD25+ cells were found to express more Foxp3 than their counterparts exposed to higher oxygen environmental oxygen levels [302]. Whole body hypoxia also increased Treg expression of CD25 and CTLA-4 [291].

When CD4+ CD25+ Tregs deficient in HIF-1α were transferred alongside CD45RBhigh effector cells in a model of colitis, the inflammation that followed was much more severe than if HIF-1α-intact Tregs were transferred, suggesting proper HIF-1α expression and function was required *in vivo* for differentiated Treg suppression [291]. Interestingly, overexpression of HIF-1α results in the upregulation of IL-10 and TGF-β in murine splenocytes, suggesting anti-inflammatory mechanisms may be promoted in hypoxic environments [297]. Despite this, HIF-1α deficiency has also been found to increase the number of Foxp3+ cells detected in lymphoid tissue [277], adding confusion to the current literature.

1.2.5.4.3 Reperfusion injury and polarisation

CD4+ T lymphocytes are known to play a role in reperfusion injury [215, 303] as the depletion of CD4+ T lymphocytes before reperfusion can protect from tissue damage [303]. T_{EM} can be detected up to 11 weeks after reperfusion, and are most probably pro-inflammatory in nature [303, 304]. IL-12 deficient mice are protected from reperfusion injury, whereas IL-4 deficient mice develop exacerbated tissue damage

[305], suggesting intermittent hypoxia promotes the development of Th1-associated inflammatory responses via APC priming. Additionally, increased IFNγ has been detected in reperfusion models, and Th2 phenotypes have been described as protective [304, 305]. However, in naïve T lymphocytes differentiated under different polarising conditions, an inhibition in both IL-17A and IFNγ production resulted after culture at 1% oxygen with reperfusion injury (by being moved to 21% oxygen) compared with 5% oxygen and constant 21% oxygen [236].Therefore, the effects of reperfusion injury on CD4+ T lymphocyte polarisation needs further investigation.

1.3 Hypothesis

CD4+ T lymphocytes will experience many different oxygen levels physiologically, and low oxygen levels are prevalent in the inflammatory environments such as those seen in RA. In this thesis, CD4+ CD45RO+ T lymphocytes cells were chosen for most of the investigation as they are the CD4+ subsets most commonly observed in the RA joint [40, 156, 306], and will be the majority of cells that migrate out of the lymph node and into inflamed tissues [148]. The effect of various, physiologically-relevant oxygen levels on the activation and function of this cell population has not been previously determined.

It is **hypothesised** that environmental oxygen levels regulate CD45RO+CD4+ T lymphocyte proliferation, cytokine production and polarisation. Furthermore, it is predicted that this is caused by fundamental alterations in T lymphocyte activation due to differences in cellular homeostasis and signalling machinery. Five different oxygen levels were selected for analysis; 21% as an example of what most *in vitro* work is performed in; 8.5% as a potential example of the average healthy joint [199,

208, 307]; 3% as an example of the average of a RA joint and some physiological environments such as the lymph node [199-202, 208, 239]; constant 1% oxygen as an example of a more severely inflamed joint [200]; and reperfusion injury as an example of a tissue where oxygen levels fluctuate [219, 220].

2. Methods

The effect of various oxygen levels on CD4+ CD45RO+ T lymphocytes was assessed by using a hypoxystation that allowed close control of environmental oxygen levels. CD4+ CD45RO+ T lymphocytes were isolated from healthy donors and frozen until required. Cells were equilibrated to the desired oxygen level for 24 hours before being stimulated and assessed for different outcomes, such as surface marker expression, cytokine secretion, and intracellular cytokine production. Treatment with deferoxamine mesylate (DFX) and H_2O_2 allowed for further investigation of HIF-1α stabilisation and ROS on these outcomes, respectively.

2.1 Antibodies and Reagents

Antibody	**Company**	**Catalogue Number**	**Use**
Purified anti-CD3	Immunotools, Friesoythe, Germany	21330280	Plate stimulation
Purified anti-CD28		21270030	
PE anti-human CD45RO	Biolegend (London, UK)	304206	Flow cytometry
PE mouse IgG2a k isotype control		400212	
PE anti-human CD3		300308	
PE Mouse IgG2b k isotype control		400312	
APC Cy7 anti-human CD69		310914	
APC/Cy7 Mouse IgG1 k isotype control		400128	
APC Cy7 anti-HLA-DR		307618	
APC/Cy7 Mouse IgG2a k Isotype control		400230	
Pacific Blue anti-human IFNγ		502522	

Pacific Blue Mouse IgG1 k isotype control		400151	
PE anti-human IL-17A		512306	
PE Mouse IgG1 k isotype control		400140	
FITC anti-human IL-4		500806	
FITC Rat IgG1 k isotype Ctrl		400406	
PE/Cy7 anti-human IL-10		501420	
PE/Cy7 Rat IgG1 k isotype control		400415	
PerCP/Cy5.5 anti-T-bet		644806	
PerCP/Cy5.5 anti-human CD194 (CCR4)		335403	
PerCP/Cy5.5 mouse IgG2b k isotype ctrl		400338	
APC anti-human CD183 (CXCR3)		353708	
APC Mouse IgG1 K isotype control		400120	
PE anti-human CD152 (CTLA-4)		349906	
Alexa-Fluor 647 mouse IgG1 k isotype control		400130	
Anti-Human CD196 (CCR6) PE/Cy7	eBioscience (Hatfield, UK)	25-1969	
Anti-human Foxp3 eFluor 450		48-4777	
Mouse IgG1 K isotype control eFluor 450		48-4714	
Mouse IgG1 k isotype control Pe-Cy7		25-4714	
Mouse IgG1 K Isotype Control PerCP-Cy5.5		45-4714	
PE mouse anti-human Lck (pY505)	BD Biosciences (Oxford, UK)	558552	

Alexa Fluor 647 Mouse anti-GATA3		560068	
Anti-ROR gamma antibody ; (4G419) (FITC)	Abcam (Cambridge, UK)	ab104906	
Goat anti-rabbit IgG (H +L) FITC conjugated	Southern Biotech (Birmingham, AL, USA)	4050-07	
Purified anti-IL12 (p70)	Biolegend (London, UK)	511001	Polarising naïve T lymphocytes
Purified anti-human IL-4		500701	
Purified anti-IFNγ pure	Miltenyi (Surrey, UK)	Cat. No. 130-095-743	
Recombinant human IL-4 stock 10µg/ml	peprotech	200-04	
Recombinant human IL-12 stock 10µg/ml		200-12	
Hydrogen peroxide (H_2O_2)	Sigma-Aldrich (St. Louis, MO, USA)	H-1009	Treating cells
Deferoxamine mesylate (DFX)		D9533-1G	
N-acetyl-*L*-cysteine (NAC)		A9165	

Table 2.1. Antibodies and reagents used experimentally, including their sources and function

2.2 CD4+ memory T lymphocyte isolation and purification

PBMCs were obtained from buffy coats from the National Blood Service or from healthy controls under ethics code 12/WM/0077. Ficoll paque density gradient centrifugation was used to obtain PBMCs. Blood was layered above 10ml Ficoll paque (GE Healthcare, Buckinghamshire, UK) (Cat. No. 17-1440-03) and centrifuged for 30 minutes with no brakes or acceleration at 21°C. Memory CD4+ T cell isolation kit (human) (Miltenyi MACs, Surrey, UK) (Cat. No. 130-091-893) was used to isolate CD4+ memory T lymphocytes for experimentation from the whole PBMC population.

Briefly, PBMCs were incubated with a biotin-labelled antibody cocktail against CD8, CD14, CD16, CD19, CCD45RA, CD56, CD123, TCRγδ and glycophorin A. Cells were then washed in sterile 0.5% bovine serum albumin (BSA)/ 2mM ethylenediaminetetraacetic acid (EDTA)// phosphate buffered saline (PBS) and incubated with anti-biotin microbeads. When placed through a separation column placed on a magnet, labelled cells were retained and unlabelled CD4+ CD45RO+ cells passed through the column and collected. Double isolations were routinely performed to obtain optimum purity. Populations over 95% pure, as assessed by CD45RO and CD3 expression, were used experimentally. Cell populations were stored at -80˚C before use in 10% dimethyl sulfoxide (DMSO)/heat-inactivated fetal calf serum (FCS). Naïve CD4+ T lymphocytes were isolated using the same technique with the miltenyi MACs Naïve CD4+ T cell isolation kit II (human) (Cat. No. 130-094-131).

2.3 CD4+ memory T lymphocyte cell culture at varying oxygen levels

Cell populations were removed from the freezer and defrosted rapidly at 37°C. Cells were washed in culture medium (RPMI-1640 (Sigma Aldrich (St. Louis, MO, USA) (R0883)/ 10% FCS (Labtech (East Sussex, England) (10454) / 1% L-Glutamine-Penicillin-Streptomycin solution (Sigma-Aldrich (St Louis, MO, USA) (G1146)) and cell counted using trypan blue to exclude dead cells. Cells were again washed in culture medium and plated at $1x10^6$ cells/ml in 200µl culture medium in 96-well round-bottomed tissue culture plates. Cells were placed on ice before being placed in either a normal CO_2 incubator (Function Line, Thermo scientific) or H35 Hypoxystation (Don Whitley, West Yorkshire, England). Both incubators maintained

37˚C and 5% CO_2, and in the hypoxystation oxygen levels were balanced using nitrogen gas. Cells were equilibrated to the desired oxygen level in the correct incubator for 24 hours before stimulation with plate bound 2µg/ml anti-CD3// 5µg/ml anti-CD28. 96-well round-bottomed stimulation plates were prepared by placing 50µl 2µg/ml anti-CD3// 5µg/ml anti-CD28 in sterile PBS over night at 4°C. The following day the stimulation mix was removed and replaced with 100µl sterile PBS. Plates were stored frozen at -20˚C before use.

After cells had been stimulated for the required amount of time, they were placed on ice for 4-5 minutes at the oxygen level they had been cultured at to avoid the effects of reperfusion injury at 37˚C. For the reperfusion injury model, a Mini Galaxy A hypoxystation (Wolf Laboratories (York, England)) was sometimes used for culture at 1% oxygen. With all reperfusion experiments, cells were equilibrated at 1% oxygen but stimulated at 21% oxygen levels and kept there for a further 24 hours before being removed back to 1% oxygen for 48 hour stimulations. For 4 day stimulations, there was an additional 24 hour stint at 21% oxygen levels. This regimen was selected as a more physiological relevant oxygen level, such as 8.5%, could not be used due to other hypoxystation users.

For treatment of cultures with *N*-acetyl-*L*-cysteine (NAC), cells were placed directly in medium containing 1mM NAC and equilibrated to the indicated oxygen level. For treatment with DFX or H_2O_2 , cells were set up in 100µl culture medium at $2x10^6$ cells/ml and equilibrated to the indicated oxygen levels. 50µl of 10µM DFX or 40µM H_2O_2 were added, and the wells were made up to 200µl with oxygen-equilibrated culture medium.

Figure 2.1. The Don Whitley H35 hypoxystation. *This hypoxystation allows for set regulation of internal oxygen levels by balancing oxygen and nitrogen levels. These oxygen levels are maintained even when in use by the use of rubber sleeves and an airlock chamber that equilibrates to the desired oxygen level before allowing access to the inner oxygen-regulated chamber. Any oxygen level between 0-21% oxygen can be selected for and maintained over time in 0.1% increments.*

2.4 CD4+ memory T lymphocyte CFSE staining for proliferation

To assess proliferation, cells were incubated with 1μM carboxyfluorescein diacetate succinimidyl ester (CFSE) /PBS (Invitrogen (Camarillo, CA, USA) C34554) for 15 minutes at 37˚C before being quenched with ice-cold culture medium. Three washes in culture medium were performed before cells were equilibrated at the intended oxygen level for 24 hours. Cells were stimulated as described above for four days before proliferation was assessed on a CyAn ADP Analyser, Beckman Coulter (High Wycombe, UK). Stimulated cells were gated on for assessment of proliferation.

2.5 Cell viability assessment using Sytox

Cell viability can be assessed by using a dead cell exclusion DNA-stain, Sytox-Blue (Invitrogen, (Camarillo, CA, USA)) (Cat. No. S34857). Sytox-Blue associated fluorescence is detected when cells have a compromised membrane, and therefore is an excellent marker of dead cells and assessor of overall cell viability. 1ul Sytox was added to 500μl PBS, and then 30μl of this was subsequently added to 300μl cell suspension in 2% BSA/PBS for 30 seconds before analysis by flow cytometry using a 405nm laser. Sytox-Blue was kept away from light sources until used to limit degradation.

2.6 Naïve CD4+ T lymphocyte polarisation

Naïve CD4+ T lymphocytes were polarised to a Th1, Th2 or Th0 phenotype at the different oxygen levels investigated in this thesis. Cells were plated at 5x 10^5 cells/ml // 100μl/ well of a round-bottomed 96 well plate. After equilibration to the desired

oxygen level for 24 hours, the cells were stimulated with plate bound anti-CD3/ anti-CD28, as described, and 100µl 2x polarisation mix (made up in culture media) was added. Final concentrations for each polarisation condition were as follows:

- Th1; 5ng/ml recombinant IL-12// 2.5µg/ml anti- IL-4
- Th2; 50ng/ml recombinant IL-4// 20µg/ml anti-IFNγ// 5µg/ml anti-IL-12

Recombinant IL-2 was also added to a final concentration of 10ng/ml to each condition, including the Th0 control. Cells were stimulated for 36 hours before being assessed for surface marker expression.

2.7 Flow cytometry

For intracellular cytokine staining, cells were treated with 2µg/ml Brefeldin A (Sigma-Aldrich (St. Louis, MO, USA) Brefeldin A Ready Made Solution (B5936)) 3 hours before the end of the indicated stimulation period to inhibit extracellular cytokine release. Cells were stained for flow cytometry after being washed in 2% BSA/ PBS, and all surface-marker antibodies were diluted in 2% BSA/PBS. Surface marker staining was performed for 30 minutes on ice in 50µl antibody cocktail.For intracellular staining, cells were subsequently fixed (Invitrogen (Camarillo, CA, USA) Fixation Medium (A) (GAS001S100)), washed twice in 2% BSA/PBS and further stained in intracellular antibody cocktails diluted in permeabilisation buffer ((Invitrogen (Camarillo, CA ,USA) Permeabilisation Medium (B) (GAS002S100)) for 30 minutes on ice. In all flow cytometry panels, isotype controls were used at the matched concentration.

For assessment of Lck tyrosine-394 phosphorylation, cells were fixed at the oxygen level they were maintained at. This was achieved by adding paraformaldehyde/PBS to the final concentration of 4% directly to the 200µl cell populations. Cells were then spun at 300g and stained for Lck-394 in permeabilisation buffer as before.

A CyAn ADP Analyser, Beckman Coulter(High Wycombe, UK) was used to determine expression of markers. Countbright Absolute Counting Beads (Life Technologies (Eugene, OR, USA) (C36950) were used to maintain consistent MFI values over time. Several different panels were used, including a pro-inflammatory panel, an anti-inflammatory panel, a cytokine panel, a CD69/CD45RO panel and a HLA-DR/CD3 panel. For compensation, BD CompBeads Anti-mouse Ig, k/Negative Control (FBS) Compensation Particles Set (552843), and Anti-Rat IgG/ Negative Control (FBS) Compensation Particles Set (552844) were used. For the larger panels (of six colours), an experiment employing fluorescence-minus-one was done to determine the isotype allowance of each colour. Briefly, the panel was run on a population of CD4+ CD45RO+ T lymphocytes, but each colour was removed sequentially from a test and replaced with the equivalent isotype, thereby allowing the amount of crossover colour bleed (after compensation) within the panel to be determined and accounted for in further analysis. Details of the larger panels can be found in the appendices. Summit v4.3 software was used to analyse results.

2.8 Enzyme-linked immunosorbent assay ELISA

Supernatants were collected from 48 hour stimulated samples (with 3 hours Brefeldin A treatment at the end of the stimulation period) and stored at -80˚C until required. eBioscience (San Diego, CA, USA) Ready-SET-Go! ELISA kits were used to assess

supernatant cytokine levels; IL-4 (88-7046), IL-5 (88-7056), IL-17A (88-7176), IL-10 (88-7106), IFNγ (88-7316) and TNFα (88-7346). Briefly, Nunc maxisorp immunoplates (sigma Aldrich, Cat. No. M9410-1CS) were incubated with capture antibody diluted in coating buffer overnight at 4°C, were washed with 0.05% Tween-20/PBS. Wells were then incubated for 2 hours at room temperature with supernatant or standard prepared as according to the protocol. Wells were washed as before and incubated with detection antibody diluted in assay diluent. Wells were again washed and incubated with Avidin-HRP diluted in assay diluent before being washed and incubated with Substrate Solution. Development of ELISAs was stopped by using the half-volume of 2M H_2SO_4. Plates were read at 450/550nm using a plate reader and supernatant pg/ml values interpolated from the standard curve using Graph Prism 5 software.

A titration was performed on one supernatant to find the optimum dilutions to run the supernatants on (figure 2.2). The supernatants were diluted as follows; IFNγ 1/160; TNFα 1/80; IL-17A 1/4; IL-4 1/8; IL-5 1/4; and IL-10 1/40.

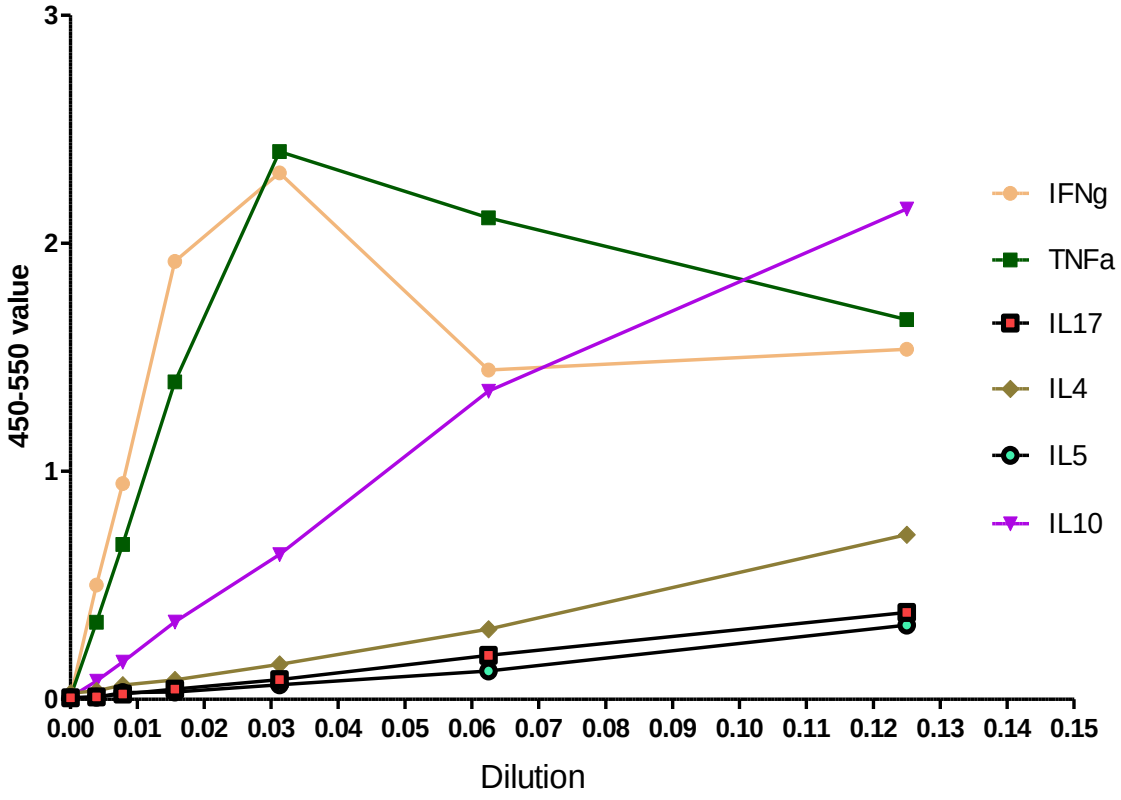

Figure 2.2 Determining the dilution factor required for ELISA. *One supernatant from a 48 hour stimulation (with the last 3 hours brefeldin A treated) was selected and diluted from between 1/8 and 1/256 with RPMI. ELISAs were done on the different dilutions for IFNγ, TNFα, IL-17A, IL-4, IL-5 and IL-10, and colorimetric results were read at 450/550nm using a plate reader. The readings were plotted against their corresponding dilution factor.*

2.9 Confocal microscopy

Confocal microscopy was performed on CD4+ CD45RO+ T lymphocytes that had been treated with several concentrations of DFX. Briefly, 1x 10^6 cells were left to equilibrate for 24 hours before being stimulated with 2µg/ml anti-CD3// 5µg/ml anti-CD28 whilst simultaneously being treated with concentrations of DFX varying

between 2.5μM to 10μM. 18 hours later the cells were directly fixed with paraformaldehyde to a final concentration of 4%. Fixed cells were washed twice in PBS at 4°C and then spun onto cytospins at 300g for 5 minutes. Slides were air dried and stored at -20°C until required for staining.

Staining for confocal microscopy was carried out as follows. Cytospin slides were defrosted and blocked with 1% BSA/ PBS for 30 minutes. Anti-HIF-1α (Thermoscientific, Hemel Hempstead, UK) (Cat. No. MA1-516) diluted 1/100 in 1% BSA/ PBS and incubated on the slides overnight at 4°C. Appropriate isotype controls were also used. Slides were washed three times in PBS and incubated with secondary antibody conjugated to Cy5 (diluted 1/250) for 1 hour at room temperature. Slides were washed as before and placed in 20μg/ml Hoescht solution for 1.5 minutes to stain the nucleus. Slides were mounted with a coverslip using DABCO and assessed for staining using a Zeiss LSM 510 confocal microscope. To analyse slides, ImageJ software was used. Lines were drawn through the nucleus and the number of Cy5-positive pixels determined.

2.10 Microarray

CD4+ CD45RO+ T lymphocytes, derived from the same donor, were stimulated at 8.5% and 1% oxygen as in section 2.3. RNA was isolated from 48 hour stimulations using a Qiagen RNeasy Mini RNA isolation kit. (Qiagen, Manchester, UK) (Cat. No. 74104). Briefly,cells were pelleted at 300g at then lysed using Buffer RLT. Equal volume of 70% ethanol was added and the lysis solution was placed on a silica membrane in a spin column. The membrane was washed with Buffer RW1 and Buffer

RPE, and the RNA was eluted from the membrane using 30ul RNase-free dH2O. RNA in solution was stored at -80°C until required.

To run the microarray, equal µg of RNA was first converted to cDNA using the Qiagen RT^2 First Strand Kit (Cat. No.330401). 96µl cDNA was then mixed with 1350µl Qiagen RT² SYBR® Green qPCR Mastermix (Cat. No 330520) and 12µl dH2O and 25µl mix was added to each well of a microarray plate (two plates were used, one for the 8.5% oxygen sample, and the other for the 1% oxygen sample). The microarray kit used was Qiagen's Human Th17 for autoimmunity and inflammation (Cat. No. 330221), of which the details can be found in the appendices. The plates were run on an Applied Biosystems 7500HT rtPCR machine running 1 cycle for 10 minutes at 95°C, followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. GAPDH was selected for as the housekeeping gene control.

2.11 Statistical analysis

For statistical analysis, the Mann Whitney U test was used as a test of non-parametric, non-paired statistical differences. Graph Prism 5 software was used for the generation of graphs and performing statistical analysis.

2.12 Method development

Fresh/frozen analysis

Due to the nature of the project, it was necessary to isolate CD4+ CD45RO+ T lymphocyte populations from several donors and then freeze them down before using them experimentally. The reasons behind this were twofold: Firstly, it allowed one

donor's T lymphocytes to be assessed at several oxygen levels. As only one Don Whitley hypoxystation was available for use, the number of oxygen levels that could be assessed at one time was limited. Therefore, freezing down samples allowed for fractions of an original donation to be used at different times, allowing for some homogeneity of donors across the data set. Secondly, it allowed more than one donor to be investigated at one time. As the hypoxystation worked by a booking system, oxygen levels had to be selected in advance. Due to this time constraint, and overall time constraints, it was necessary to have several donors ready to assess for each session at a specified oxygen level. As the hypoxystation maintained oxygen levels well, experimental variance was limited when investigating the same oxygen level at two difference time points.

The effects of freezing on the CD4+ CD45RO+ T lymphocyte populations on cytokine production and markers associated with polarisation was therefore investigated after 24 hours stimulation at 21% oxygen. As the cells investigated in this thesis were left for 24 hours to equilibrate to their oxygen environment, this additional equilibration step before stimulation was also included.

IL-17A, TNFα and IL-10 cytokine secretion appeared to be depressed in the frozen population (figure 2.3), whereas IL-4 and IL-5 cytokine production appeared to be less affected by freezing. In contrast, IFNγ cytokine production appeared increased in the frozen population.

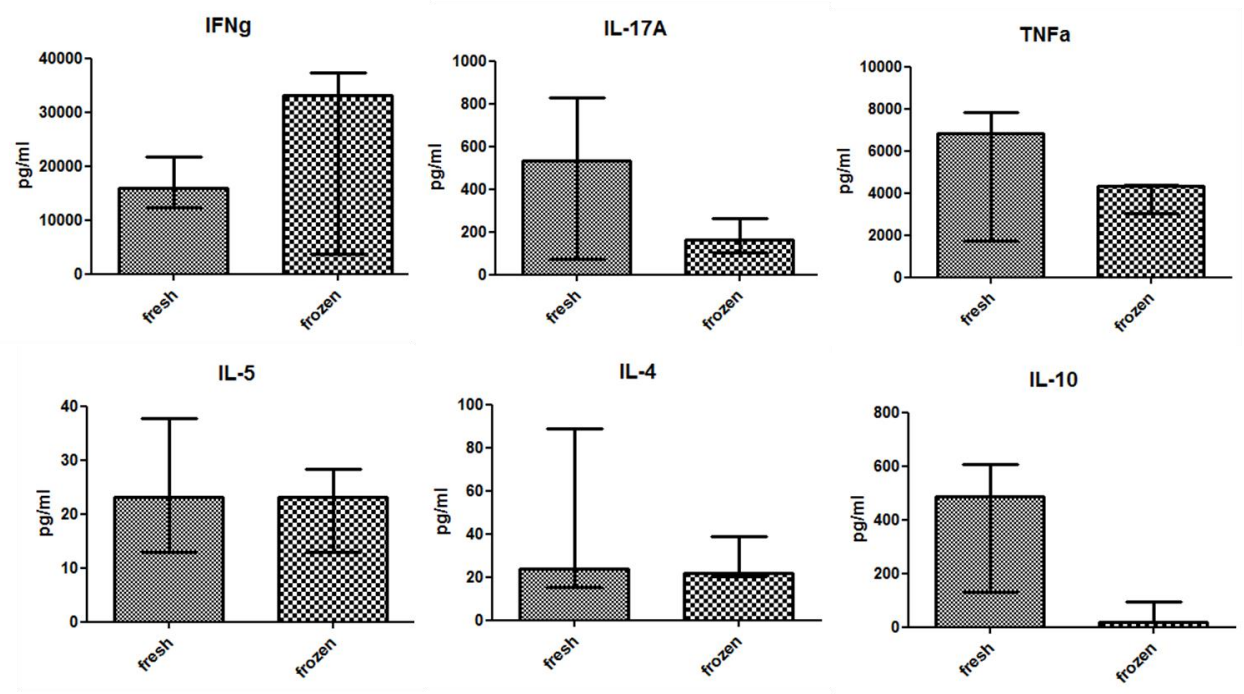

Figure 2.3. Assessment of cytokine production from fresh cells and those that had been frozen. *CD4+ CD45RO+ T lymphocytes were isolated using a Miltenyi MACs CD4+ memory isolation kit and either directly assessed or frozen at -80°C for later analysis. ELISA was performed on supernatants of T lymphocytes stimulated for 24 hours at 21% oxygen (with the last 3 hours brefeldin A treated concurrent with subsequent intracellular staining). 2x10^5 cells were equilibrated to the stated oxygen level for 24 hours before stimulation. Supernatant pg/ml was determined before further analysis was performed. Six cytokines were chosen for investigation. Cells were either assessed directly after isolation from PBMC populations (fresh), or after isolation and freezing at -80˚C (frozen). Data shows median values and range. n=3 per experiment. No significant differences were observed by Mann Whitney statistical analysis. Differential effects of the freezing process were observed depending on the cytokine concerned.*

Intracellular cytokine staining was also performed on the two polarisation panels described in section 2.7 and the Appendices. A large difference in the FS/SS plots was observed between the fresh and frozen cells, with the frozen population appearing to split into two different populations – one that did expand in size in response to stimulation (as expected), and one that did not (figure 2.5). These distinct populations could also be seen in non-stimulated controls (figure 2.4) and suggested that freezing cells resulted in a population of cells of diminishing size that subsequently appeared to not respond to stimulation (figure 2.5). In these non-stimulated controls no monocytic populations were observed, further confirming that the cells in the stimulation gate in stimulated populations were of lymphocytic origin. These non-stimulated controls therefore instructed further selection of FS/SS stimulation gates.

In comparison, most fresh cells all appeared to respond to stimulation, albeit in a varied fashion. Therefore, gates were used that selected the cells increasing in size in the frozen cell populations, and this also selected the majority of stimulated cells within the fresh population. This alteration in cellular responses to stimulation could be the reason that a drop in cytokine secretion was observed for IL-17A, TNFα, IL-4 and IL-10. It is possible that the freezing process inhibited some cells response to TCR stimulation.

Intracellular cytokine staining showed that IFNγ production was again increased in cells that had undergone freezing, and, in contrast to the secretion data, so was IL-17A production (figure 2.5). However, IL-10 production was again depressed in frozen cells. With regards to surface markers, CXCR3 and CCR4 also showed a mild increase in expression in frozen cells. In contrast, the expression of CCR6 and

CTLA-4 was reduced in cells that had been frozen. Freezing, therefore, may select for cells that are likely to express certain markers experimentally, or may reduce the expression of certain markers within a population. These data, alongside the cytokine stimulation data, suggests that frozen T lymphocytes may be altered in their response to stimulation compared to freshly isolated T lymphocytes, and the differences observed need to be considered in relation to the results presented in subsequent chapters.

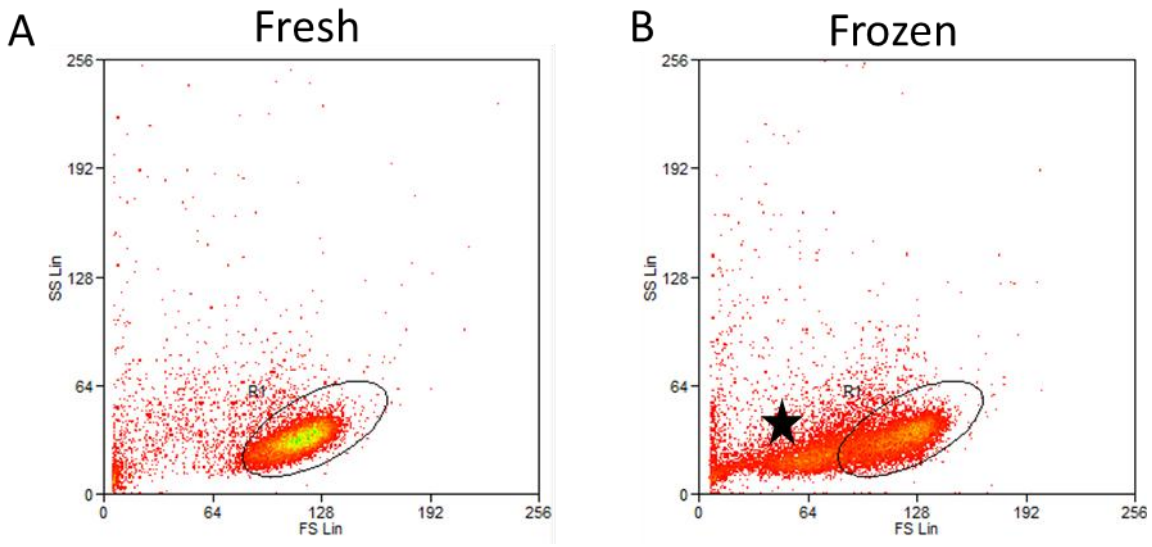

Figure 2.4. The FS/SS side profiles of non-stimulated fresh and frozen lymphocytes are different. *CD4+ CD45RO+ T lymphocytes were isolated using a Miltenyi MACs CD4+ memory isolation kit and either directly assessed or frozen at -80°C for later analysis. Forward scatter (FS)/ side scatter (SS) plots are shown here for non-stimulated cells freshly isolated (A) and cells that underwent the freezing process (B). Cells were plated at 2x10^5 and left to equilibrate for 24 hours to the desired oxygen levels. They were then left for a further 24 hours before analysis on a flow cytometer. The star symbol indicates the extra population of lymphocytes that have a low forward scatter values in the cells that had undergone freezing.*

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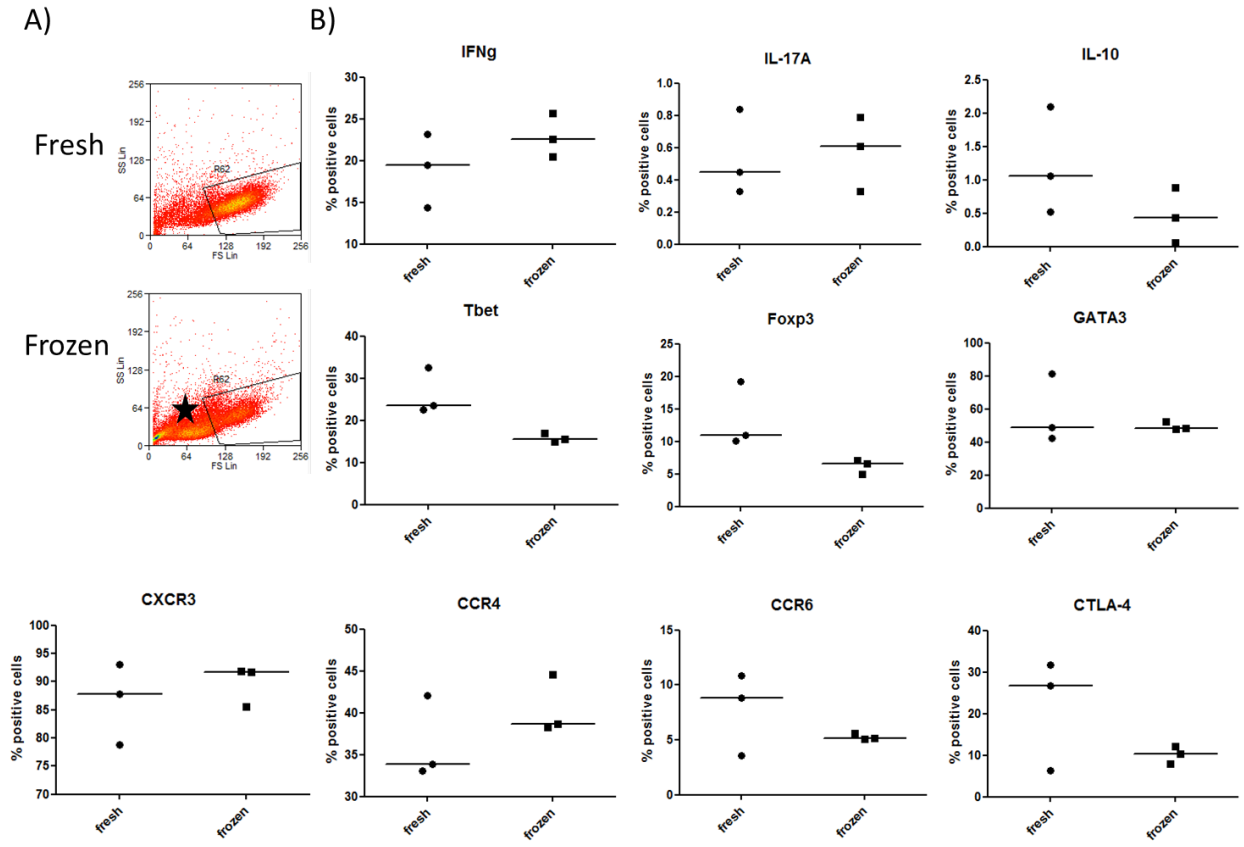

Figure 2.5. Markers associated with T helper subsets were assessed by intracellular staining and flow cytometry at 21% oxygen. *CD4+ CD45RO+ T lymphocytes were isolated using a Miltenyi MACs CD4+ memory isolation kit and either directly assessed or frozen at -80°C for later analysis. 2x10^5 cells were equilibrated to the stated oxygen level for 24 hours before stimulation with 2µg/ml anti-CD3// 5µg/ml anti-CD28 for 24 hours. 2µg/ml brefeldin A treatment was given for the last three hours of stimulation for cytokine analysis. FS/SS plots for the fresh and frozen cells are shown in A) showing the selected gate for analysis. As fresh and frozen cells differed in their FS/SS plots after stimulation, a gate around the stimulated cells in the frozen population was selected for analysis, as this also selected the majority of stimulated cells in the fresh populations. The star symbol highlights the population observed in cells that had undergone the freezing process*

that was reduced in size and that did not respond to stimulation. The results from staining using the two polarisation staining panels are shown in B). n=3 per experiment. No significant differences were observed by Mann Whitney statistical analysis. Differences in the responses of several markers were observed when looking at fresh and frozen cells.

3. RESULTS: DO ENVIRONMENTAL OXYGEN LEVELS INFLUENCE CD4+ MEMORY T LYMPHOCYTE ACTIVATION AND PROLIFERATION?

3.1 Introduction

As T lymphocytes experience several different oxygen levels physiologically, it was important to determine whether these alterations had any effect on their biology. Within this first chapter the effect of varying oxygen levels on basic CD4+ CD45RO+ T lymphocyte biology was examined.

3.1.1Proliferation

One of the cardinal responses of T lymphocytes to antigenic stimulation is proliferation [63, 81]. This serves to increase the number of antigen-specific T lymphocytes available, amassing a population of effector cells able to respond to a specific antigen, and improving the chances of disease resolution. Central memory T lymphocytes are expected to proliferate more in response to stimulation than effector memory T lymphocytes [54].

3.1.2 Markers of T cell activation

Activation markers have been identified that enable the assessment of activated T lymphocytes within a population. These markers are generally upregulated on the cell surface post-stimulation and are classified on the timings of their expression. CD69 is a very early marker of activation, whereas intermediate markers include CD25 and CD71. HLA-DR is a late activation marker [308]. Within the rheumatoid joint CD69 and HLA-DR expression has been observed on CD4+ T lymphocytes [170], whereas

intermediate markers are not as well expressed. This is somewhat confusing, and suggests that CD69 expression may be sustained in the rheumatoid joint abnormally. We therefore initially investigated the effect of differing oxygen levels on CD69 and HLA-DR expression.

3.1.2.1 CD69

CD69 is a heavily glycosylated C-type II lectin present on the surface of cells as a homodimer with no known extracellular ligand or receptor. The *cd69* gene is found within the natural killer gene complex on chromosome 12 [169, 309-311]. In T lymphocyte biology, CD69 is one of the first activation markers upregulated in response to T lymphocyte stimulation. Time taken for expression can be dependent on the stimulation method used. Following PHA stimulation, CD69 expression is initially detected 3 hours post-activation with a peak in expression at around 15 hours [308]. After TCR engagement, CD69 transcripts can be detected within the first hour post stimulation, and surface expression peaks between 18-24 hours [312]. Thymocytes express CD69 [313], and a role for CD69 has been identified in the regulation of lymphocyte egress from the thymus and lymph nodes [314] by binding the sphingosine-1-phosphate receptor resulting in its internalisation. Sphingosine-1-phosphate (S1P) is expressed at increasing gradients in lymph nodes and the thymus. When bound to its receptor on thymocytes and lymphocytes, S1P promotes egress, for example, by promoting migration into cortical sinuses in the lymph node. CD69 internalisation of the S1P receptor therefore inhibits this process, maintaining the lymphocyte's presence within the lymphoid organ [314, 315].

CD69 expression is also found in chronic inflammatory environments. The rheumatoid joint and infiltrating tumour lymphocytes have all been shown to express high levels of CD69, suggesting the cells within these environments have been recently or persistently activated [40, 170, 311, 316]. One study found that around 60% of CD4+ T lymphocytes from RA synovial tissue were CD69 positive [40]. As CD69 expression is observed alongside late activation markers in the RA joint [170], its expression may not only be a marker for recently-activated T lymphocytes, but also be associated with a particular population of T lymphocyte found in chronic inflammatory environments. Furthermore, anti-CD69 autoantibodies are found in some patients with RA [317, 318] but the role CD69 may play in chronic inflammatory sites is somewhat controversial, with opposing results reported [317, 319, 320].

3.1.3 General surface markers of CD4+ T lymphocytes

3.1.3.1 CD45RO

CD45 is a key molecule involved in the regulation of T lymphocyte signalling. CD45 can be extensively glycosylated and is therefore a relatively large molecule on the lymphocyte cell surface [160]. It comprises around 10% of the total surface protein in T lymphocytes but has no known ligand [160, 161]. However, it has several biological functions, including the dephosphorylation of both the inhibitory and activatory tyrosine residues on the Lck molecule [73, 85, 90, 160, 161]. Indeed, CD45 dephosphorylation of Y394 is associated with the termination of signalling in the IS [70, 73]. CD45 therefore plays a key role in T cell signalling, and its knockout results in an increase in both positive and negative selection [73]. Despite this, CD45 is

thought to be excluded from the IS due to its large size, potentially limiting its contact with its substrates during stimulation [67, 70].

There are several different isoforms of CD45 differing in their extracellular domains [160]. Naïve and memory T lymphocytes can be separated by their expression of the CD45RA and CD45RO isoforms, respectively. Indeed, these markers are used for the magnetic isolation of these different cell populations. CD45RO isoforms have smaller extracellular domains than their RA counterparts due to exon splicing [160]. CD45RA to RO isoform switching begins within the first 48 hours post-stimulation of naïve T lymphocytes, and CD45RO is also a marker for recently activated CD4+ T lymphocytes [148]. Isotype switching may have several effects – it may allow the CD45RO monomers to dimerise more easily, potentially resulting in the downregulation of phosphatase activity. Alternatively, the smaller space occupied by the CD45RO molecule in the extracellular milieu may allow it to migrate more easily into the signalling synapse formed between a T lymphocyte and an APC [85].

3.1.3.2 CD3

CD3 is regularly used to identify T lymphocyte populations. There are four CD3 subunits that form hetero- or homo- dimers within the cell: CD3ε, CD3γ,CD3 δ and CD3ζ. [67]. As described in chapter 1, the CD3 subunits complex with the TCR and provide intracellular cytoplasmic domains for downstream signalling from the TCR:CD3 protein complex post-TCR ligation. Through mechanisms still being elucidated, CD3 ITAM motifs are phosphorylated, allowing for docking of downstream signalling molecules [67, 321]. After T lymphocyte activation, TCR:CD3 complex expression is reduced [322-325] limiting any further TCR ligation and subsequent

stimulation. T lymphocytes start to re-express the TCR:CD3 complex around two days after stimulation, but rates of re-expression vary and depend on the length of initial stimulation [322].

3.1.4 This chapter's aims

In this chapter the effect of hypoxia on basic T lymphocyte biology has been investigated. CD4+ CD45RO+ T lymphocytes were isolated from PBMC populations and frozen at -80˚C until required. Proliferation, expression of activatory and identifying markers, cell size and complexity, and viability were examined to provide initial clues as to the effect of varying oxygen level on the biology of CD4+ CD45RO+ T lymphocytes. Immunofluorescent staining and flow cytometry were utilised in these assessments. The FS/SS stimulation gates were determined by comparison to non-stimulated controls, as in chapter 2, and were used for assessment of these different parameters.

3.2 Results

3.2.1 Physiologically healthy oxygen levels promote increased proliferation in CD4+ effector memory T lymphocytes

CD4+ memory T lymphocytes were isolated from healthy control PBMC populations and frozen at -80°C. When required, they were defrosted and loaded with CFSE and equilibrated at the desired oxygen level for 24 hours. They were then stimulated with plate-bound anti-CD3/ anti-CD28 and left to proliferate for four days. Proliferation was assessed by flow cytometry and the stimulated cells were gated upon for analysis (figure 3.1B). Equal sized bars were applied to each peak representing a division. Non-stimulated controls were also left for four days to confirm CFSE integrity over time (figure 3.1A). This also showed that it was the lymphocyte population that had expanded into the stimulation gate selected in figure 3.1B. The cells that had not responded to stimulation have previously been shown to be lymphocytes that respond differently to the freezing process (figure 2.4 and figure 2.5).

Proliferation at 21% oxygen was significantly lower than at 8.5%, 3% and 1% oxygen (figure 3.1E). Fewer cells initiated cell division at 21% oxygen, despite their cell size increasing into the stimulation gate and hence resulting in their inclusion within the results (figure 3.1C). Proliferation at 21% oxygen appeared inhibited after 3 rounds of division, as a large drop was observed in the number of cells completing four rounds of division compared to those completing three (figure 3.1D). This is also revealed in the proliferative index (PI) (figure 3.1E), which highlights the depressed rounds of divisions. This index is generated by dividing the total number of cells in the stimulation gate by the estimated number of starting cells, and so gives an arbitrary measurement of proliferation.

Significantly enhanced proliferation was observed at 8.5% oxygen, and a step-wise decrease occurred as oxygen became less available (figure 3.1C). At 8.5% oxygen, more cells entered 4 or 5 rounds of division compared to the other oxygen levels, (figure 3.1D) and the PI was highest (figure 3.1E). This therefore suggested that cells were most likely to divide at this oxygen level.

At lower oxygen levels the number of cellular divisions was reduced in comparison but 3% oxygen provided a relatively good environment for proliferation, having the second highest number of cells entering five rounds of division compared to 8.5% oxygen. Reperfusion injury and constant 1% oxygen differed slightly in their proliferation profiles, with the exposure to higher oxygen in reperfusion injury treatments resulting in slightly more cellular divisions than constant 1% oxygen in which proliferation appeared to be more depressed. These results therefore suggested that physiological variances in oxygen level can influence downstream proliferative responses. Consequently, lymphocytes may proliferate more readily in a well-perfused tissue compared to a hypoxic inflamed tissue.

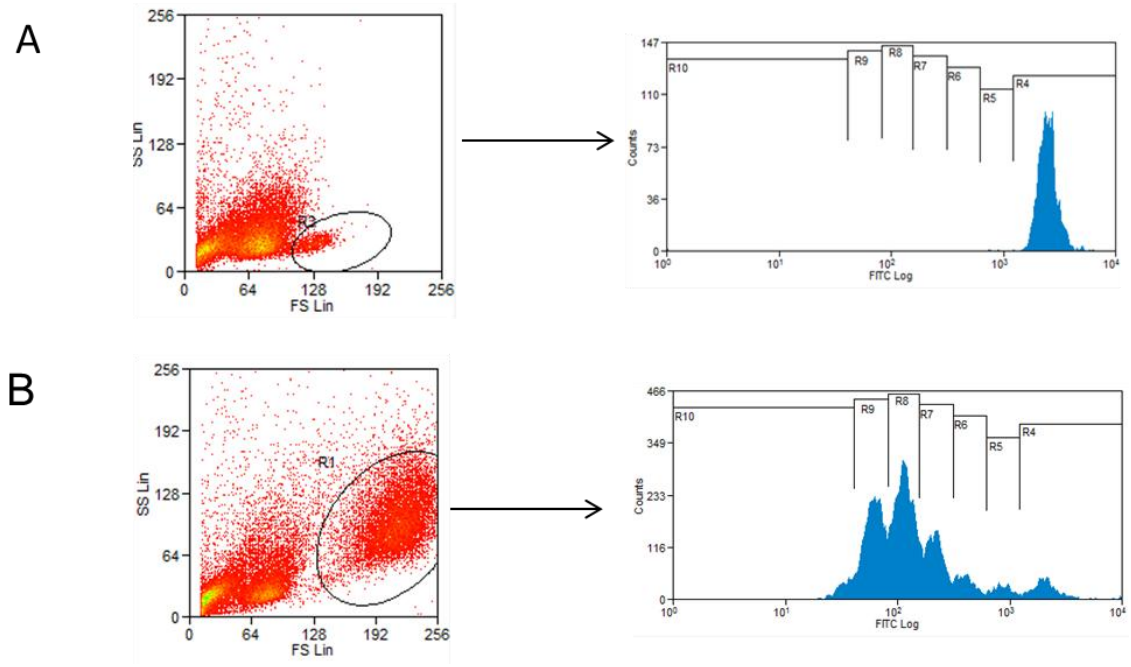

Figure 3.1 (Part 1) Proliferation of CD4+ memory T lymphocytes is increased at physiologically healthy oxygen levels. *CD4+ CD45RO+ T lymphocytes were isolated using a Miltenyi MACs CD4+ memory isolation kit and were frozen at -80°C for later analysis. Once required, CD4+ memory T lymphocytes were defrosted and loaded with 1μM CFSE and plated at 2x10^5 cells before being equilibrated to the stated oxygen level for 24 hours. They were then stimulated with plate bound anti-CD3/ anti-CD28 and left for 4 days when proliferation was determined. A) Non-stimulated lymphocytes after 4 days stimulation and their corresponding CFSE loading as assessed by the 488 laser. B) Stimulated lymphocytes from the same donor as in (A) after 4 days stimulation. Gating strategy used showing selection of stimulated cells and assessment of proliferation using the 488 laser.*

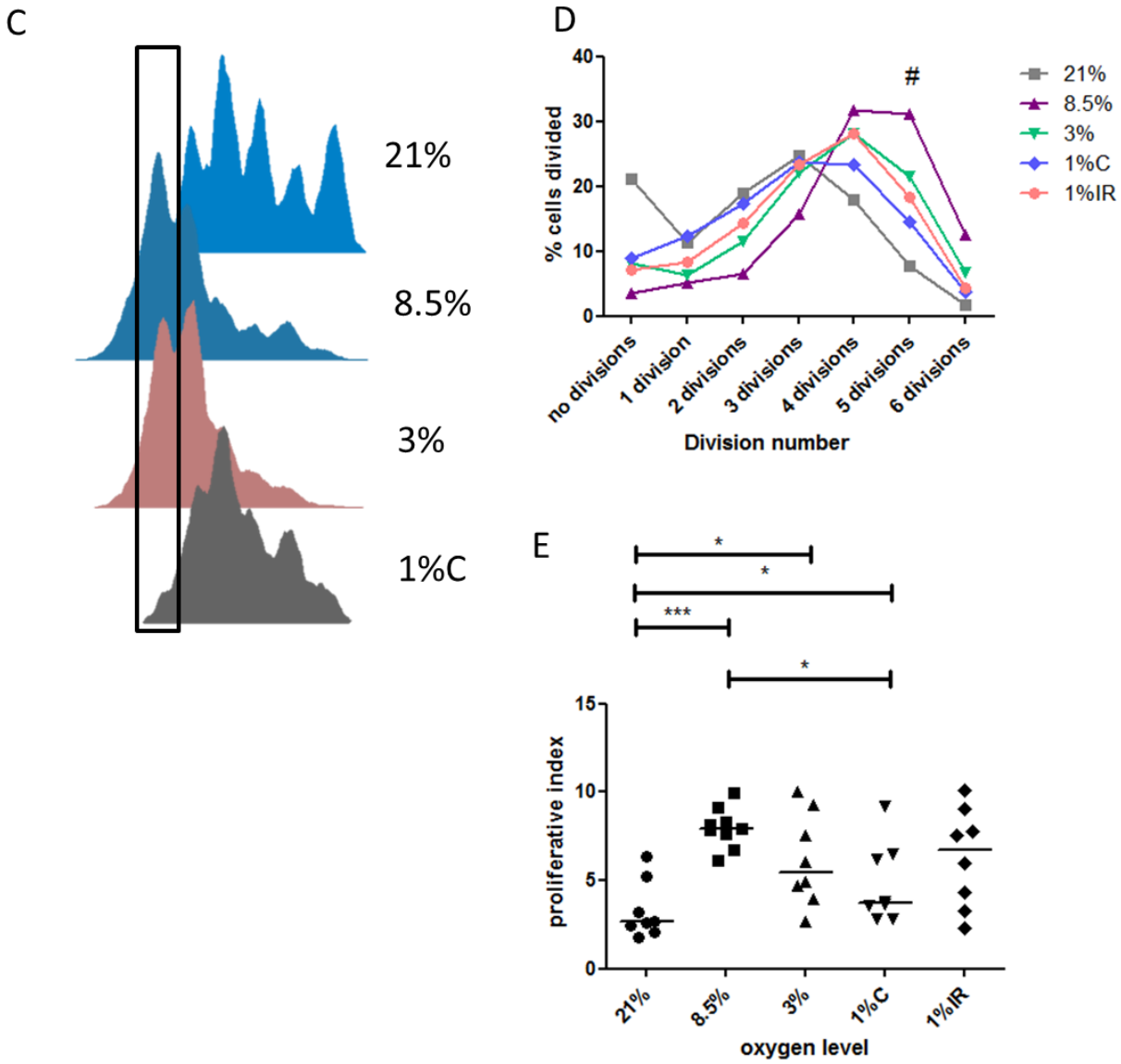

Figure 3.1(Part 2) Proliferation of CD4+ memory T lymphocytes is increased at physiologically healthy oxygen levels. *C) Example CFSE plots after 4 days stimulations at different oxygen levels. Black box shows 5 cell divisions. D) A line graph to depict the number of cell divisions at each oxygen level. All data obtained were included in this summarisation. E) The proliferative index was determined by dividing the total number of cells with the estimated number of original cell (as determined from the gates applied). Data shows the median values. Analysis was performed using Mann Whitney statistical test. *=p<0.05, ***=p<0.001. # p<0.05 for 8.5% oxygen compared with 1% oxygen, and p<0.005 for 8.5% compared to 21%,*

after 5 divisions. 1%C = constant 1% oxygen. 1% IR = 1% oxygen with reperfusion injury. Data is representative of a minimum of 7 donors per oxygen level, from 3 or more separate experiments and is representative of at least 2500 cells in the stimulation gate. An increase in proliferation at 8.5% oxygen is suggested from this data.

3.2.2 Cell viability after four days stimulation is reduced at 8.5% oxygen

Cell viability was assessed by Sytox staining after four days stimulation (the same duration of stimulation used for assessment of proliferation). At 8.5% oxygen, an increase in the number of Sytox-positive cells was observed compared to the other oxygen levels (figure 3.2). This suggested that the cells were the least viable at this oxygen level. At the other oxygen levels there were no significant differences in viability suggesting that the difference in oxygen availability does not influence cell viability in hyper- or hyp- oxia, atleast in pure CD4+ CD45RO+ T lymphocyte cultures, after 4 days stimulation.

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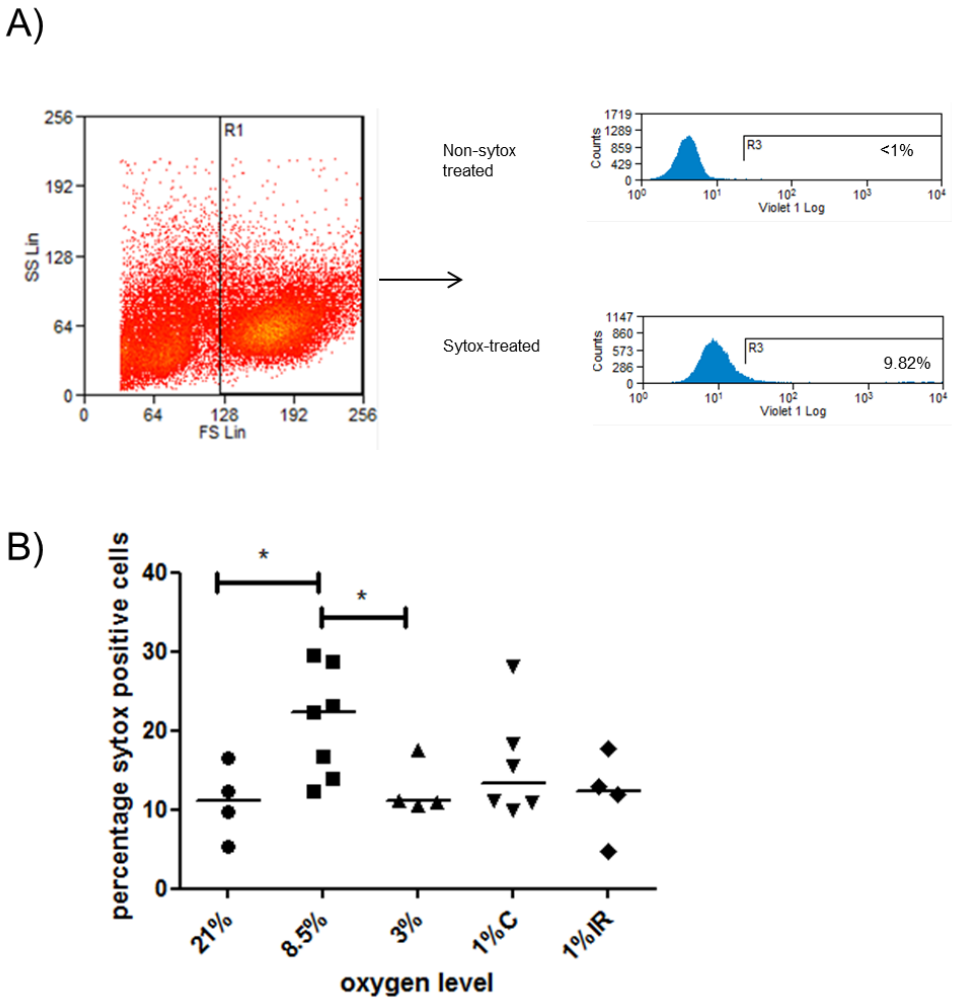

Figure 3.2. The number of Sytox positive cells is increased at 8.5% oxygen. *CD4+ CD45RO+ T lymphocytes were isolated using a Miltenyi MACs CD4+ memory isolation kit and were frozen at -80°C for later analysis. Once required, cells were defrosted and 2x10^5 T lymphocytes were equilibrated for 24 hours at the desired oxygen level before being stimulated with plate bound anti-CD3/anti-CD28. After four days the cells were stained with Sytox Dead Cell stain and assessed by flow cytometry using the 405 laser. A) shows the gate selection applied to stimulated cells (stimulated cells were selected as these were the cells assessed in other analysis and therefore made the data comparable) and the histograms obtained from this data. Bars in histograms were set within <1% of non-sytox treated cells. B)*

Summarisation of sytox assessment at different oxygen level. Analysis was performed using Mann Whitney statistical test.=p<0.05. 1%C = constant 1% oxygen. 1% IR = 1% oxygen with reperfusion injury. Data is representative of at least 2500 cells in the stimulation gate and median values are shown. A minimum of 4 donors were examined at each oxygen level. An increase in the number of Sytox positive cells was observed at 8.5% oxygen, suggesting that viability was reduced at this oxygen level.*

3.2.3 The expression of the activation marker CD69 is increased in hypoxia

The expression of the early activation marker, CD69, was altered at varying oxygen levels. Both the percentage of CD69 positive cells and the intensity of CD69 expression, as determined by the mean fluorescence intensity (MFI), were upregulated at constant 1% oxygen after 48 hours stimulation (figure 3.4 and figure 3.3A, respectively). Both 3% oxygen and reperfusion injury treated cells showed a slight drop in CD69 expression in comparison, having equivalent average CD69 MFI values.

At the physiologically healthy oxygen levels of 8.5% oxygen for the joint, both the percentage of CD69 positive cells and intensity of CD69 expression were the most reduced compared to other oxygen levels, although a similar drop in expression was observed at 21% oxygen (figures 3.3 and 3.4).

CD69 is an early activation marker normally downregulated 24 hours post-stimulation [310, 312]. Cell cycle inhibition in hypoxic conditions has been reported previously [282] and therefore the increased CD69 expression detected at 48 hours at 1% oxygen could have been due to the CD4+ T lymphocytes being 'stuck' in their progression when exposed to hypoxia. The delay in proliferation at lower oxygen levels also suggested that this could be the case. CD69 expression was therefore further examined after 24 hours stimulation. Constant 1% oxygen and 8.5% oxygen were selected for these additional experiments as these two oxygen levels revealed the extremes in CD69 expression after 48 hours stimulation.

An upregulation in expression at the lower oxygen level was again observed (figure 3.5), suggesting that the upregulation at 48 hours observed was not necessarily due to the cells in hypoxia falling behind their 8.5% oxygen counterparts in cell cycle and

expression profiles, but because CD69 upregulation was an intrinsic response of CD4+ T lymphocytes to hypoxia. After 24 hours stimulation at 8.5% oxygen, CD69 expression MFI values did not reach the values obtained at 1% oxygen after 48 hours stimulation, further revealing the decreased CD69 expression at 8.5% oxygen.

No differences in the percentage of the HLA-DR positive cells after 48 hours stimulation were observed at varying oxygen levels (figure 3.4B), suggesting that late activation markers were unaffected by varying oxygen levels.

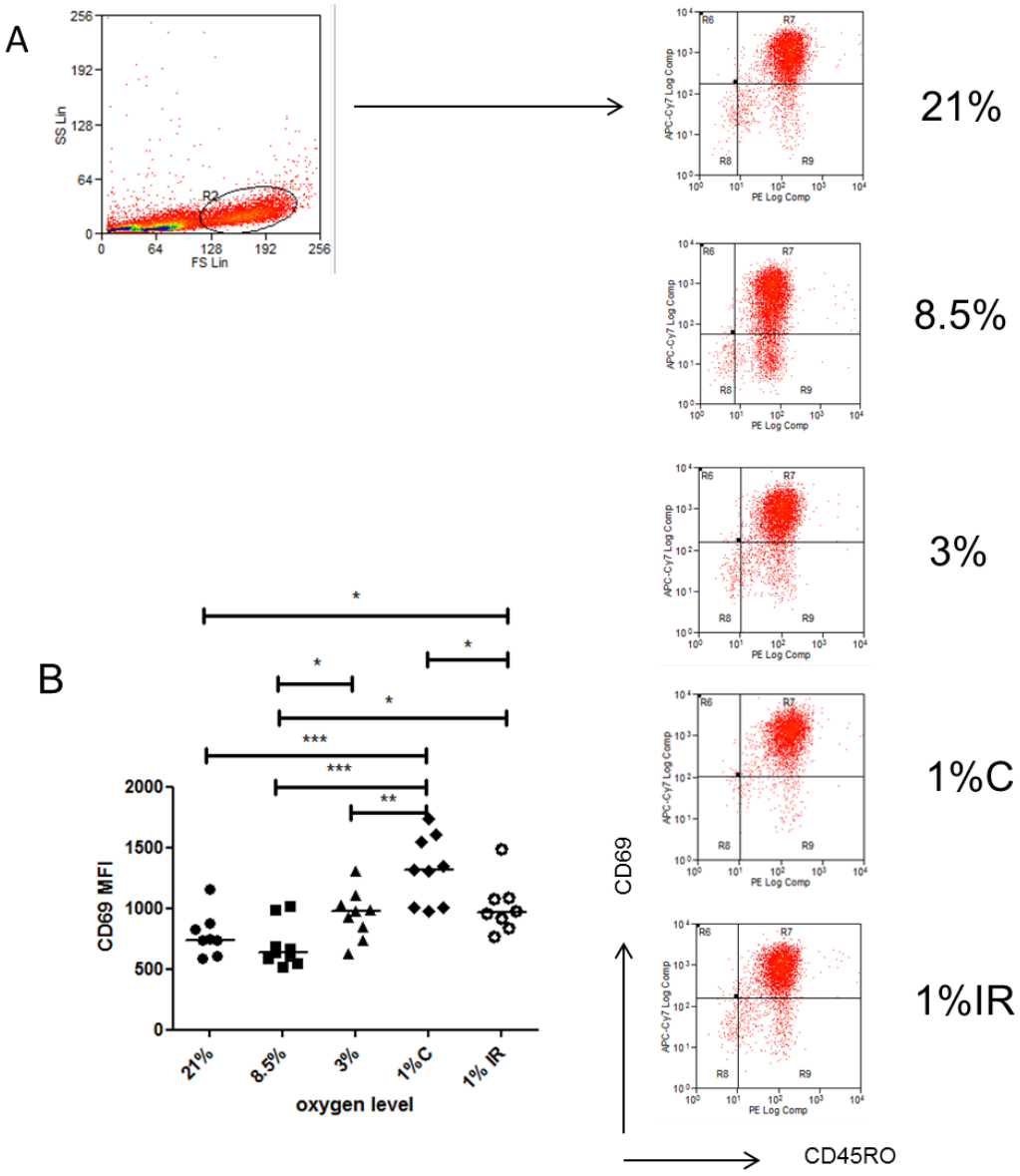

Figure 3.3. Varying responses of the activation marker CD69 associated with CD4+ T lymphocytes stimulated at different oxygen levels. *CD4+ CD45RO+ T lymphocytes were isolated using a Miltenyi MACs CD4+ memory isolation kit and were frozen at -80°C for later analysis. Once required, cells were defrosted and 2x10^5 T lymphocytes were equilibrated for 24 hours at the desired oxygen level before being stimulated with plate bound anti-CD3/anti-CD28. T lymphocytes were*

*stimulated for 48 hours at the stated oxygen levels. Cells were stained for expression of CD69 (APC-Cy7) and CD45RO (PE) and assessed by flow cytometry. A) Example forward scatter (FS)/ side scatter (SS) plots for analysis. The SS was depressed as cell counting beads were occasionally used alongside this protocol. Example dot plots of CD45RO vs CD69 expression are shown from one donor at the different oxygen levels investigated. Quadrants were applied with respect to the respective isotype control ran on the same day at the same oxygen level. B) shows the CD69 MFI values at the different oxygen levels investigated. Median values are shown. Statistical analysis was performed using Mann Whitney test. 1%C = constant 1% oxygen. 1% IR = 1% oxygen with reperfusion injury. * p< 0.05, **p< 0.01, ***p<0.001. Data is representative of at least 2500 cells in the stimulation gate. A minimum of 8 donors were investigated at each oxygen level.*

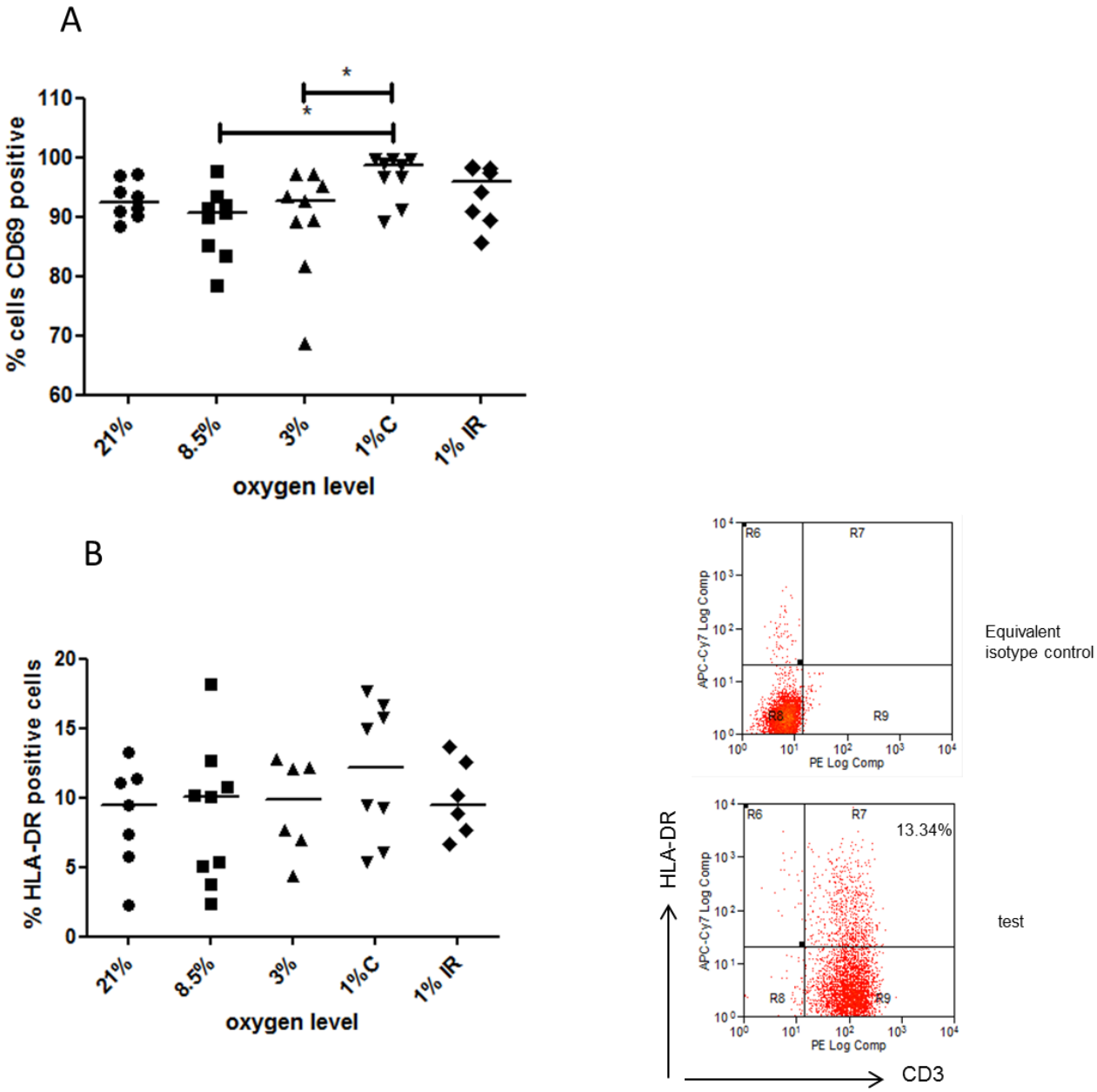

Figure 3.4 The percentage of CD69 and HLA-DR positive cells at different oxygen levels. *CD4+ CD45RO+ T lymphocytes were isolated using a Miltenyi MACs CD4+ memory isolation kit and were frozen at -80°C for later analysis. Once required, cells were defrosted and 2x10^5 T lymphocytes were equilibrated for 24 hours at the desired oxygen level before being stimulated with plate bound anti-CD3/anti-CD28. T lymphocytes were stimulated for 48 hours at the stated oxygen*

*levels. Cells were stained for expression of CD69 (APC-Cy7) and CD45RO (PE) or HLA-DR (APC-Cy7) and CD3 (PE) and assessed by flow cytometry using stimulation gates as in figure 3.3. The percentage of A) CD69 and B) HLA-DR positive cells are shown. Example dot plots of HLA-DR vs CD3 are shown in B) against the equivalent isotype control (the same donor ran on the same day after stimulation at the same oxygen level). Median values are shown. Statistical analysis was performed using Mann Whitney test. * p< 0.05, **p< 0.01, ***p<0.001.1%C = constant 1% oxygen. 1% IR = 1% oxygen with reperfusion injury. Data is representative of at least 2500 cells in the stimulation gate. A minimum of 6 donors were investigated at each oxygen level. The percentage of CD69 positive cells was increased at lower oxygen levels, whereas no differences in HLA-DR expression were observed.*

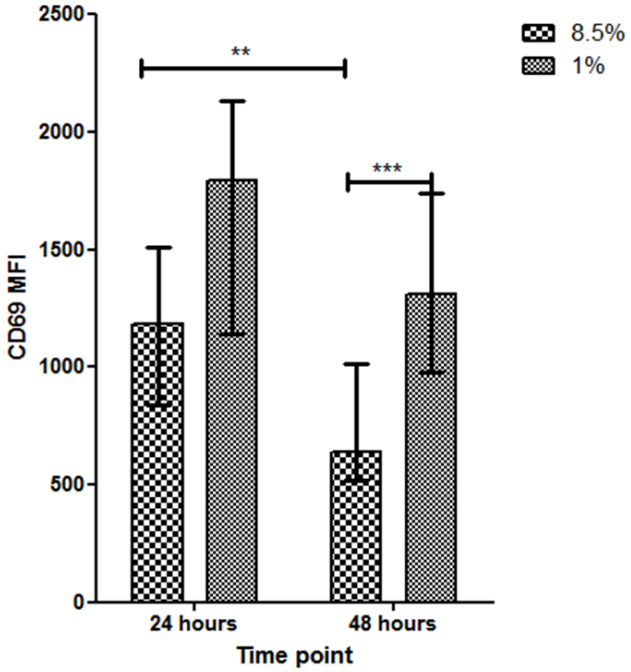

Figure 3.5. CD69 expression is upregulated at 24 and 48 hours in hypoxia compared to 8.5% oxygen. *CD4+ CD45RO+ T lymphocytes were isolated using a Miltenyi MACs CD4+ memory isolation kit and were frozen at -80°C for later analysis. Once required, cells were defrosted and 2x10^5 T lymphocytes were equilibrated for 24 hours at the desired oxygen level before being stimulated with plate bound anti-CD3/anti-CD28. T lymphocytes were stimulated for 24 and 48 hours at 8.5% and constant 1% oxygen. Cells were stained for expression of CD69 and assessed by flow cytometry, with stimulated cells selected for as in figure 3.3. Median values are shown in the data with the range. Statistical analysis was performed using the Mann Whitney test. ***= p<0.001 at 48 hours between 1% and 8.5% oxygen. **=p<0.01 between 24 and 48 hours in 8.5% oxygen treatments. Data is representative of at least 2500 cells in the stimulation gate and a minimum of n=6 at each treatment at each time point. A drop in CD69 expression was observed over time at both oxygen levels, and was higher at both time points at 1% oxygen.*

3.2.4 The expression of other surface markers is altered at different oxygen levels

3.2.4.1 CD45RO

CD45RO expression was expressed on all of the cells used experimentally in this chapter. Over 95% CD45RO positivity was therefore a precondition of every culture, therefore the intensity of its expression per oxygen level could be assessed by determining the MFI after flow cytometry.

CD45RO expression intensity is not reported to change in response to antigenic stimulation [73]. However, a greater intensity in CD45RO expression at 3% oxygen compared to the other oxygen levels investigated was observed, significantly in comparison to 8.5% oxygen. At 21% oxygen, constant 1% oxygen and 1% oxygen with reperfusion injury, CD45RO levels appeared similar (dot plots figure 3.3 and graphs figure 3.6B).

3.2.4.2 CD3

The TCR:CD3 complex is thought to be internalised and recycled as a complete complex after antigenic stimulation [322-324] and therefore any component of the complex can be assessed to make general investigations into its overall surface expression level. We selected an antibody against CD3ε to examine the expression of the complex on the membrane surface, as it has been used as a marker to observe the cycling of the TCR:CD3 complex previously [323].

After 48 hours expression of CD3ε expression at constant 1% oxygen was increased compared to the other oxygen levels assessed (figure 3.6A). This result was similar

to the pattern of CD69 expression and therefore led us to also examine the expression of CD3ε after 24 hours stimulation, again at constant 1% and 8.5% oxygen. Results from these experiments revealed a pattern of expression that differed from the surface expression of the CD69 molecule (figure 3.7). Expression at 8.5% oxygen was slightly increased compared to 1% oxygen treatments after 24 hours. Stimulation at 8.5% oxygen followed the expected pattern of downregulation of CD3ε expression over time. However, the pattern of CD3ε surface expression at constant 1% oxygen was significantly increased at 48 hours compared to 24 hours.

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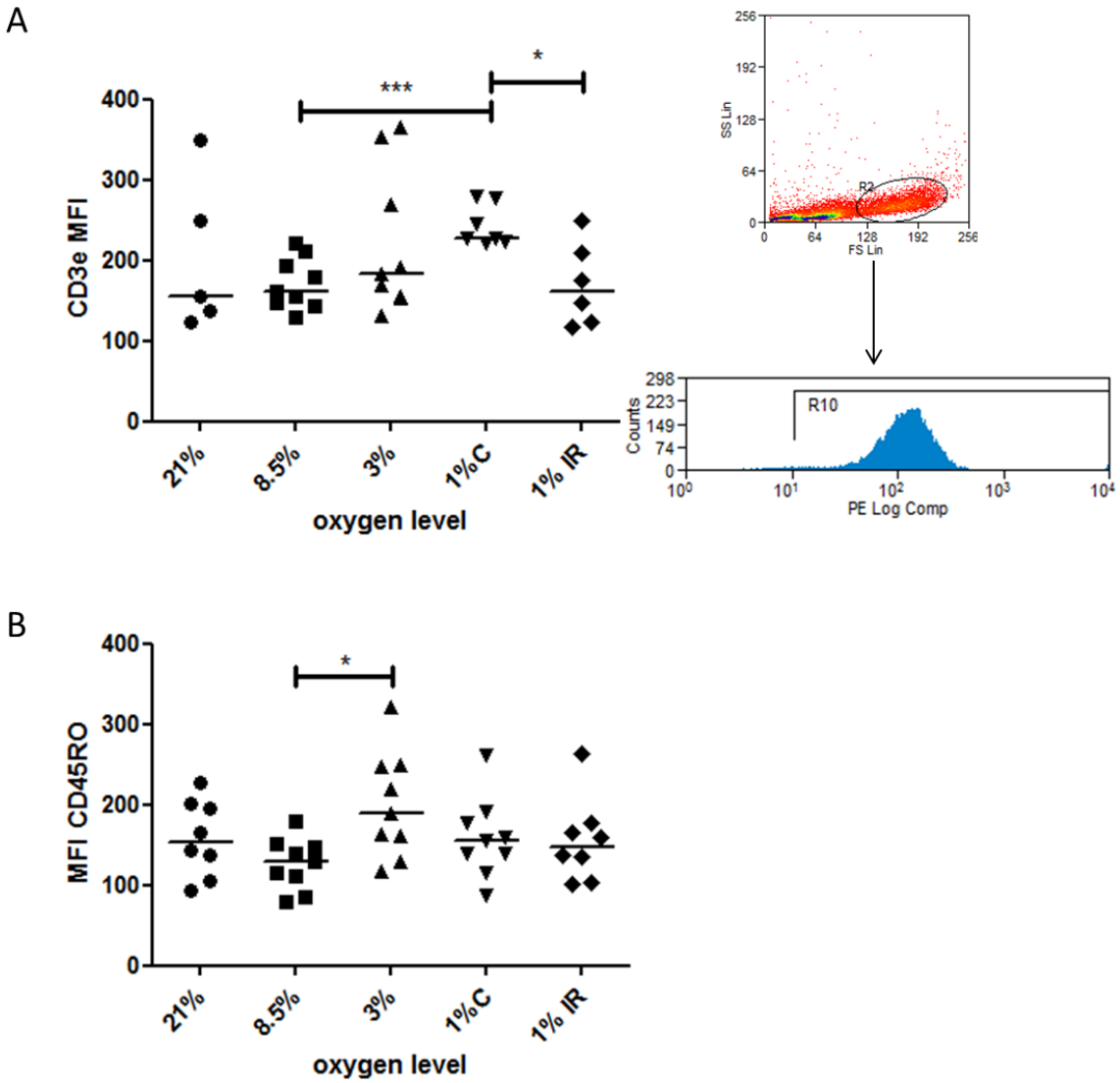

Figure 3.6. Alternative markers of CD4+ memory T lymphocytes reveal variable expression at different oxygen levels. *CD4+ CD45RO+ T lymphocytes were isolated using a Miltenyi MACs CD4+ memory isolation kit and were frozen at -80°C for later analysis. Once required, cells were defrosted and 2x10^5 T lymphocytes were equilibrated for 24 hours at the desired oxygen level before being stimulated with plate bound anti-CD3/anti-CD28. T lymphocytes were stimulated for 48 hours at the stated oxygen levels. Cells were stained for expression of CD45RO (PE) and CD3 (PE) and assessed by flow cytometry using stimulation gates as in figure 3.3 and*

*shown in (A). The mean fluorescence intensity (MFI) of A) CD3ε and B) CD45RO expression are shown with median values. 1%C = constant 1% oxygen. 1% IR = 1% oxygen with reperfusion injury. Statistical analysis was performed using the Mann Whitney test * p< 0.05. ***p<0.001. Data is representative of at least 2500 cells in the stimulation gate and a minimum of n=5 per oxygen level. Differences in CD3ε expression were observed at different oxygen levels.*

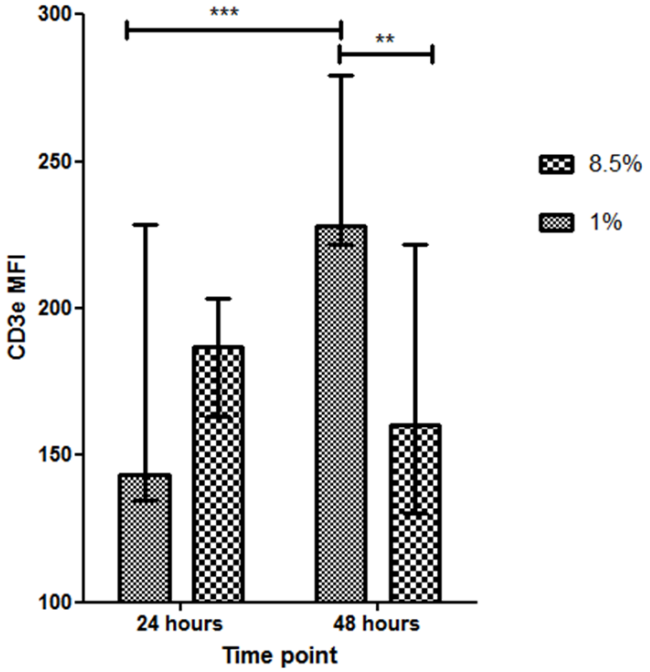

Figure 3.7. CD3ε expression over time has different patterns of expression at different oxygen levels. *CD4+ CD45RO+ T lymphocytes were isolated using a Miltenyi MACs CD4+ memory isolation kit and were frozen at -80°C for later analysis. Once required, cells were defrosted and 2x10^5 T lymphocytes were equilibrated for 24 hours at the desired oxygen level before being stimulated with plate bound anti-CD3/anti-CD28. T lymphocytes were stimulated for 24 and 48 hours at 8.5% and constant 1% oxygen. Cells were stained for expression of CD3ε and assessed by flow cytometry, with stimulated cells selected for as in figure 3.3. Median values are shown in the data with the range. Statistical analysis was performed using the Mann Whitney test. ***= p<0.001 at 48 hours between 1% and 8.5% oxygen. **=p<0.01 between 24 and 48 hours in 8.5% oxygen treatments. Data is representative of at least 2500 cells in the stimulation gate and a minimum of n=5 at each treatment at each time point. A drop in CD3e expression was observed over time at 8.5% oxygen levels, but was increased over time at 1% oxygen.*

3.2.5 Cell size and granularity is altered at different oxygen levels

To determine whether the alterations in surface molecule expression were due to differences in cell size, the forward scatter (FS) results obtained from the flow cytometry assessment of activation marker expression were analysed. This revealed a pattern of significantly decreased cell size at 3% oxygen compared to the oxygen levels (figure 3.8A). Highest cell size was observed at 8.5% and constant 1% oxygen. However, there was no correlation between CD69 MFI, which was greatest at 1% oxygen, and FS mean (figure 3.8B). As this pattern also did not correlate with CD3ε or CD45RO expression, it is likely that differences in surface marker expression intensity were not due to differences in cell size.

Differences in the granularity of the cells could also be assessed by using the side scatter (SS) data from the flow cytometry assays due to the fact that the cell populations were over 95% pure. Granularity values varied somewhat over time due to equipment variability, but a pattern was apparent after stimulation at different oxygen levels (figure 3.8C). Granularity increased as oxygen level decreased, with the highest granularity observed at constant 1% oxygen after 48 hours stimulation suggesting that the cells in this oxygen environment contain more intracellular structures.

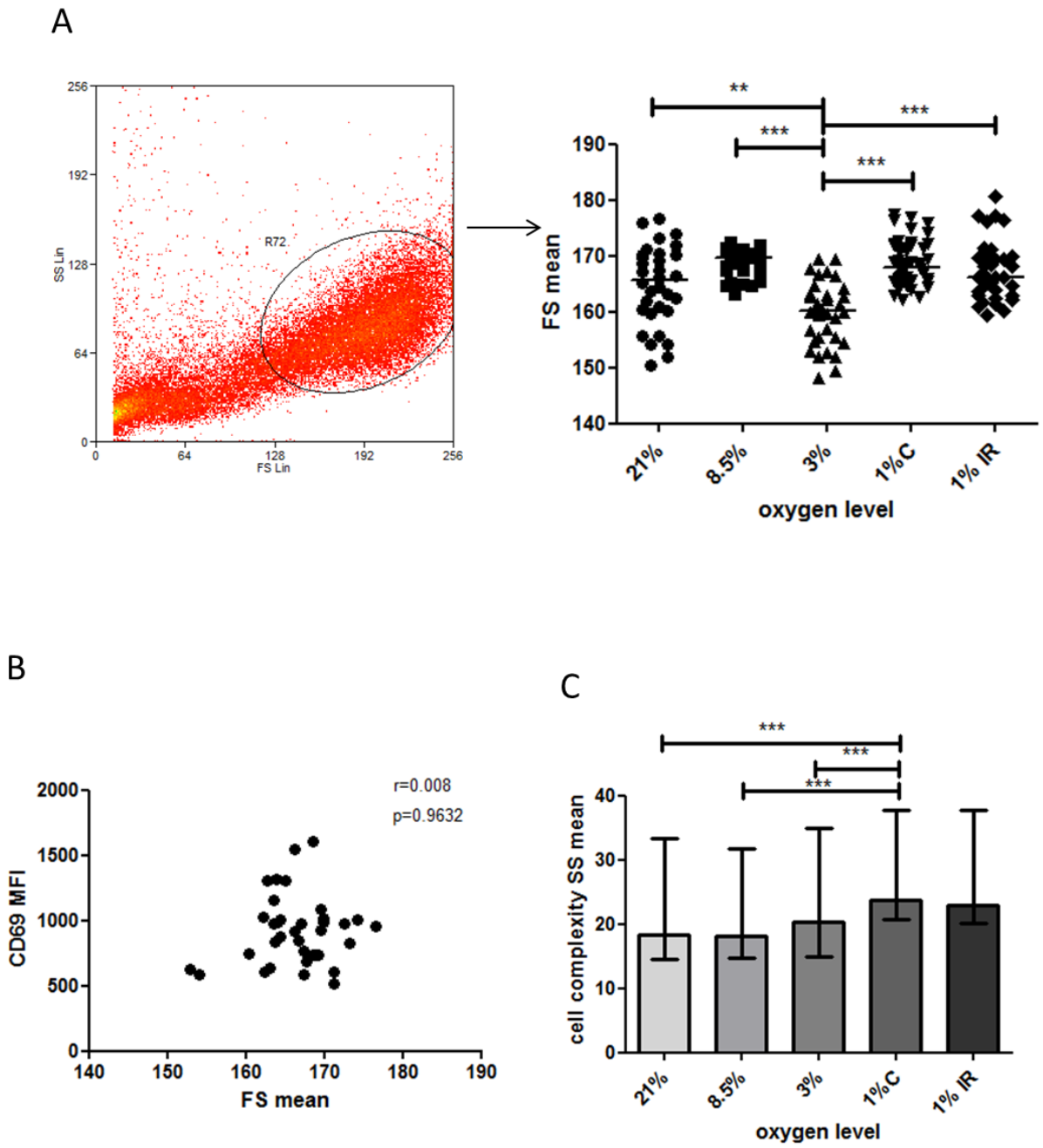

Figure 3.8. Cell size varies considerably at different oxygen levels after 48 hours stimulation, but does not correlate with CD69 expression. *CD4+ CD45RO+ T lymphocytes were isolated using a Miltenyi MACs CD4+ memory isolation kit and were frozen at -80°C for later analysis. Once required, cells were defrosted and 2x10^5 T lymphocytes were equilibrated for 24 hours at the desired oxygen level before being stimulated with plate bound anti-CD3/anti-CD28. T*

*lymphocytes were stimulated for 48 hours at the stated oxygen levels. Flow cytometry was used to assess cell size according to the linear forward scatter (FS) mean (A) and cell granularity according to linear side scatter (SS) mean (C). Stimulated cells were selected on from the lymphocyte populations. Statistical analysis was performed using Mann Whitney test and median values are shown. **=p<0.01, ***=p< 0.001. Cell size varies considerably depending on the oxygen level stimulated at, with a reduction in cell size observed at 3% oxygen. B) Correlation of CD69 MFI vs linear forward scatter mean was analysed by determining the Spearman Rank Correlation Coefficient. No correlation was observed. Data is representative of at least 2500 cells in the stimulation gate. A minimum of 28 FS/SS plots were assessed for analysis.*

3.3 Discussion

The data presented within this chapter reveal the varied response of CD4+ T lymphocytes to different environmental oxygen levels. Proliferation and surface protein expression were altered at different oxygen levels and basic cellular biology, such as size and granularity, also appeared to differ. Hence, the data supports the hypothesis that environmental oxygen influences CD4+ CD45RO+ T lymphocyte function.

3.3.1 Proliferation

Memory T lymphocytes proliferate more readily than naïve T lymphocytes due to their lower threshold for activation [63], and within the CD4+ CD45RO+ memory T lymphocyte population it is expected that the central memory compartment would proliferate the most readily compared to effector memory T lymphocytes [54, 58]. Therefore, it is possible the data obtained in this study regarding proliferation is mainly descriptive of the central memory response to different oxygen levels. Proliferation appeared inhibited in 21% oxygen, and this was contradictory to previous reports. However, much of this literature has focussed on naïve and mixed CD4+ T lymphocyte populations. Memory T lymphocytes are generated from, and are thus adapted to, previous infectious stimuli, and therefore may be more tailored to function in an oxygen environment much lower than 21%.

At the physiological healthy oxygen level of 8.5% an increase in proliferation was observed compared to both hyper- and hypo- oxic conditions (figure 3.1), suggesting that this may be close to the optimum oxygen level required for maximal CD4+ T lymphocyte proliferation. Indeed, the increase in the number of cells produced after

four days stimulation could have contributed to the decrease in cell viability observed at 8.5% oxygen (figure 3.2), as visually the cultures were larger at 8.5% oxygen (as observed with a brightfield microscope) and became more acidic (as observed by the colour of the culture medium), most probably due to increased lactic acid caused by increased cellularity. Further studies where cell medium is replenished mid-experiment may help to determine whether this was the reason for decreased cell viability at physiologically healthy oxygen levels. This would also replenish nutrients such as the essential amino acid tryptophan, which when lost from the local environment can result in T lymphocyte apoptosis [326].

The greater proliferation observed at 8.5% oxygen may highlight the ‘healthiness’ of this oxygen level, as it may provide the cells with the right amount of oxygen for cellular division, but not place too much of an oxidative strain to inhibit cellular processes. Investigation of proliferative responses at 8.5% oxygen is, to our knowledge, novel, and this condition may represent what is present when T lymphocytes are stimulated in well-perfused tissues. However, this is unlikely physiologically, as CD4+ memory T lymphocyte stimulation will normally occur in oxygen-low tissues such as the thymus [243, 245, 246], lymphoid organ [239, 245] and inflamed tissue [9]; tissues not thought to reach 8.5% oxygen. The reduction in proliferation at lower oxygen levels, however, provides further evidence that the oxygen environment regulates CD4+ T lymphocyte responses. This limitation of proliferation may be beneficial, as overt proliferative responses can be associated with pathology [327]. Significant differences in cell size at different oxygen levels may also be symptomatic of differences in proliferation and cell cycle at different oxygen levels.

3.3.2 The activation marker CD69

In this study, an increase in CD69 expression was observed in constant 1% oxygen after both 24 and 48 hours stimulation. This upregulation appeared to be much greater than that observed at 8.5% oxygen (figure 3.4). Increasing CD69 expression at 5% oxygen compared to 21% oxygen has been previously reported, [244, 288] which is in slight contrast with the results found in this study, as both 8.5% and 3% oxygen revealed a slight drop in CD69 expression compared to 21% oxygen. The reasons for this difference may be due to the use of CD4+ CD45RO+ T lymphocytes experimentally. The increase in the percentage of cells expressing CD69 at constant 1% oxygen suggested that the cells at this oxygen level may have been more activated, but the proliferation data suggested otherwise. Therefore, it could be possible that cells at 1% oxygen immediately 'fall behind' higher oxygen level treatments after stimulation, resulting in a lengthier expression of CD69, and a lower proliferative response. Although CD69 is a marker of activation *per se*, the intensity of its expression may therefore not reflect a cells level of activation.

The increased CD69 expression observed in the RA joint alongside late markers of activation corroborates with this *in vitro* data, as both environments would have been hypoxic. Therefore, low environmental oxygen levels may result in altered CD69 expression compared to what is normally observed in 21% oxygen *in vitro* experiments. CD69 does not seem to play a large role in T lymphocytes activation (although cross-linking can result in cell proliferation [313]) but its upregulation in hypoxia may still influence T lymphocyte downstream biological functions. For

example, CD69 is involved in inhibiting cell egress out of tissues [314, 315] and therefore may contribute to the ‘stuck’ phenotype of T lymphocytes in the RA joint [306]. A second potential role for CD69 is in cell contact between T lymphocytes and macrophages [28, 169, 195], particularly in inducing TNFα production [194, 195]. Synovial T lymphocytes have been found to be able to elicit TNFα production from monocytes, and this cell-contact dependent activity has been implicated as the main source of TNFα in the RA joint [40, 194, 195]. Blocking CD69 significantly reduces this production [195].

3.3.3 General markers of CD4+ memory T lymphocytes

In resting cells, the maintenance of the TCR:CD3 expression at the membrane is an active process, with constant recycling of the complex [323]. The detection of the TCR:CD3 complex on the surface of CD4+ T lymphocytes is reduced after stimulation [322-325] but there is some disagreement as to how. Some studies observe a decrease in exocytosis [323], others an increase in endocytosis [325], whereas another described a decrease in the TCR:CD3 complex half-life post-stimulation [324]. Recent data suggesting TCR-containing microvesicles are released from the immunological synapse post-stimulation may also provide an additional mechanism by which TCR:CD3 complex expression is reduced post-stimulation [72].

In this study the effect of hypoxia on the TCR:CD3 complex was investigated by assessing surface expression of CD3ε. Significant differences in the expression of CD3ε after 48 hours stimulation at different oxygen levels were observed (figures 3.5 and 3.6), with an increase at lower oxygen levels compared to physiologically healthy oxygen levels. Further investigations revealed that the pattern of CD3ε over time was

altered dependent on which oxygen level they were maintained at. At 8.5% oxygen CD3ε expression continued to decrease to 48 hours, which would be expected with the duration of stimulation provided [322]. At constant 1% oxygen, however, expression rose significantly from 24 to 48 hours (figure 3.6). This could be due to an increase in TCR:CD3 recycling in hypoxia, with more CD3 being returned to the surface of the cell after the initial downregulation. An increase in cellular granularity was observed at 1% oxygen (figure 3.7C), but unfortunately could not be correlated with CD3 expression due to equipment variance over time. However, this granularity could be due to increased vesicle cycling, and this could result in increased expression of recycled proteins on the surface membrane. This pattern could also be due to an upregulation in expression in hypoxia or an increase in TCR:CD3 release over time at 8.5% oxygen. Functionally, these results could mean that re-stimulation may occur more rapidly at lower oxygen levels, whereas re-stimulation may be limited at 8.5% oxygen. Furthermore, it has been previously reported that a longer stimulation period results in a more delayed re-expression of the TCR:CD3 complex [322] and in this study cells were not removed from stimulation plates. Future investigations where cells are removed from stimulation plates after a shorter amount of time may provide more clarity as to the effect of different oxygen levels on TCR:CD3 expression.

CD45 is one of the most highly expressed molecules on the surface of lymphocytes [160]. In the current study the CD45RO isotype was assessed as it is expressed on the memory T lymphocytes present within the populations [160]. At 21% oxygen, constant 1% oxygen and reperfusion treatments, the oxygen levels thought to most upregulate ROS (due to over-exposure to oxygen, hypoxic-mitochondrial leak, and

mitochondrial over-production post-ischemia, respectively), CD45RO levels were similar. However, an increase in CD45RO expression at 3% oxygen was observed and may give some clues as to the effect of further re-stimulations would have on cells in this environment.

As CD45RO is smaller than other isotypes, it may be able to diffuse more easily into the IS formed between an APC and a lymphocyte, and thereby may be one of the reasons for the differences in activation threshold between naïve and memory T lymphocytes. Knock-down and over-expression studies have given some insight into how it regulates lymphocyte activation [88, 160, 328] and suggest that CD45RO expression levels may need to be tightly controlled to allow for proper T lymphocyte signalling and function. Overexpression of CD45RO resulted in increased downstream signalling after TCR activation and increased proliferation [328]. Furthermore, an increase in negative selection was observed in CD4+CD8+ double positive thymocytes overexpressing CD45RO, suggesting that a larger proportion of the thymocyte population had received larger intracellular signals in response to thymic antigen presentation, and that these signals had been higher than the threshold required to initiate cell apoptosis [328]. Therefore the upregulation at 3% oxygen may be a regulatory response if further stimulation should occur. However, the CD45RO pattern of expression would need to be maintained until re-stimulation for this to play any role in deciding cell fate. This cannot be confirmed from the current data and CD45RO expression would need to be examined over a longer time period to further assess whether this is the case.

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4. RESULTS: DO ENVIRONMENTAL OXYGEN LEVELS INFLUENCE CD4+ MEMORY T LYMPHOCYTE POLARISATION AND CYTOKINE PRODUCTION?

4.1 Introduction

In the previous chapter the basic biology of CD4+ T lymphocyte responses to stimulation were assessed. In this chapter another cardinal response to stimulation is examined; CD4+ CD45RO+ T lymphocyte cytokine production and polarisation. As CD4+ central memory T lymphocytes are not expected to produce much effector cytokine immediately post-stimulation in response to artificial stimulation (and in the absence of exogenous polarising conditions), except for IL-2 [64, 98], it has been assumed that most of the data obtained is relevant to effector memory T lymphocytes present within our populations. However, central memory T lymphocytes may immediately produce cytokine when stimulated with the antigen they are specific for, or with bacterial toxins [329]. Therefore, in a less artificial system, central memory T lymphocytes may have the ability to rapidly contribute to the cytokine milieu present in the lymph node.

Pro-inflammatory Th1 and Th17 cells play an important role in controlling infection but are also thought to be involved in the chronic inflammation observed in autoimmune diseases [31, 176, 180, 184] - physiological environments that are often hypoxic [199, 200, 202, 204, 208]. Th2-like responses are more commonly associated with allergic immune responses. This can involve tissues that are well oxygen-perfused, such as the airway [47, 115, 116], but also hypoxic sites such as the skin [330]. It therefore is interesting to examine whether environmental oxygen levels can influence and regulate the development of different T helper cell subsets.

Cytokines are often used to aid the classification of T helper cells, but there are alternative markers also associated with the distinct subsets, including chemokine receptors. Chemokines and their receptors are of vital importance in the organisation of T lymphocyte biology [331] and their expression varies on T helper effector and effector memory cells. CXCR3 is a chemokine receptor upregulated on naïve T lymphocytes post-stimulation [332]. Its expression is lost over time on cells polarised to a Th2 phenotype, but is maintained on Th1 cells [332]. CXCR3 expression is associated with autoimmune disease infiltrates [63, 333, 334], and the expression of its chemokine ligands have also been observed in autoimmune sites such as the inflamed RA synovium [175, 334]. These ligands include CXCL9, CXCL10 and CXCL11 [331, 333, 335] and are expressed highly by DCs and macrophages [332].

CXCR3+ lymphocytes have been found to preferentially migrate into inflammatory sites in graft-versus-host-disease (GVHD) with high serum levels of CXCL9 and CXCL10 [336]. This increased migration resulted in a reduction in peripheral blood CXCR3+ cells, highlighting that examinations of the cellular content of peripheral blood cannot always be diagnostic or conclusive about ensuing inflammatory responses [336]. CXCR3 is thought to be important in the specific generation of Th1 cells in the lymph node, especially via CXCL10 [335], which may provide some explanation for its sustained expression on this particular cell type. Within the lymph node, IFNγ positive cells have a higher CXCR3 MFI, and CXCR3 expression is required for proper IFNγ production in response to viral antigens [331]. With regards to pro-inflammatory Th17 cells, CCR6 was originally identified as being associated as a Th17 marker from an animal model of RA, and responds to the chemokine ligands CCL20 [32]. CCR6 expression has been observed in human RA [31] and has also

been detected in Reactive Arthritis [188]. However, Th17 cells differentiating to a more Th1-like phenotype are also thought to maintain their CCR6 expression [123], so the use of it as a marker for Th17 cells has to be done with caution.

CCR4 is a marker associated with Th2 cells [331, 337], and is a chemokine receptor for the ligands CCL17 and CCL22 [331]. CCR4 has been used to identify Th2 cells in many previous studies [100, 118, 337], and more recently an upregulation of CCR4 in peripheral blood T lymphocytes was found associated with hyper-immunoglobulin E syndrome, where overt IgE production results in an atopic phenotype [338], again suggesting its expression is associated with a Th2 phenotype. Interestingly, an increase in CCR4 expression has been observed in RA, ankylosing spondylitis and systemic lupus erythematosus patient's blood [179]. As all three of these conditions are associated with antibody production [4, 339, 340] it is possible that an increase in circulating CCR4-positive cells correlates with an increase in Th2-support for pathologic B lymphocyte antibody production.

Further non-chemokine receptor surface markers also aid the delineation of T helper subsets, including markers associated with a regulatory phenotype, such as CTLA-4, that aid their suppression of inflammatory responses [74, 163]. CTLA-4 is a particularly interesting molecule as it appears to have multiple effects. Firstly, it competes for binding to the co-stimulatory molecules CD80 and CD86 on APC, therefore reducing full activation of naïve T lymphocytes [74]. It also 'rips' these molecules off the surface of APCs, internalising them and therefore further reducing the magnitude of APC co-stimulation [74, 163]. Additionally, CTLA-4 promotes the production of indoleamine 2,3-dioxygenase (IDO) from APC, which is associated with the promotion of Treg activities but inhibits effector T lymphocyte proliferation by

degrading tryptophan [74, 341, 342]. CTLA-4 expression has been found to not be restricted to only Tregs as recently activated effector cells also upregulate it [74, 138, 343], potentially limiting local inflammation.

However, these surface markers do not completely distinguish different subtypes. For example, CCR4 can also be expressed on both Th17 [119] and Treg cells [344]. Therefore, it can be of benefit to look at the proteins involved in regulating cytokine production and polarisation. ‘Master regulators’ have been identified for each of the main T helper subsets. These regulators are transcription factors whose expression has been found to be necessary for a specific T helper subset differentiation. T-bet has been identified for Th1 cells, GATA-3 for or Th2, RORγt for Th17 and Foxp3 for regulatory T lymphocytes [47, 108]. The expression of T-bet is induced mainly as a response to IFNγ receptor binding via STAT1 signalling [47, 108, 345] but also as a response to IL-12 signalling via STAT4 [107, 110, 346]. IL-12 and IFNγ can both be produced by primed DCs [94] and IFNγ production by CD4+ T helper cells can act in an autocrine manner. T-bet has been detected bound to DNA at the site of the start of transcription for many Th1-associated genes [99]. Its most well-documented role is the remodelling of the *ifnγ* gene promoting increased IFNγ expression [347, 348]. This is further emphasised by experiments in which forced expression of T-bet in Th2 cells results in IFNγ expression [98, 345, 348]. However, T-bet is not absolutely required for IFNγ transcription [110, 345] as T-bet deficient CD4+ T lymphocytes are still able to produce reasonable amounts of this cytokine. This is probably due to the activity of STAT4 [345, 347].

T-bet is also involved in the upregulation of Th1-associated molecules such as CXCR3 [47] and IL-12Rβ [47, 345]. Increased expression of IL-12Rβ promotes

further IL-12 signalling which promotes Th1 differentiation by signalling via STAT-4. Activated STAT-4 induces the expression of T-bet and other Th1-associated molecules, and thus T-bet induced expression of IL-12Rβ further propagates the development of a Th1 phenotype [47]. A vital role T-bet may have in Th1 development could involve its ability to antagonise the expression and function of GATA-3, [47, 108, 110, 129], and RORγt [108, 129, 349]. The inhibition of GATA3 by T-bet is a result of phosphorylation of tyrosine residue 525 on T-bet, which allows it to interact with GATA-3 and inhibits its binding to target promoters, such as IL-5 [350]. Similarly, T-bet-mediated inhibition of RORγt activity is achieved by inhibiting the binding of Runx1 to the *Rorc* locus, thus halting transcription of the Th17 master regulator [349]. Therefore, an important function of T-bet may be to limit the differentiation of CD4+ T lymphocytes into alternative subsets and therefore by default promote Th1-differentiation programs [110].

Alongside its role in Th2 cells, GATA-3 expression is involved in CD4+ maintenance in single positive thymocytes [47, 351], and is expressed at detectable levels in Th1 cells [98, 99]. In Th2 cells it acts to open up the chromatin at the *il4-il13* locus [111, 112], allowing for easy transcription of cytokine genes associated with the Th2 phenotype. It itself also binds to the promoters for *il5* and *il13* expression [117, 352], and is able to bind to non-promoter *il4* regulatory regions [99]. GATA-3 also antagonises the generation of the Th1 phenotype by inhibiting the expression of key molecules involved in early Th1 induction [111].

Foxp3 mutation or deletion is associated with a severe autoimmune phenotype, and its re-introduction into the immune system can drastically alleviate inflammatory manifestations [353]. As well as being associated with nTreg, Foxp3 expression is

also linked to a population of inducible Tregs - Th3s - that produce TGF-β and also express CTLA-4 [139]. Thymic-derived Treg were initially identified as the adaptive immune system's main suppressive cells, but more recent data has highlighted the important role that iTregs play in regulating peripheral immunity [137, 139]. However, Foxp3 is not expressed in all iTreg populations [139]. The mechanisms behind Foxp3-mediated induction of a regulatory phenotype is still not fully understood. Foxp3 appears to regulate two important mechanisms of suppression – the inhibition of IL-2 production and the induction of CTLA-4 expression [354]. Both of these mechanisms may be by an interaction with NFAT1, a transcription also involved in the activation of *il2* gene expression [355]. Foxp3 is an incredibly complex molecule, with three separate domains that can interact with both DNA and proteins in many different ways [356, 357]. There is increasing evidence that conventional CD4+ T lymphocytes may upregulate regulatory markers, including Foxp3, post-stimulation, and that this upregulation could be functional to limit exaggerated immune responses [138]. All cells with a suppressive phenotype will need to be able to function in low oxygen levels in parallel to their effector counterparts, and it is interesting to note that less Foxp3 expression has been observed in RA [167]. Therefore markers associated with suppressive function have been evaluated in this study.

Most of the understanding about the effects of hypoxia on CD4+ T lymphocyte phenotype and function have come from studies looking at naïve or mixed CD4+ T lymphocyte populations [204, 235, 236, 238, 289, 292]. However, it will be CD4+ CD45RO+ cells that enter an inflammatory site responding to re-exposure to the same antigen such as in the RA joint [148, 160, 306]. If these cells are re-activated in the tissue they will assert regulation over the inflammatory process and it is therefore

important to understand the effects of differing oxygen levels on this important CD4+ T lymphocyte population.

CD4+ CD45RO+ T lymphocytes used in this study were isolated from PBMC populations. Predominantly these cells will have been generated from a previous pathogen/antigen exposure, and will have circulated between the blood and tissue as effector and central memory cells. Immune responses in which T_{EM} cells are generated determine their subsequent phenotypic preference, laying down epigenetic markers that dictate gene transcription after further stimulations [63, 95, 144]. Therefore, the effector memory T lymphocytes present most likely would have been generated from a Th1, Th2 or Th17-like response, and would have continued to carry this identity into the experiments performed. This is a beneficial trait of the mammalian immune system, since if a Th1 response was the most desirable means of limiting a pathogen in a previous immune response, it is best to have a Th1 response at the ready in the memory population for identical exposures in the future. In contrast, central memory T lymphocytes have been described as losing epigenetic markers associated with certain T helper subsets [98].

Despite the described 'set' nature of CD4+ memory T lymphocytes after differentiation from naïve T lymphocytes, *in vivo* it is possible they do have some phenotypic plasticity [95, 146] as already discussed in the main introduction. This plasticity is mainly understood with regards to recently generated effector T lymphocytes thus far [95]. However, there are some suggestions that both central and effector memory T lymphocytes do also have the potential for plasticity from an *in vivo* murine study [154] and an *in vitro* human study [98]. Further aspects

associated with T lymphocyte polarisation will be introduced within the various sub-headings of this chapter.

4.1.1 This chapter's aims

The aim of this chapter is to investigate whether differing oxygen levels can influence cytokine production and polarisation of CD4+ CD45RO+ T lymphocyte populations, and to examine whether certain subsets are selected for at specific oxygen levels. CD4+ CD45RO+ T lymphocytes were again frozen at -80˚C before use experimentally. Markers associated with different T helper subsets were assessed to aid these observations. ELISA was used to assess cytokine production, and flow cytometry was utilised to assess cytokines and markers associated with different T helper subsets. In flow cytometric analysis, the FS/SS stimulation gates were determined by comparison to non-stimulated controls, as in chapter 2, and were used for assessment of these different parameters.

4.2 Results

4.2.1 The cytokine population is more pro-inflammatory at lower oxygen levels

To assess the effect of varying oxygen levels on CD4+ CD45RO+ T lymphocytes, cytokine secretion was examined by ELISA on CD4+ CD45RO+ T lymphocytes isolated from healthy control PBMC populations and frozen at -80°C until required. Six cytokines were selected for investigation; IFNγ, TNFα and IL-17A to examine pro-inflammatory effector functions; IL-4, IL-5 and IL-10 to investigate Th2-associated and anti-inflammatory effector functions. Supernatants from 45 hour stimulated populations (plus 3 hour brefeldin A-treated as cells were used for intracellular staining) were frozen at -80˚C until analysis.

Cytokine production at different oxygen levels did not differ greatly and only IL-10 production was significantly increased at 21% oxygen compared to constant 1% oxygen ($P<0.05$). However, certain trends became evident. IFNγ and TNFα production showed a trend for increased production at constant 1% oxygen and in reperfusion injury treatments. In contrast, at 8.5% oxygen the lowest IFNγ and TNFα was detected. IL-17A production did not fit the same pattern as IFNγ and TNFα with a drop in production occurring at constant 1% oxygen, although again this was not significant. IFNγ and TNFα were the most prominent cytokines produced at all oxygen levels with much less IL-4 and IL-5 detected in comparison.

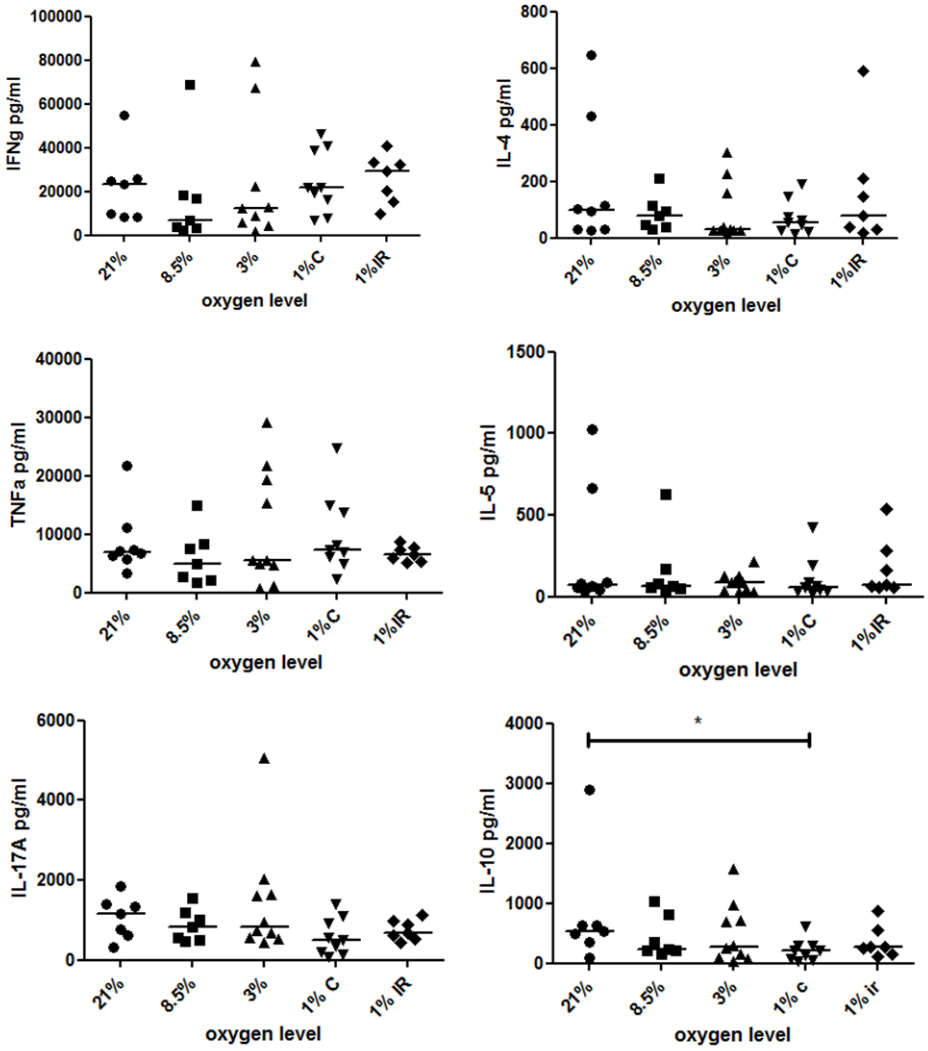

Figure 4.1. Cytokines show a significant increase in IL-10 at 21% oxygen and a slight increase in pro-inflammatory expression at 3%. *CD4+ CD45RO+ T lymphocytes were isolated using a Miltenyi MACs CD4+ memory isolation kit and frozen at -80°C for later analysis. ELISA was performed on supernatants of T lymphocytes stimulated for 48 hours at the stated oxygen levels (with the last 3 hours brefeldin A treated concurrent with subsequent intracellular staining). 2x10^5 cells were equilibrated to the stated oxygen level for 24 hours before stimulation. Supernatant pg/ml was determined before further analysis was performed. Six cytokines were chosen for investigation. A minimum of 7 donors were investigated at*

*each oxygen level. Data shows medians. Statistical analysis was performed using Mann Whitney test. *=p.0.05. 1%C = constant 1% oxygen. 1% IR = 1% oxygen with reperfusion injury. Slight trends in cytokine production could be observed, with an upregulation in IFNγ and TNFα at constant 1% oxygen, but a drop in IL-10 at this same oxygen level.*

Despite observing no statistical differences in overall cytokine production, the data from ELISAs suggested that there may be subtle patterns of cytokine expression that could be important in the microenvironment of a chronic inflammatory site. The overall equilibrium of a cytokine population may be important in determining an inflammatory response. It was therefore decided to investigate the effect of varying oxygen levels on cytokine production more closely, examining the results on a per-supernatant basis. Each cytokine was expressed as a percentage of the total population of the six cytokines investigated for each donor at each oxygen level. This helped eliminate the effects of donor-specific differences in volume of cytokine production. This analysis revealed a more pro-inflammatory pattern of cytokine production at constant 1% oxygen. Indeed, the combination of IFNγ, TNFα and IL-17A at constant 1% oxygen was significantly higher than at 8.5% oxygen. It was further apparent that IL-17A production was reduced at constant 1% oxygen compared to 8.5%, suggesting that it was mainly IFNγ and TNFα that were promoting the pro-inflammatory phenotype (figure 4.2).

This data was probed further by directly comparing two cytokines in the same supernatant. When IFNγ or TNFα were compared to IL-10, IL-4 or IL-5, the generated ratios again revealed a favouring of pro-inflammatory cytokine production in hypoxia with a decrease as oxygen increased to 8.5%. This further suggests that IFNγ and TNFα contribute more to the overall cytokine population when oxygen is increasingly scarce, whereas anti-inflammatory cytokines appear to play more of a role at physiologically healthy oxygen levels of 8.5% oxygen (figures 4.3, 4.4, 4.5).

IL-17A showed a different pattern of secretion. When looking at its contribution to the overall cytokine population it appears to play the greatest role at 8.5% oxygen (figure

4.2). However the highest ratios compared to the anti-inflammatory cytokines were observed at 3% oxygen, implying that its role in altering the balance between pro- and anti-inflammatory mechanisms may be more important at this oxygen level instead of at 8.5% oxygen. These ratios again dropped at constant 1% oxygen suggesting it did not play an important role in promoting pro-inflammatory processes at lower oxygen levels as its contribution to the overall cytokine population was on a par with the anti-inflammatory cytokines investigated. IL-17A levels were also reduced at constant 1% oxygen when compared to IFNγ and TNFα (figure 4.6).

Reperfusion injury at 1% oxygen appeared to counteract the effects of constant 1% oxygen to some extent. The role played by pro-inflammatory cytokines was reduced as revealed by the general trend of decreasing ratios in figures 4.3, 4.4 and 4.5. Anti-inflammatory cytokine production appeared to be more promoted in reperfusion injury treatments, as the percentage of total IL-4, IL-5 and IL-10 to the six cytokines investigated was higher than at constant 1% oxygen (figure 4.2).

IL-10 played a surprisingly large role in the overall cytokine population at 21% oxygen, and also appeared to play an important role at 8.5% oxygen (figure 4.2). When IFNγ and TNFα were directly compared to IL-10 the resulting ratios were again reduced at 21% and 8.5% oxygen, further confirming the involvement of this cytokine at these higher oxygen levels. IL-10 ratios were also non-significantly increased when compared to IL-4 and IL-5 at 21% oxygen (figure 4.6). The combination of this evidence suggests that the secretion of this anti-inflammatory cytokine is promoted in hyperoxic conditions. No significant differences in ratios between other alike cytokines (IFNγ and TNFα; IL-4 and IL-5) at different oxygen levels were observed (figure 4.6).

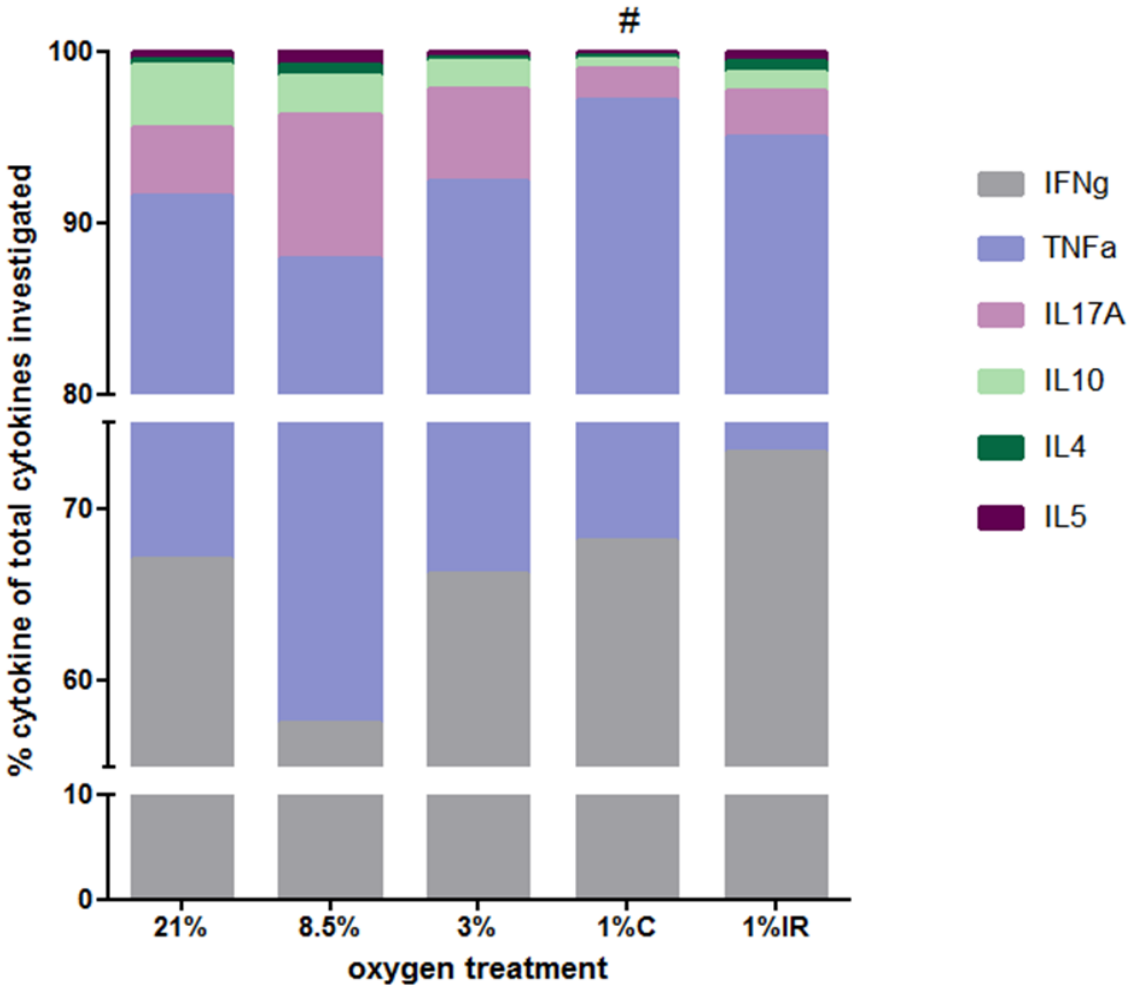

Figure 4.2. The cytokine population is more pro-inflammatory at lower oxygen levels. *CD4+ CD45RO+ T lymphocytes were isolated using a Miltenyi MACs CD4+ memory isolation kit and frozen at -80°C for later analysis. ELISA was performed on supernatants from CD4+ memory T lymphocytes stimulated for 48 hours (with the last 3 hours brefeldin A treated concurrent with subsequent intracellular staining). $2x10^5$ cells were equilibrated to the stated oxygen level for 24 hours before stimulation. Supernatant pg/ml was determined before further analysis was performed. Six cytokines were chosen for investigation. A minimum of 7 donors were investigated at each oxygen level and each cytokine was investigated in each donor. The overall contribution of the individual cytokines to the cytokine population was*

assessed as a percentage of the six cytokines investigated. Statistical analysis was performed using Mann Whitney test. # p<0.001 for cumulative percentages of pro-inflammatory cytokine IFNγ, TNFα IL-17A at constant 1% oxygen, compared to 8.5% oxygen. 1%C = constant 1% oxygen. 1% IR = 1% oxygen with reperfusion injury. Pro-inflammatory cytokines played a greater role in the overall cytokine population at constant 1% oxygen than at higher oxygen levels.

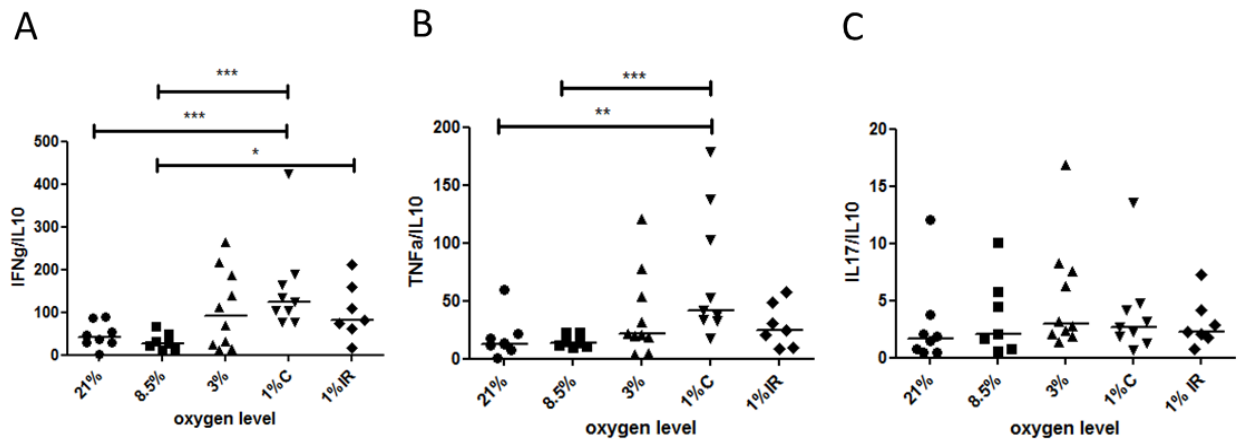

Figure 4.3. Direct pairwise comparisons of cytokine production at different oxygen levels with IL-10 cytokine production. *CD4+ CD45RO+ T lymphocytes were isolated using a Miltenyi MACs CD4+ memory isolation kit and frozen at -80°C for later analysis. ELISA was performed on supernatants from CD4+ memory T lymphocytes stimulated for 48 hours (with the last 3 hours brefeldin A treated concurrent with subsequent intracellular staining). 2x10^5 cells were equilibrated to the stated oxygen level for 24 hours before stimulation. Supernatant pg/ml was determined before further analysis was performed. Six cytokines were chosen for investigation. A minimum of 7 donors were investigated at each oxygen level and each cytokine was investigated in each donor. Ratios of IL-10 with A) IFNγ, B) TNFα and C) IL-17A were determined. Statistical analysis was performed using Mann Whitney test and medians are shown in the data. *=p<0.05. **=p<0.005. ***=p<0.001. 1%C = constant 1% oxygen. 1% IR = 1% oxygen with reperfusion injury. Pro-inflammatory cytokines IFNγ and TNFα ratio's to IL-10 are greater at lower oxygen levels.*

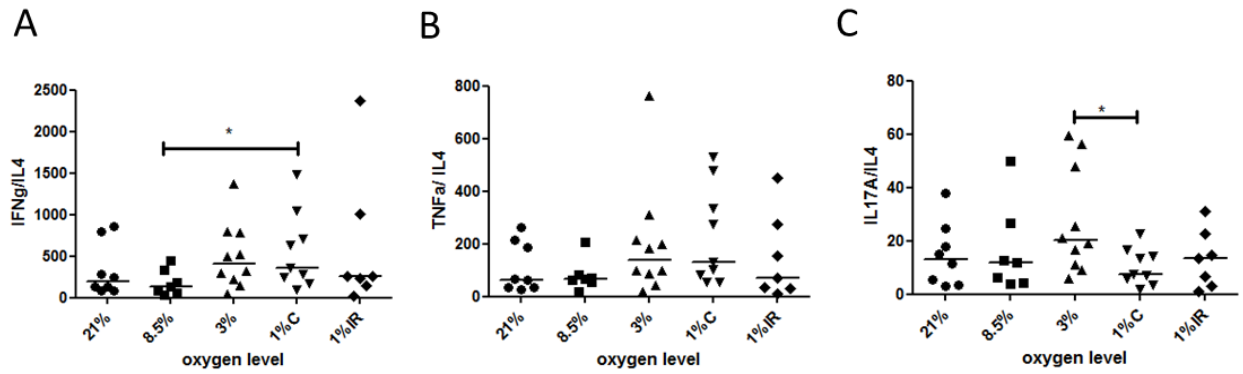

Figure 4.4. Direct pairwise comparisons of cytokine production at different oxygen levels with IL-4 cytokine production. *CD4+ CD45RO+ T lymphocytes were isolated using a Miltenyi MACs CD4+ memory isolation kit and frozen at -80°C for later analysis. ELISA was performed on supernatants from CD4+ memory T lymphocytes stimulated for 48 hours (with the last 3 hours brefeldin A treated concurrent with subsequent intracellular staining). 2x10^5 cells were equilibrated to the stated oxygen level for 24 hours before stimulation. Supernatant pg/ml was determined before further analysis was performed. Six cytokines were chosen for investigation. A minimum of 7 donors were investigated at each oxygen level and each cytokine was investigated in each donor. Ratios of IL-4 with A) IFNγ, B) TNFα and C) IL-17A were determined. Statistical analysis was performed using Mann Whitney test and medians are shown in the data. *=p<0.05. 1%C = constant 1% oxygen. 1% IR = 1% oxygen with reperfusion injury. Pro-inflammatory cytokine IFNγ ratio to IL-4 is greater at constant 1% oxygen.*

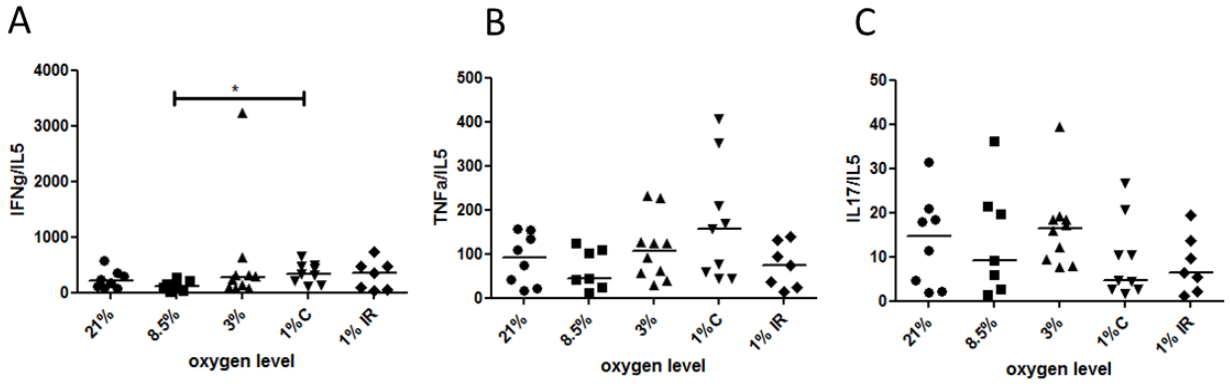

Figure 4.5. Direct pairwise comparisons of cytokine production at different oxygen levels with IL-5 cytokine production. *CD4+ CD45RO+ T lymphocytes were isolated using a Miltenyi MACs CD4+ memory isolation kit and frozen at -80°C for later analysis. ELISA was performed on supernatants from CD4+ memory T lymphocytes stimulated for 48 hours (with the last 3 hours brefeldin A treated concurrent with subsequent intracellular staining). 2x10^5 cells were equilibrated to the stated oxygen level for 24 hours before stimulation. Supernatant pg/ml was determined before further analysis was performed. Six cytokines were chosen for investigation. A minimum of 7 donors were investigated at each oxygen level and each cytokine was investigated in each donor. Ratios of IL-5 with A) IFNγ, B) TNFα and C) IL-17A were determined. Statistical analysis was performed using Mann Whitney test and medians are shown in the data. *=p<0.05. 1%C = constant 1% oxygen. 1% IR = 1% oxygen with reperfusion injury. Pro-inflammatory cytokines IFNγ and TNFα ratio's to IL-5 are greater at lower oxygen levels.*

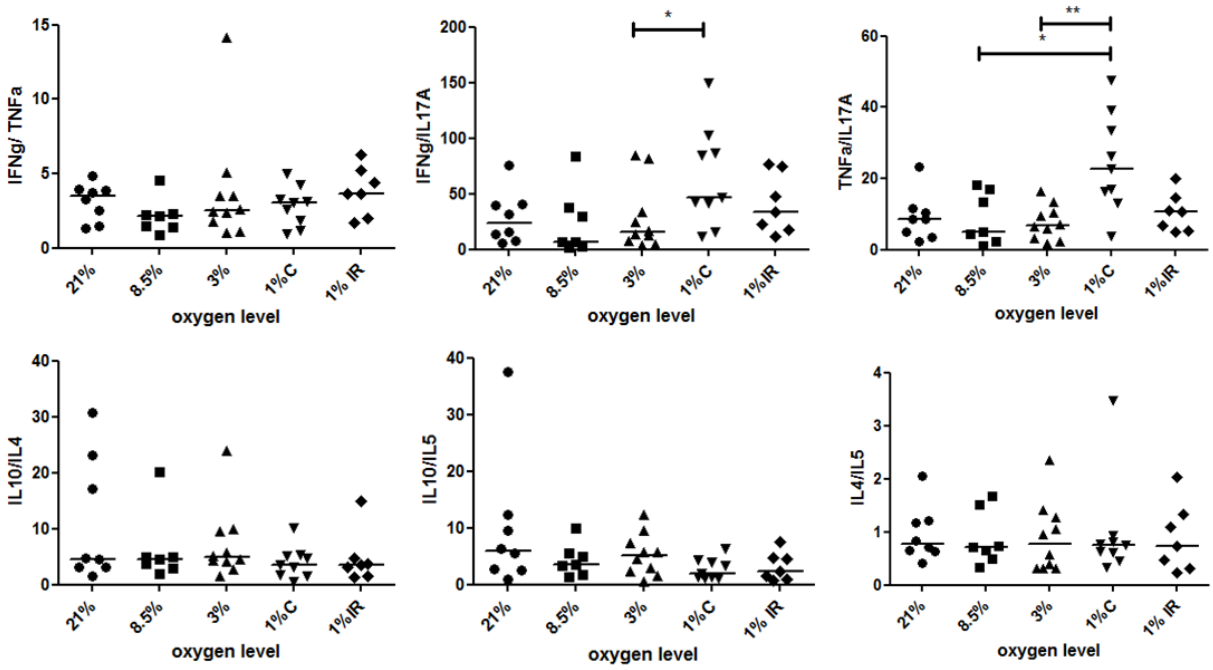

Figure 4.6. Direct pairwise comparisons of cytokine production at different oxygen levels between alike cytokines. *CD4+ CD45RO+ T lymphocytes were isolated using a Miltenyi MACs CD4+ memory isolation kit and frozen at -80°C for later analysis. ELISA was performed on supernatants from CD4+ memory T lymphocytes stimulated for 48 hours (with the last 3 hours brefeldin A treated concurrent with subsequent intracellular staining). 2x10^5 cells were equilibrated to the stated oxygen level for 24 hours before stimulation. Supernatant pg/ml was determined before further analysis was performed. Six cytokines were chosen for investigation. A minimum of 7 donors were investigated at each oxygen level and each cytokine was investigated in each donor. Ratios of were as follows A) IFNγ/ TNFα B) IFNγ/IL-17A C) TNFα/IL-17A D) IL-10/IL-4 E) IL-10/IL-5 and F) IL-4/ IL-5. Statistical analysis was performed using Mann Whitney test and medians are shown in the data. *=p<0.05. **=p<0.005. 1%C = constant 1% oxygen. 1% IR = 1% oxygen with reperfusion injury. Alike cytokines do not differ in their ratios across different oxygen levels, excepting IL-17A.*

Further analysis of T helper polarisation using flow cytometry

To further clarify the effect of varying oxygen levels on CD4+ CD45RO+ T lymphocyte cytokine production, intracellular cytokine staining was performed on the same cell populations. Three panels were employed to investigate this. The first set of panels (discussed in greater detail in later sub-chapters) investigated cytokine production alongside alternative markers of T helper cells subsets; Panel 1 assessed IFNγ and IL-17A production alongside CXCR3, CCR6, and T-bet; Panel 2 assessed IL-4 and IL-10 production alongside CCR4, CTLA-4, GATA-3 and Foxp3. These panels were assessed after both 24 and 48 hours stimulation. A third panel assessed all four cytokines simultaneously (IFNγ, IL-17, IL-4 and IL-10) and was performed after 48 hours stimulation (please see Appendices for details of the panels).

4.2.1.1 Intracellular cytokine staining reveals a decrease in cytokine expression after 48 hours at constant 1% oxygen and confirms the pro-inflammatory effect of hypoxia

Initially intracellular cytokine staining was investigated at 48 hours stimulation by using the cytokine-only staining panel. This revealed an upregulation in IFNγ and IL-17A at 3% oxygen (figure 4.7B) after 48 hours stimulation, and an upregulation in IFNγ production at 21% oxygen (figure 4.7C). However, both of these pro-inflammatory cytokines were reduced at constant 1% oxygen, with reperfusion injury resulting in a slight elevation in cytokine production in comparison. T lymphocytes stimulated at 8.5% oxygen did not show a high production of either pro-inflammatory cytokine, but showed a reasonable production of IL-10. The highest production of IL-10 was observed at 21% oxygen, but again constant 1% oxygen again showed a

depression in production. This was in agreement with previous analysis of supernatants (figure 4.7B). IL-4 did not show any significant patterns, but a slight increase in production was observed at 3% oxygen. High background was detected during IL-4 staining and additional blocking steps were employed to improve resolution.

Therefore, cytokine production at constant 1% oxygen was generally depressed by 48 hours staining which was not in agreement with the basic ELISA data. We therefore used the other two panels to assess cytokine production at both 24 and 48 hours alongside other markers of T helper cell subsets. Similar patterns of cytokine production were observed at 48 hours compared to the cytokine-only staining panel (figure 4.7C) with only minor changes probably resulting from natural experimental deviation and differences in donors between experiments. IL-10 production showed the greatest difference between the two. IL-4 staining could not be detected after 24 hours and this data was therefore excluded from analysis.

IFNγ production after 24 hours stimulation was highest at 21% oxygen, constant 1% oxygen and reperfusion injury treatments. However, IFNγ production at constant 1% oxygen and reperfusion injury treatments decreased over time, and 21% oxygen resulted in a more static percentage of IFNγ positive cells at both time points (figure 4.7C). In contrast, at 3% and 8.5% oxygen, there was a much bigger increase in the percentage of cytokine-positive cells from 24 to 48 hours.

In general, all cytokines appeared to be delayed in their production at 8.5% oxygen. IL-10 production was reduced at 8.5% oxygen at 24 hours but increased to 48 hours. Therefore, there may be a slight reduction in some cytokine production at 8.5%

oxygen, but IL-10 may be less affected by this over time compared to the other cytokines investigated.

In contrast, at 3% oxygen, IFNγ and IL-17A production at 24 hours was low compared to other oxygen levels, but expression increased to 48 hours stimulation. IL-17A production at constant 1% oxygen appeared to be depressed compared to other oxygen levels at both time points.

Intracellular staining revealed a similar ratio of IFNγ/IL-17A (figure 4.8) to the ratios attained from supernatants, further supporting the downregulation in IL-17A production at constant 1% oxygen (figure 4.8). However, the IFNγ/ IL-10 ratio was slightly altered, with a downregulation in IFNγ compared to IL-10 at constant 1% oxygen (figure 4.8). This may have been due to differences when comparing ELISA data to intracellular staining. ELISA reveals the amount of free cytokine within the supernatant that has been released over time, whereas intracellular staining reveals a snapshot of what cytokine preference a cell has at a specific time-point, therefore revealing the changeable nature of cytokine production over time.

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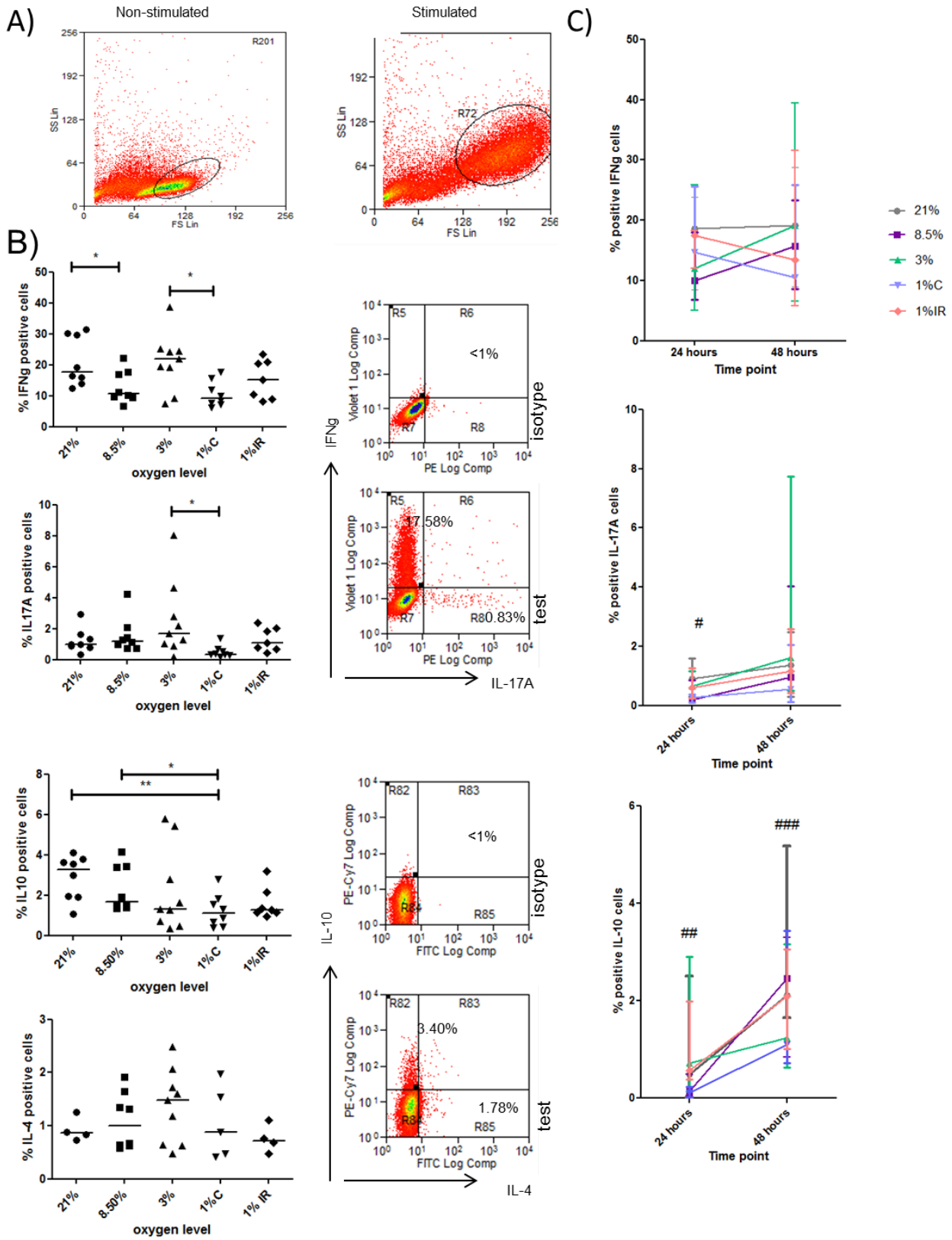
A)
Non-stimulated
Stimulated
R201
R72
SS Lin
FS Lin
B)
% IFNg positive cells
% IL17A positive cells
% IL10 positive cells
% IL-4 positive cells
oxygen level
21%
8.5%
3%
1%C
1%IR
IFNg
IL-17A
IL-10
IL-4
isotype
test
Violet 1 Log Comp
PE Log Comp
PE-Cy7 Log Comp
FITC Log Comp
<1%
17.58%
80.83%
3.40%
1.78%
C)
% positive IFNg cells
% positive IL-17A cells
% positive IL-10 cells
Time point
24 hours
48 hours
21%
8.5%
3%
1%C
1%IR

Figure 4.7

Figure 4.7. Intracellular cytokine staining over time. *CD4+ CD45RO+ T lymphocytes were isolated using a Miltenyi MACs CD4+ memory isolation kit and frozen at -80°C for later analysis. 2x10^5 cells were equilibrated to the stated oxygen level for 24 hours before stimulation with 2µg/ml anti-CD3// 5µg/ml anti-CD28. 2µg/ml brefeldin A treatment was given for the last three hours of stimulation for cytokine analysis. A) Forward scatter (FS)/ side scatter (SS) plots for non-stimulated and stimulated cells show the selected gate on stimulated cells (R72). Intracellular cytokine staining for IFNγ, IL-17A, IL-10 and IL-4 was employed to determine cytokine production on a per cell basis at the oxygen levels stated. B) Using the cytokine staining panel, CD4+ memory T lymphocytes were intracellularly stained after 48 hours stimulation and their percentage cytokine positive cells determined. Example plots, including isotype controls, from a donor stimulated at 8.5% oxygen are shown, with percentages of each cytokine stated. Isotype controls were gated to within 1% of the control except for IL-10 where a fixed gate was applied. C) Using the two marker panels, cytokine production on a per cell basis was investigated after 24 hours and 48 hours and their percentage cytokine positive cells determined. Statistical analysis was performed using Mann Whitney test, and median values are shown in the data with range where necessary. *=p<0.05 **=p<0.005. # p<0.05 between 21% oxygen with 8.5% and 1%C oxygen at 24 hours. ## p<0.05 between 3% and 1%C oxygen. ### p< 0.05 between 21% oxygen with 3% and 1%C oxygen. 1%C = constant 1% oxygen. 1% IR = 1% oxygen with reperfusion injury. Data is representative of at least 2500 cells in the stimulation gate. A minimum of 7 donors were investigated at each oxygen level for IFNγ, IL-17A and IL-10 after 48 hours, and a minimum of 4 donors for IL-4 after 48 hours. A minimum of 4 donors were investigated at each oxygen level for the 24 hour investigations in C). Although initially, pro-inflammatory, cytokine production over time at constant 1% oxygen appears to not be sustained over time.*

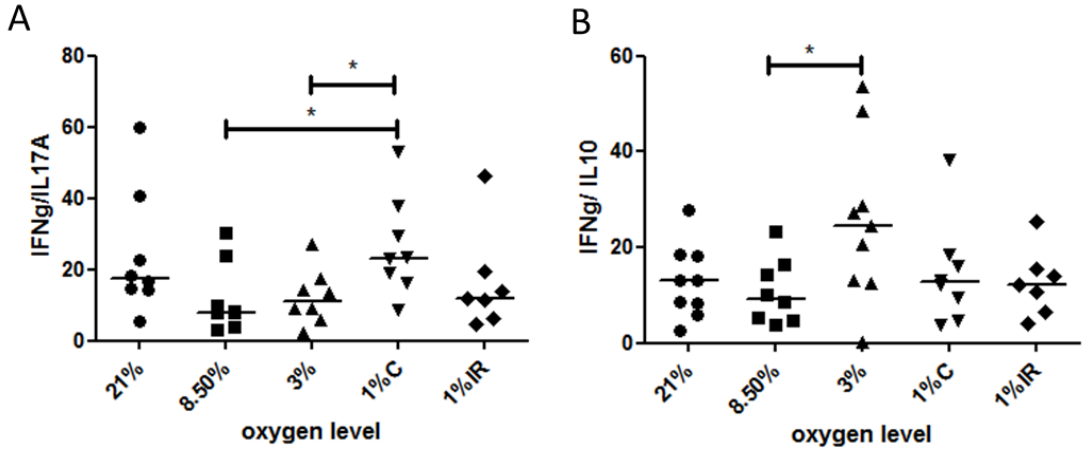

Figure 4.8. Intracellular cytokine staining revealed similar ratios of cytokines to the ELISA assessment of cytokines in supernatant. *CD4+ CD45RO+ T lymphocytes were isolated using a Miltenyi MACs CD4+ memory isolation kit and frozen at -80°C for later analysis. 2x10^5 cells were equilibrated to the stated oxygen level for 24 hours before stimulation with 2µg/ml anti-CD3// 5µg/ml anti-CD28 for 48 hours. Intracellular cytokine staining was used to determine cytokine production on a per cell basis at the oxygen levels stated. All stimulations were treated for the last 3 hours with 2µg/ml brefeldin A to inhibit cytokine secretion. A) Ratios of IFNγ % positive cells with IL-17 % positive cells were investigated. B) Ratios of IFNγ positive cells with IL-10 were investigated. Statistical analysis was performed using Mann Whitney test and median values are shown in the data. *=p<0.05. 1%C = constant 1% oxygen. 1% IR = 1% oxygen with reperfusion injury. Data is representative of at least 2500 cells in the stimulation gate. A minimum of 7 donors were investigated at each oxygen level. IFNγ plays more of a prominent role at constant 1% oxygen compared to IL-17A. The same gating strategy was used as in figure 4.7.*

4.2.1.2 Increase in the percentage of cytokine double-positive cells at 3% oxygen

The cytokine-only panel allowed for investigation into the presence of cytokine double-positive cells within CD45RO+CD4+ cell populations after 48 hours stimulation. Double positive cytokines were mainly observed as another cytokine being simultaneously expressed alongside IFNγ. Combinations of other cytokines were rarely observed (data not shown).

A significant increase in the number of IFNγ+IL-17A+ cells was observed at 3% oxygen, although there was quite a spread of data depending on donor (figure 4.9B). Additionally IFNγ+IL-10 cells were increased at 21%, 8.5% and 3% oxygen compared to both low oxygen treatments (figure 4.9A). The spread of data at 3% oxygen meant that this was not significant, whereas data from 21% and 8.5% oxygen cultures were significantly increased compared to constant 1% oxygen. The numbers of IFNγ+IL-4+ CD4+ T lymphocytes was consistently low, but were increased at 21% and 3% oxygen and reduced at 8.5% oxygen and in cells exposed to reperfusion injury (figure 4.9C).

At constant 1% oxygen, the numbers of IFNγ+IL-17A+ and IFNγ+IL-10+ double-positive cells were significantly reduced. Reperfusion treatment alleviated this reduction slightly. This reflected the overall reduction in IL-17A and IL-10 intracellular staining observed at constant 1% oxygen (figure 4.9).

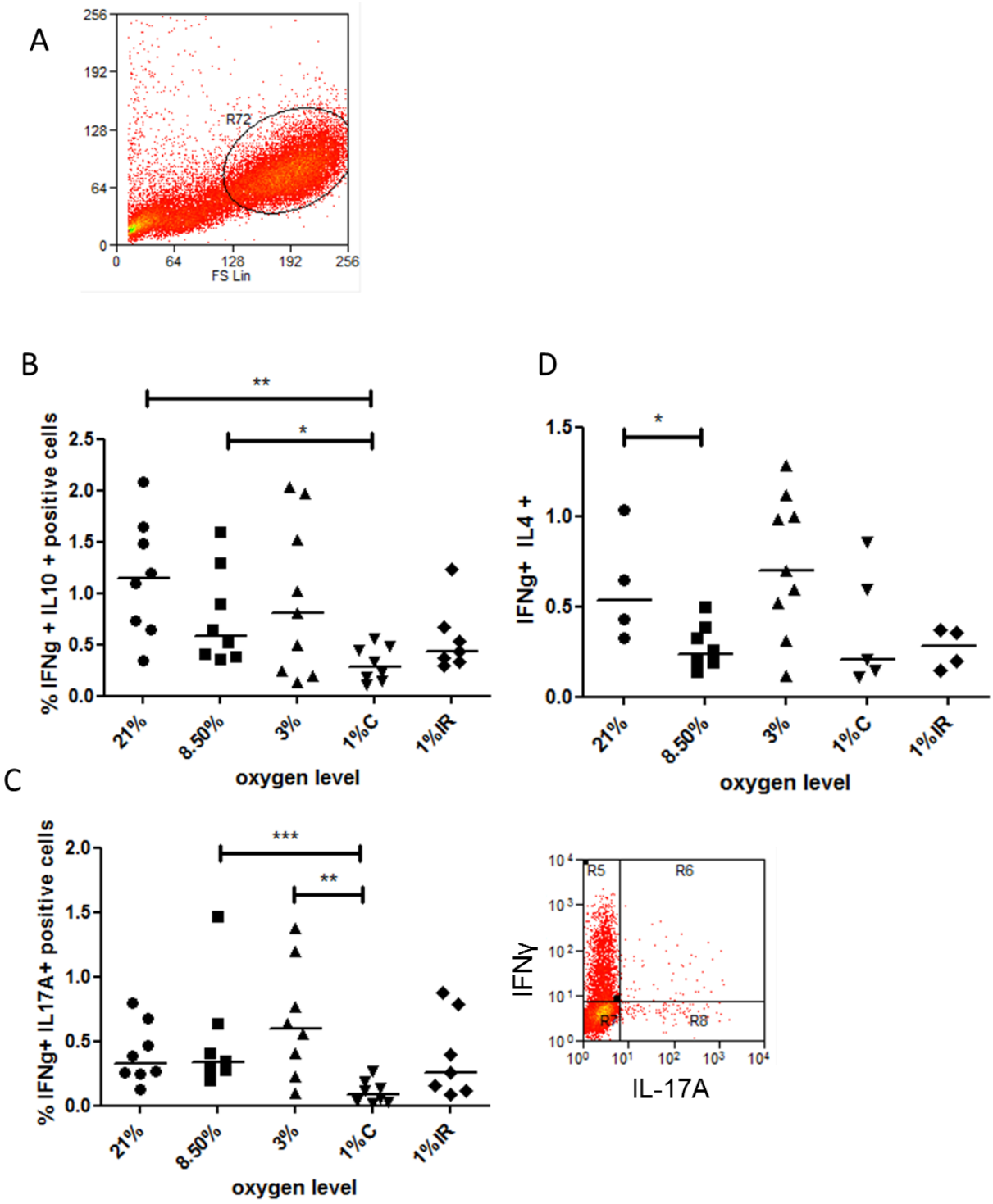

Figure 4.9

Figure 4.9. Increased cytokine double positives at 3% oxygen. *CD4+ CD45RO+ T lymphocytes were isolated using a Miltenyi MACs CD4+ memory isolation kit and frozen at -80°C for later analysis. 2x10^5 cells were equilibrated to the stated oxygen*

level for 24 hours before stimulation with 2µg/ml anti-CD3// 5µg/ml anti-CD28. Intracellular cytokine staining was used to determine cytokine production on a per cell basis at the oxygen levels stated. All stimulations were treated for the last 3 hours with 2µg/ml brefeldin A to inhibit cytokine secretion. Using the cytokine staining panel, CD4+ memory T lymphocytes were stimulated for 48 hours and the percentage of cytokine double positive cells was determined. A) An example forward scatter (FS)/ side scatter (SS) plot showing gating on stimulated cells. B) IFNγ+IL-10+ cells C) IFNγ+IL-17+ cells with an example plot. D) IFNγ+IL-4+ cells. Statistical analysis was performed using Mann Whitney test and median values are shown.=p<0.05. **=p<0.005. ***=p<0.001. 1%C = constant 1% oxygen. 1% IR = 1% oxygen with reperfusion injury. Data is representative of at least 2500 cells in the stimulation gate. A minimum of 7 donors was investigated at each oxygen level excepting where IL-4 was involved, where a minimum of 4 donors was investigated. An increase in IFNγ+IL-17A+ double positive cells was seen at 3% oxygen, whereas double positive cells were not observed frequently at constant 1% oxygen.*

4.2.2 Markers associated with different subsets are differentially expressed in varying oxygen levels

Historically, cytokine production was the cardinal marker for T lymphocyte polarisation. Due to the importance of the cytokines' role within the immune response this output is still considered when evaluating different subsets in contemporary science. However, as shown, cells expressing two cytokines associated with different subsets are present [145, 154, 358]. Additionally, stimulation methods do not always result in detectable cytokine production from every cell at the time point of analysis, therefore alternative markers that define T lymphocyte subsets is of additional benefit.

4.2.2.1 Pro-inflammatory markers are upregulated at lower oxygen levels

4.2.2.1.1 Assessment of surface markers associated with a pro-inflammatory phenotype

In this study, the percentage of CXCR3 positive cells remained similar across the different oxygen levels investigated with a large majority of CXCR3+ CD4+ memory T lymphocytes (figure 4.10B). When intensity of expression was investigated, using the MFI, a pattern emerged of decreased expression at 3% oxygen but an increase at constant 1% oxygen (figure 4.10C). However, this pattern correlated with the FS values obtained at different oxygen levels (figure 4.10D and fig 3.8) and the differences in CXCR3 MFI are therefore most probably due to the differences in cell size at different oxygen levels.

The Th17-associated marker, CCR6, showed no statistical significant increase or decrease in expression at any particular oxygen level, and appeared to be quite donor-specific in its expression on CD4+ CD45RO+ T lymphocytes (figure 4.10B).

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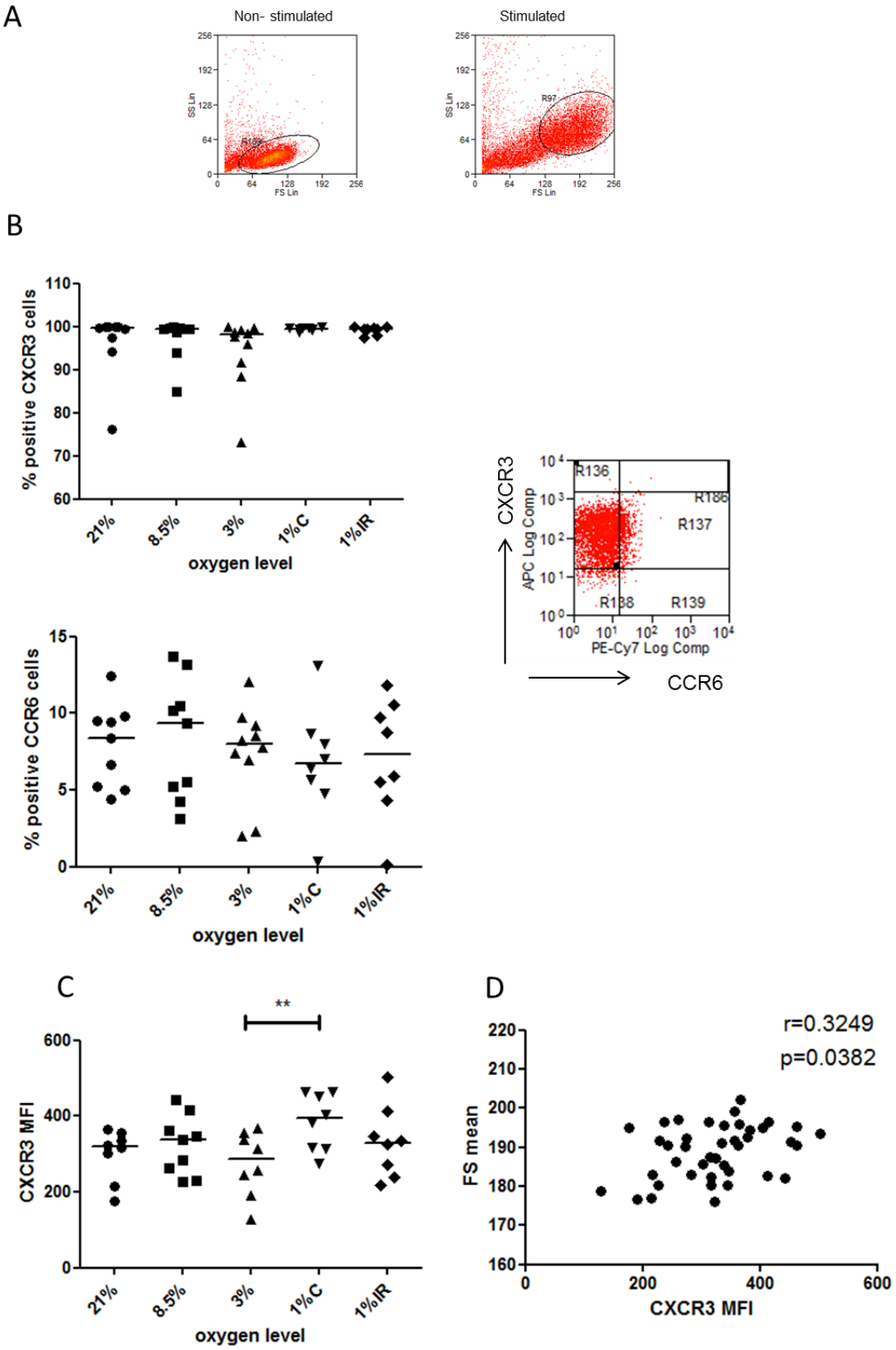
A
Non- stimulated
Stimulated
SS Lin
FS Lin
R97
B
% positive CXCR3 cells
oxygen level
21%
8.5%
3%
1%C
1%IR
% positive CCR6 cells
CXCR3
APC Log Comp
PE-Cy7 Log Comp
CCR6
R136
R186
R137
R138
R139
C
CXCR3 MFI
**
D
FS mean
r=0.3249
p=0.0382

Figure 4.10

Figure 4.10. Surface markers associated with pro-inflammatory CD4+ memory T lymphocytes do not differ at varying oxygen levels. *CD4+ CD45RO+ T lymphocytes were isolated using a Miltenyi MACs CD4+ memory isolation kit and frozen at -80°C for later analysis. 2x10^5 cells were equilibrated to the stated oxygen level for 24 hours before stimulation with 2µg/ml anti-CD3// 5µg/ml anti-CD28. All stimulations were treated for the last 3 hours with 2µg/ml brefeldin A. Using the pro-inflammatory marker panel, surface markers associated with Th1 and Th17 phenotypes were investigated at different oxygen levels using flow cytometry. A) Forward scatter (FS)/ side scatter (SS) plots for non-stimulated and stimulated cells show the selected gate on stimulated cells (R97). B) Percentage positive CXCR3 and CCR6 cells were assessed at different oxygen levels after 48 hours stimulation. An example dot plot is shown. C) CXCR3 MFI at the different oxygen levels after 48 hours stimulation. Mann Whitney statistics were used for analysis and median values are shown. **=p< 0.01. D) Correlation between CXCR3 and cell size as assessed by Spearman Rank Correlation. R= 0.3249, p= 0.0382. Data is representative of at least 2500 cells in the stimulation gate. A minimum of 8 donors was investigated at each oxygen level. CXCR3 expression appeared to correspond to cell size, but no other effect of environmental oxygen level could be seen on CXCR3 and CCR6 expression.*

4.2.2.2 Pro-inflammatory Transcription Factors

In this study, T-bet expression initially appeared highest in reperfusion injury treatments after 24 hours (figure 4.11B). However, after 48 hours stimulation this pattern altered, with T-bet expression being highest at 3% oxygen, and a reduction in expression in reperfusion treatments occurring (figure 4.11C).

Furthermore, two of the three oxygen levels thought to expose cells to the most ROS – 21% and 1%IR - contained the highest numbers of T-bet+IFNγ+ cells after 24 hours stimulation (figure 4.11B), but this was not sustained overtime to 48 hours. The reperfusion injury treatment particularly resulted in a drop in double positive cells further clarifying the T-bet only data. At 8.5% oxygen, the expression of T-bet+IFNγ Th1-associated markers was low at both 24 and 48 hours, suggesting they played less of an overall role within the cell population at physiologically healthy oxygen levels.

Flow cytometric data acquired from staining CD4+ CD45RO+ cells for RORγt, the master regulator of Th17 cells, was eliminated from analysis due to its variability of staining. Time constraints did not allow for further analysis.

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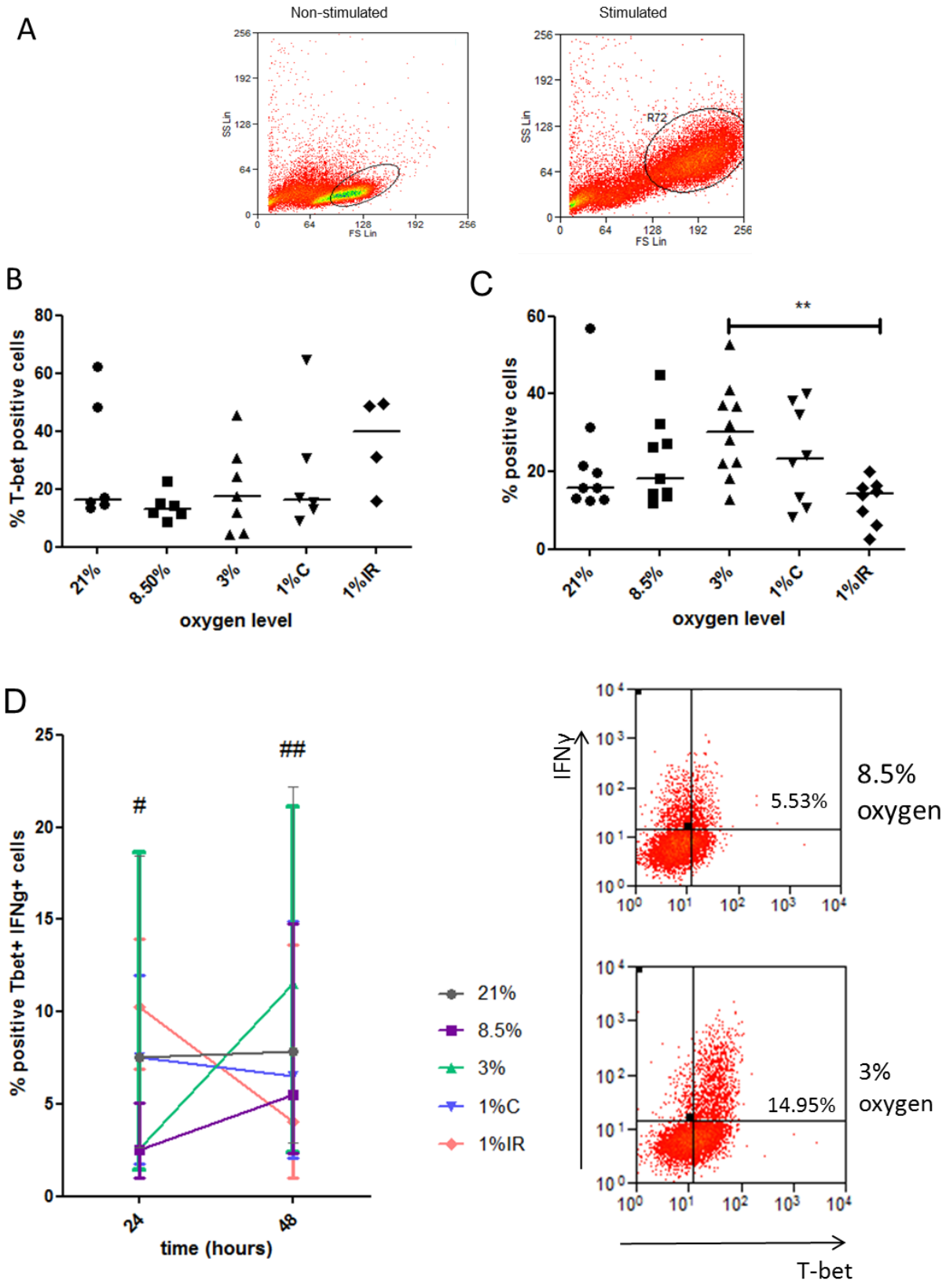
A
Non-stimulated
Stimulated
SS Lin
FS Lin
R72
B
% T-bet positive cells
21%
8.50%
3%
1%C
1%IR
oxygen level
C
% positive cells
**
21%
8.5%
3%
1%C
1%IR
oxygen level
D
% positive Tbet+ IFNg+ cells
#
##
24
48
time (hours)
21%
8.5%
3%
1%C
1%IR
IFNγ
5.53%
8.5%
oxygen
14.95%
3%
oxygen
T-bet

Figure 4.11

Figure 4.11. Alternative pro-inflammatory markers are upregulated in hypoxia. *CD4+ CD45RO+ T lymphocytes were isolated using a Miltenyi MACs CD4+ memory isolation kit and frozen at -80°C for later analysis. 2x10^5 cells were equilibrated to the stated oxygen level for 24 hours before stimulation with 2µg/ml anti-CD3// 5µg/ml anti-CD28. 2µg/ml brefeldin A treatment was given for the last three hours of stimulation for cytokine analysis. A) Forward scatter (FS)/ side scatter (SS) plots for non-stimulated and stimulated cells show the selected gate on stimulated cells (R72). Using the pro-inflammatory marker panel, T-bet and IFNγ expression were investigated at different oxygen levels by intracellular staining and flow cytometry. B) The percentage of T-bet positive cells was assessed at the stated oxygen levels after 24 hours stimulation. C) The percentage of T-bet positive cells was assessed at the stated oxygen levels after 48 hours stimulation. D)The percentage of Tbet+IFNγ+ cells was assessed at both 24 and 48 hours post-stimulation. Example plots of T-bet+IFNγ+ staining after 48 hours stimulation. Statistical analysis was performed using Mann Whitney test, and median values are shown in the data with range where necessary. **=p<0.005. # p<0.05 1%C compared to 8.5% oxygen, and p<0.005 21% compared with 8.5% oxygen at 24 hours. ## p<0.05 3% compared to 8.5% oxygen at 48 hours.1%C = constant 1% oxygen. 1% IR = 1% oxygen with reperfusion injury. Data is representative of at least 2500 cells in the stimulation gate. A minimum of 4 donors was investigated at each oxygen level. The percentage of T-bet+IFNγ+ cells at 3% oxygen increased over time.*

4.2.2.3 Th2 associated markers are increased at physiologically healthy oxygen levels

The highest percentage of CCR4 positive cells and the highest CCR4 MFI was observed at 8.5% oxygen (figure 4.12B and C). A stepwise decrease in MFI values to 1% oxygen treatments was observed with stimulation at constant 1% oxygen resulting in the lowest CCR4 expression intensity. As constant 1% oxygen contained the largest cells (figure 3.8) it can be assumed that the upregulation in CCR4 expression was a more direct response to the environmental oxygen levels experienced by the cells and not due to cell size as observed for CXCR3 expression.

The transcription factor GATA-3 has been labelled the 'master-regulator' of Th2 cells after it was discovered that its deletion can inhibit the generation of this particular subset in CD4+ cells [47, 117]. Similar to the increase in CCR4 expression, the highest GATA-3 expression was also observed at 8.5% oxygen. However, high GATA-3 expression was also observed at constant 1% oxygen after 48 hours stimulation contrary to what would be expected from the CCR4 data. Nonetheless, GATA-3 was again reduced in reperfusion injury (figure 4.12D).

The combination of these markers, at the two different time points being investigated, provided a clearer picture of what was occurring at different oxygen levels (figure 4.12E). At 8.5% oxygen, the percentage of CCR4+GATA3+ positive cells was highest at both time points compared to all other oxygen levels. After 24 hours this was significant when compared to 3% and constant 1% oxygen. A Th2-like response may therefore be promoted at 8.5% oxygen. However, at 48 hours cells stimulated at constant 1% oxygen increased their combined expression of CCR4 and GATA3, suggesting a Th2-like response was favoured, but delayed, at this oxygen level.

CD4+ memory T lymphocytes stimulated at 3% oxygen also increased CCR4+GATA3+ combined expression over time, but not to the extent observed at constant 1% oxygen (figure 4.12E).

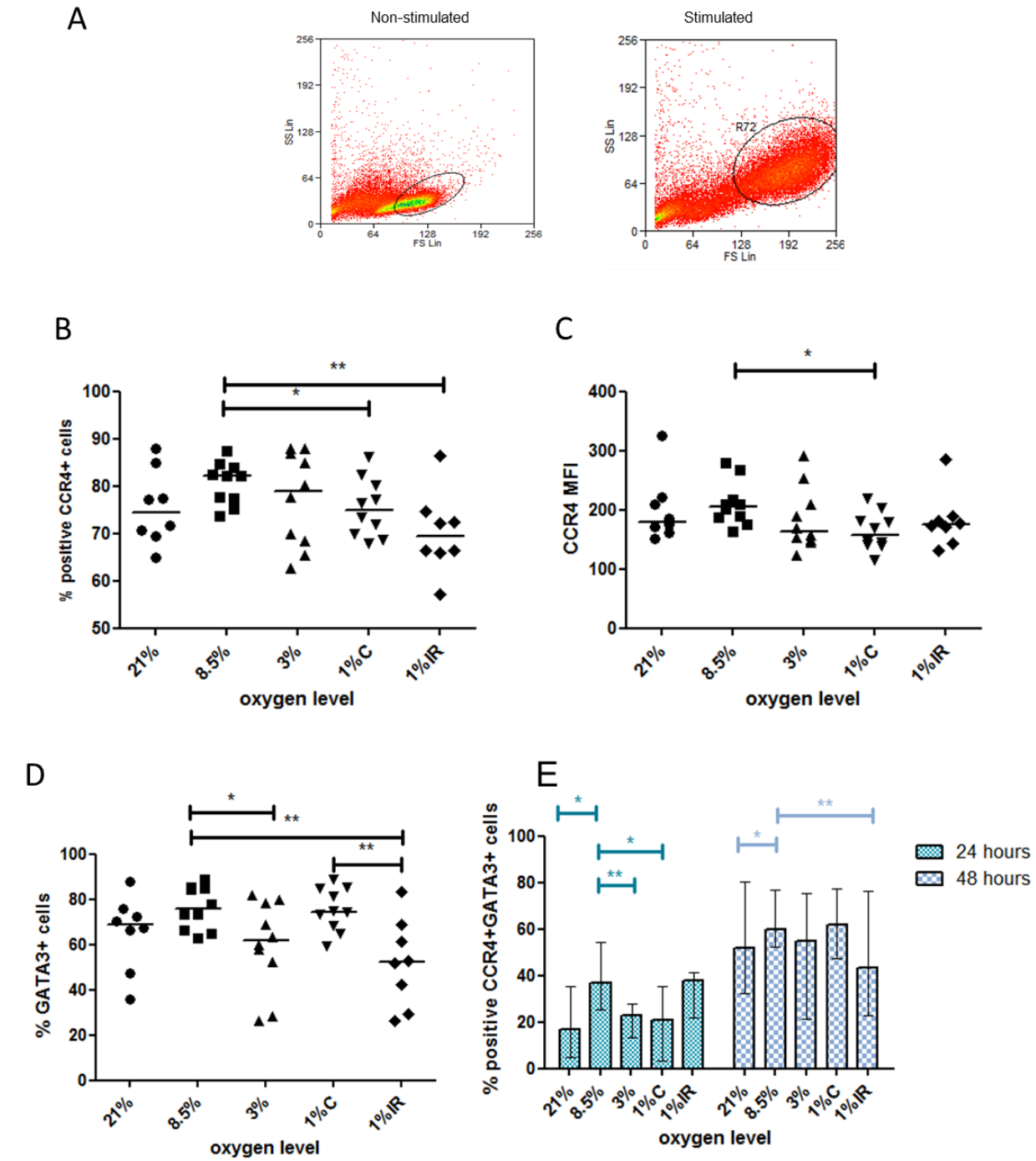
A
Non-stimulated
Stimulated
SS Lin
FS Lin
R72
B
% positive CCR4+ cells
oxygen level
21%
8.5%
3%
1%C
1%IR
C
CCR4 MFI
D
% GATA3+ cells
E
% positive CCR4+GATA3+ cells
24 hours
48 hours

Figure 4.12

Figure 4.12. Markers associated with a Th2 phenotype are upregulated at physiologically healthy oxygen levels*. CD4+ CD45RO+ T lymphocytes were isolated using a Miltenyi MACs CD4+ memory isolation kit and frozen at -80°C for later analysis. 2x10^5 cells were equilibrated to the stated oxygen level for 24 hours before stimulation with 2µg/ml anti-CD3// 5µg/ml anti-CD28. 2µg/ml brefeldin A treatment was given for the last three hours of stimulation for additional cytokine analysis. A) Forward scatter (FS)/ side scatter (SS) plots for non-stimulated and stimulated cells show the selected gate on stimulated cells (R72). Using the anti-inflammatory marker panel, surface markers and transcription factors associated with a Th2 phenotype were investigated at different oxygen levels by both surface and intracellular staining, and assessed by flow cytometry. CCR4 surface marker staining was assessed and the B) percentage of positive cells and C) MFI were assessed after 48 hours stimulation. D) GATA-3 intracellular staining was performed and assessed after stimulation at varying oxygen levels. E) The percentage of CCR4+GATA3+ cells at both 24 (turquoise) and 48 (blue) hours was assessed. Statistical analysis was performed using the Mann Whitney test and median values are shown in the data. *=p<0.05. **=p<0.005. ***=p<0.001. 1%C = constant 1% oxygen. 1% IR = 1% oxygen with reperfusion injury. Data is representative of at least 2500 cells in the stimulation gate. A minimum of 4 donors was investigated at each oxygen level. CCR4 and GATA-3 expression were highest at 8.5% oxygen.*

4.2.2.4 Markers associated with a regulatory phenotype are increased at 3% oxygen after 48 hours stimulation

Foxp3 is a transcription factor integral for the maintenance and functioning of most Treg cells, and is thought of as the 'master regulator' of nTregs and some iTregs [353, 359, 360]. After 48 hours stimulation, Foxp3 expression was highest at 3% and constant 1% oxygen, and was significantly reduced in reperfusion injury (figure 4.13C). CTLA-4, a surface marker associated with Treg function, did not reveal any significant patterns, but appeared to have reasonable expression at 21%, 3% oxygen and reperfusion injury treatments (figure 4.13B). The combination of these surface markers showed the percentage of CTLA-4+Foxp3+ cells was slightly increased at 3% oxygen at 48 hours, indicating an increase in regulatory functions may occur in physiologically low-oxygen environments such as the RA joint and the lymph node (figure 4.13D). However, at 24 hours reperfusion treatments contained the most CTLA-4+Foxp3+ cells, suggesting a regulatory phenotype may be a rapid response to re-exposure to oxygen. However, this regulatory-like phenotype severely dropped to 48 hours compared to the other oxygen treatments suggesting that it was not maintained when oxygen levels fluctuated drastically. The percentage of CTLA-4+Foxp3+ cells at 8.5% oxygen remained low compared to 3% oxygen at both time points, highlighting further the role that different environmental oxygen levels can have on this important T helper cell subset (figure 4.13D).

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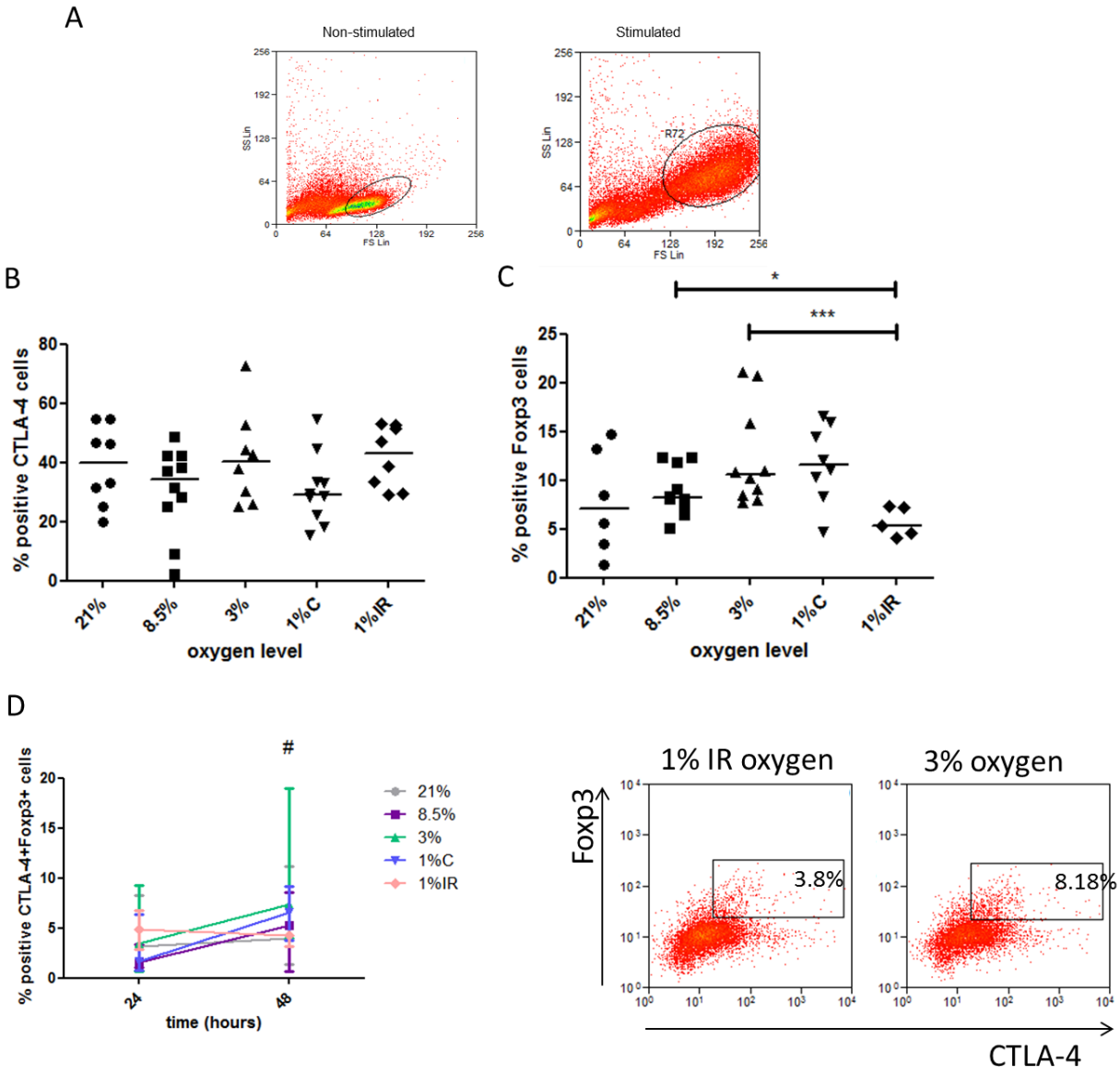
A
Non-stimulated
Stimulated
SS Lin
FS Lin
R72
B
% positive CTLA-4 cells
21%
8.5%
3%
1%C
1%IR
oxygen level
C
*
***
% positive Foxp3 cells
oxygen level
D
% positive CTLA-4+Foxp3+ cells
#
time (hours)
21%
8.5%
3%
1%C
1%IR
1% IR oxygen
3% oxygen
Foxp3
CTLA-4
3.8%
8.18%

Figure 4.13

Figure 4.13. Markers associated with regulatory functions are increased at 3% oxygen*. CD4+ CD45RO+ T lymphocytes were isolated using a Miltenyi MACs CD4+ memory isolation kit and frozen at -80°C for later analysis. 2x10^5 cells were equilibrated to the stated oxygen level for 24 hours before stimulation with 2µg/ml anti-CD3// 5µg/ml anti-CD28. 2µg/ml brefeldin A treatment was given for the last three hours of stimulation for additional cytokine analysis. A) Forward scatter (FS)/ side scatter (SS) plots for non-stimulated and stimulated cells show the selected gate on stimulated cells (R72). Using the anti-inflammatory marker panel, surface markers and transcription factors associated with a regulatory phenotype were investigated at different oxygen levels by both surface and intracellular staining, and assessed by flow cytometry. The percentage of B) CTLA-4+ and C) Foxp3+ cells was assessed after 48 hours stimulation. D) The combination of these markers was assessed by determining the percentage of CTLA-4+ Foxp3+ cells at both 24 and 48 hours, with example staining plots given in E). Statistical analysis was performed using the Mann Whitney test and median values are shown in the data. *=p<0.05. ***=p<0.001. #= p<0.005 between 3% oxygen and reperfusion injury treatments at 48 hour stimulations. 1%C = constant 1% oxygen. 1% IR = 1% oxygen with reperfusion injury. Data is representative of at least 2500 cells in the stimulation gate. A minimum of 4 donors was investigated at each oxygen level. 3% oxygen upregulated expression of both CTLA-4 and Foxp3 after 48 hours stimulation compared to reperfusion injury treatments.*

4.2.2.5 IL-10 production is associated with different T helper cell subsets at different oxygen levels.

The marker staining panels also allowed assessment of whether IL-10 was being produced by Th2 or Treg cells by assessing its expression in either CCR4+GATA3+ or CTLA-4+Foxp3+ cells. IL-10 positive cells were gated upon, as seen in the gating plan in figure 4.14, and assessed for the expression of the indicated markers. A greater majority (averages > 40% vs < 30%) of IL-10 was expressed in the CCR4+GATA3+ cells compared to the CTLA-4+Foxp3+ cells highlighting that it was mainly Th2-like cells producing IL-10 in these culture conditions. Furthermore, the patterns of CCR4+GATA3+ and CTLA-4+Foxp3+ expression in the IL-10 gates reflected the patterns observed for the overall stimulated population (figures 4.12 and 4.13) suggesting that IL-10 production in CD4+ memory T lymphocytes may be associated with multiple T helper subsets and that oxygen may not influence which T helper subset IL-10 is produced in.

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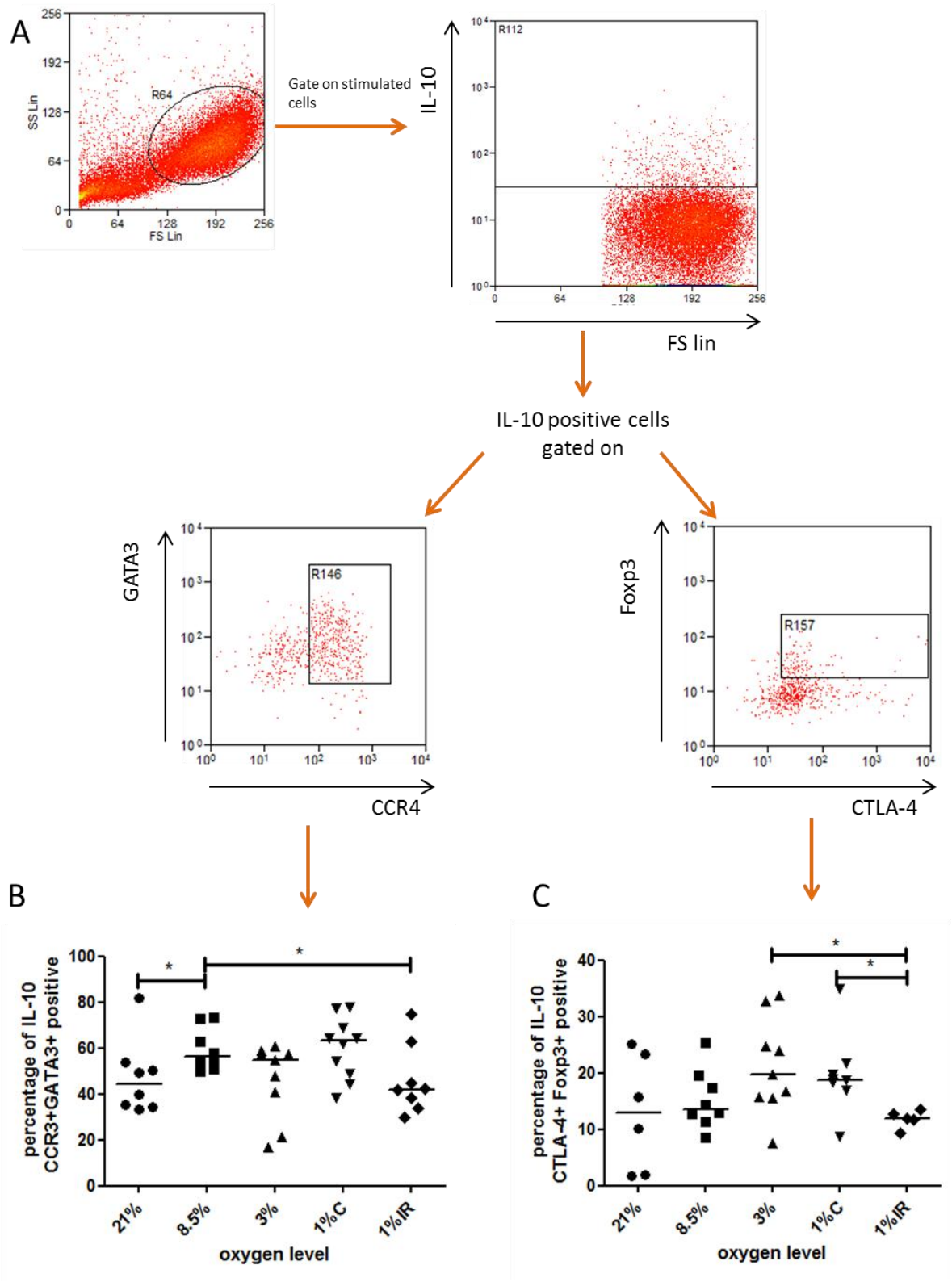
A
SS Lin
FS Lin
R64
Gate on stimulated cells
IL-10
R112
FS lin
IL-10 positive cells gated on
GATA3
R146
CCR4
Foxp3
R157
CTLA-4
B
percentage of IL-10 CCR3+GATA3+ positive
21%
8.5%
3%
1%C
1%IR
oxygen level
C
percentage of IL-10 CTLA-4+ Foxp3+ positive
21%
8.5%
3%
1%C
1%IR
oxygen level

Figure 4.14

Figure 4.14. The production of IL-10 varies between different subsets at different oxygen levels. *CD4+ CD45RO+ T lymphocytes were isolated using a Miltenyi MACs CD4+ memory isolation kit and frozen at -80°C for later analysis. $2x10^5$ cells were equilibrated to the stated oxygen level for 24 hours before stimulation with 2µg/ml anti-CD3// 5µg/ml anti-CD28. 2µg/ml brefeldin A treatment was given for the last three hours of stimulation for cytokine analysis. A) Forward scatter (FS)/ side scatter (SS) plots for non-stimulated and stimulated cells show the selected gate on stimulated cells (R64). Using the anti-inflammatory marker panel, IL-10 production was assessed in conjunction with surface markers and transcription factors associated with a Th2 and a regulatory phenotype by flow cytometry after stimulation for 48 hours at different oxygen levels. A) shows the gating used for analysis of the flow cytometry data. Stimulated cells were selected for analysis and the IL-10+ cells gated upon. This gate was applied to the B) CCR4+GATA3+ and C) CTLA-4+Foxp3+ graphs and the percentage of positive cells assessed. Mann Whitney test was used for statistical analysis and median values are shown. *=p<0.05. Data is representative of at least 2500 cells in the stimulation gate. A minimum of 5 donors was investigated at each oxygen level. When considering overall expression Th2 and regulatory marker expression, IL-10 expression does not seem to be preferentially associated with either subset.*

4.2.3 A limited microarray analysis gives clues as regulatory mechanisms instigated in hypoxia

Given the differences in protein and transcription factor expression observed it was decided to undertake a preliminary examination of mRNA associated with the different T helper subsets (for details please see Appendices). cDNA was generated from mRNA isolated from stimulations for 48 hours at 8.5% (treated as a control) and constant 1% oxygen. Along with the limited numbers of samples being investigated, the 8.5% oxygen RNA isolation was apparently contaminated with human genomic DNA according to in-built quality control checks in the plate. Therefore no conclusions can be drawn from this data but it gives an interesting indication of further trends that may be occurring in physiologically healthy oxygen levels compared to hypoxia.

Despite the limitations of these data, the results obtained did reflect some of the results gained thus far. Firstly, the microarray showed that GATA-3 expression was upregulated at constant 1% oxygen compared to 8.5% oxygen. Our flow cytometry data suggested that GATA-3 expression increased at 1% oxygen over time whereas at 8.5% oxygen the increase observed from 24 to 48 hours was more limited. It is therefore possible that the increase in GATA-3 mRNA observed in hypoxia may reflect the delayed upregulation in Th2-associated markers as implied previously. CCL7 mRNA was also downregulated at 1% oxygen.

Gene Symbol	Protein/description	Fold upregulation at 1%	Fold Downregulation at 1%
GATA3	GATA binding protein 3	153.4311	
IL23A	Interleukin 23, alpha subunit p19	95.1049	
CCL7	Chemokine (C-C motif) ligand 7		-65.7333
IL17RD	Interleukin 17 receptor D		-44.8972
IL17C	Interleukin 17C		-44.5871
IL4	Interleukin 4		-24.9083
HGDC	Human Genomic DNA Contamination		-52.657

Table 4.1. Genes upregulated (red) and downregulated (blue) after 48 hours stimulation at constant 1% oxygen compared to control oxygen level of 8.5%.

4.2.4 Naïve T lymphocyte polarisation reveals a similar pattern to memory cells

While the focus of the project is on CD4+ CD45RO+ cells, given their role in regulating process in inflammatory issues, limited experiments on naïve T lymphocytes polarised to Th1, Th2 or Th0 phenotype at different oxygen levels were also undertaken. CCR4 and CXCR3 expression were examined to assess the success of polarisation at different oxygen levels. At 8.5% oxygen the highest CCR4 expression was observed for all polarisations whereas at 3% and constant 1% oxygen CCR4 expression was reduced even in Th2 polarisations (figure 4.15). Conversely, CXCR3 expression was the most increased at constant 1% oxygen. Interestingly 3% oxygen did not upregulate CXCR3 as much as may be expected from the previous memory cytokine data (figure 4.10). The Th1/Th2 polarisations appeared particularly distinct in the 21% cultures, with high CCR4 expression in Th2-polarised cells and high CXCR3 expression in Th1-polarised cells. As the *in vitro* protocols used for polarisation in many laboratories were generated in 21% oxygen, it may be of no surprise that these observations were made. Th0 cells (no polarisation) appeared to favour CXCR3 expression over CCR4 expression at all the oxygen levels investigated, indicating that our environmental conditions may favour a Th1 phenotype, or that Th1 differentiation is generally more favoured in the naïve T lymphocyte differentiation process. At 8.5% oxygen both CXCR3 and CCR4 was reduced in the Th1-polarised cells. This may have been due to their inhibition of forming a Th2-like phenotype due to the factors added to their environment, but inhibition of a Th1-like phenotype due to the oxygen conditions. Due to time constraints, these experiments could only be performed once.

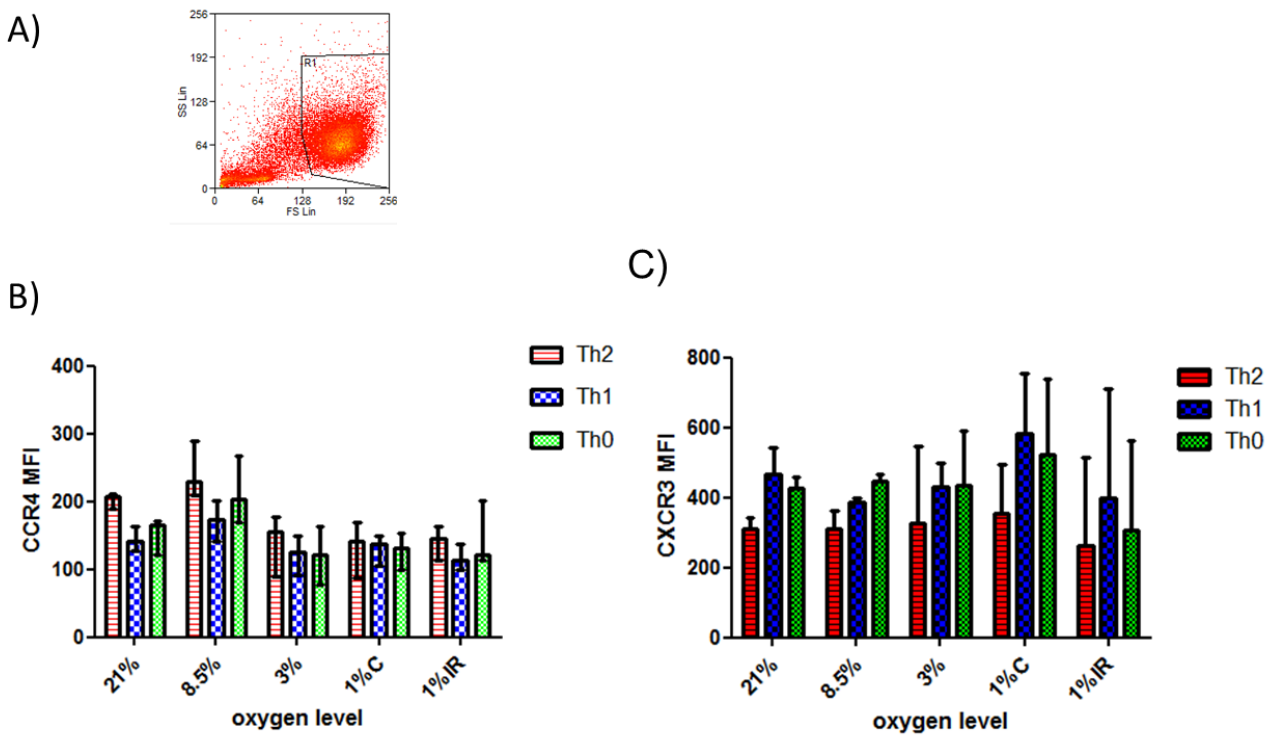

Figure 4.15. Naïve CD45RA+T lymphocytes CCR4 and CXCR3 expression at different oxygen levels. *CD45RA+CD4+ T lymphocytes were isolated using a Miltenyi naïve T lymphocyte isolation kit and frozen at -80°C until required. Cells were then defrosted and 5x 10^4 cells were equilibrated to the stated oxygen level for 24 hours and then stimulated using plate-bound anti-CD3/ anti-CD28 under Th0, Th1 or Th2 differentiating conditions. A) shows the gate applied to stimulated cells for analysis by flow cytometry (R1). B) CCR4 and C) CXCR3 MFI were assessed by flow cytometry after 72 hours stimulation. 3-4 donors were run at each oxygen level for each treatment in one experiment. Statistical analysis was performed using Mann Whitney test but no results were significant. Median values are shown with the range. CCR4 appeared to be better expressed at 8.5% oxygen, and CXCR3 at constant 1% oxygen.*

4.3 Discussion

In this chapter the phenotype of CD4+ memory T lymphocytes at different oxygen levels has been assessed. An array of different cytokines can be observed in chronic inflammatory environments such as the rheumatoid joint [29, 33, 35], and the effectiveness of anti-TNFα in RA treatment [35] has shown that depleting a certain cytokine within a tissue, which will alter the overall balance of cytokines, can be of benefit. Therefore, the balance of cytokine production was investigated at different oxygen levels by both ELISA and intracellular cytokine staining.

The data presented in this chapter suggest that cytokine production can vary depending on environmental oxygen levels. ELISA assessment of stimulated CD4+ CD45RO+ T lymphocytes supernatants revealed that IFNγ and TNFα were the most prominent cytokines produced at every oxygen condition, contributing to between 87-97% of the overall cytokines produced over 45 hours. This is the expected trend in *in vitro* stimulated peripheral blood CD4+ memory T lymphocytes [58, 62, 361]. However, at constant 1% oxygen these more pro-inflammatory cytokines were in greater proportion when compared to higher oxygen levels. In contrast, cytokines deemed to be more Th2-associated or anti-inflammatory (IL-4, IL-5 and IL-10) were greatest in the overall cytokine population at higher oxygen levels.

4.3.1 CD4+ CD45RO+ T lymphocyte cytokine production and polarisation at constant 1% oxygen

Intracellular cytokine staining further confirmed the previous results that low environmental oxygen promotes a pro-inflammatory phenotype. After 24 hours stimulation a high percentage of IFNγ-positive cells was detected at constant 1%

oxygen. This suggested that low environmental oxygen may promote a more pro-inflammatory T helper cell phenotype in CD4+ CD45RO+ T lymphocyte populations. As pro-inflammatory cytokines may play a greater role in chronic inflammatory tissues [29, 35], it is possible that the oxygen environment contributes to a more aggressive immune response. However, highest IFNγ production was also detected at 21% oxygen at this time point, suggesting that it is not only lack of oxygen that results in an initial pro-inflammatory response.

Despite good IFNγ intracellular cytokine staining at 24 hours in constant 1% oxygen, the percentage of cytokine-positive cells dropped to 48 hours stimulation compared to 21%, 8.5% and 3% oxygen treatments, suggesting that sustained hypoxia may result in a reduction in cytokine production over time. As supernatants were only assessed by ELISA from 48 hour stimulations (with 3 hours treatment with brefeldin A) this may not have been fully reflected in the ELISA data, due to the detection of cytokines produced in the earlier stages of stimulation. Alongside this, T-bet expression also dropped overtime, and this may provide one mechanism for the drop in IFNγ production overtime in constant 1% oxygen.

Whereas T-bet expression appeared to drop in constant 1% oxygen over time, GATA-3 expression appeared to increase overtime compared to other oxygen levels (as indicated by both intracellular staining and the limited microarray). It is possible that the later increase in GATA-3 expression observed at 48 hours stimulation may be due to a delayed promotion of humoral immunity, or may be result of the lack of T-bet expression which normally antagonises GATA-3 function [110]. Despite the increase in GATA-3 expression after 48 hours, IL-4 cytokine production did not increase alongside it.

In contrast to IFNγ, IL-17A and IL-10 cytokine production was slightly increased over time at constant 1% oxygen. However, this increase was not as pronounced as those observed at higher oxygen conditions. With regards to these two cytokines, examinations of RORγt expression were fraught with problems, and MAF, a possible transcriptional regulator of IL-10 [362], was not assessed. The data regarding IL-17A is somewhat contradictory to that described in the literature. The stabilisation of HIF-1α in low environmental oxygen levels is thought to promote a Th17-like phenotype as already discussed [120, 236, 276, 298], and therefore it was hypothesised that an increase in IL-17 production may be observed. However, less IL-17 production was observed at constant 1% oxygen compared to other oxygen levels. This was observed in both analyses of supernatants and from intracellular cytokine staining. IL-17A was also reduced when assessing double-cytokine expressing cells, as the IFNγ+IL-17A+ population was reduced at constant 1% oxygen compared to other oxygen levels. As the cytokine production observed was produced by CD4+ memory T lymphocytes in the cell population, it may be that the response of this specific cell type to hypoxia is altered compared to naïve T lymphocytes with respect to IL-17 production. As the literature on hypoxia and Th17 differentiation mainly employs naïve CD4+ T lymphocyte populations [120, 236, 276], the observation that CD4+ CD45RO+ T lymphocytes depress IL-17 production is novel.

4.3.2 Constant 1% oxygen with 3% oxygen differ in their effect on CD4+ T CD45RO+ lymphocytes

In assessing the effect of environmental oxygen levels on memory T lymphocyte IL-17A production, it is interesting to compare the results from constant 1% oxygen to those from 3% oxygen. The differences between the effect of 1% and 3% oxygen

environmental oxygen on CD4+ memory T lymphocytes appeared quite profound, especially when considering that it is possible for a tissue to fluctuate between these oxygen levels. At 3% oxygen cytokine production appeared much more maintained over time, with both IFNγ and IL-17A production increasing over time more than at other oxygen levels. As HIF-1α is stabilised at 3% oxygen [230], it is possible the expected effect of HIF-1α stabilisation on IL-17A expression occurs at 3% oxygen, but that 1% oxygen depresses IL-17A production due to additional influences on cellular processes.

IFNγ+IL-17A+ double positive cells have been detected in inflamed joints [145, 358] , and are associated with worse pathology in EAE compared to IL-17A-only producing cells [349]. An increase in IFNγ+IL-17A+ double positive cells was detected at 3% oxygen compared to all the other oxygen levels. This suggests that cytokine expression is favoured at this oxygen level, but also promotes the notion that Th17 plasticity is upregulated at this oxygen level compared to other oxygen levels, including constant 1% oxygen. As T-bet expression was also observed to be increased at this oxygen level, it is possible that the Th17 cells present within the original population favour this oxygen level, and also undergo a phenotypic switch to a more Th1-like phenotype. However, IL-17A was more regularly found in T-bet negative cells than T–bet positive (data not shown).

Despite the observation of IFNγ+IL-17+ cells in the rheumatoid joint, T lymphocyte cytokine production has been reported to be limited in this environment [175, 176]. As 3% oxygen is the average oxygen level detected in the rheumatoid joint, the results obtained in this chapter contradict what may be hypothesised as cytokine production appears to be more supported at this oxygen level. As this study only

examined cytokine production over 48 hours, it is possible the data is limited, and more longitudinal studies may be of benefit in the future. Previous studies investigating CD4+ T lymphocyte cytokine production in the joint provide only a snap-shot view of lymphocyte behaviour in the joint, and it is not known how long the lymphocytes examined actually have existed within the joint environment. As RA T lymphocytes express CD45RB only at low levels, it is possible that they get 'stuck' in the joint [306]. Therefore, it is difficult to decipher how they behave when they initially migrate into the synovial tissue, and it would be interesting to investigate whether long-term culture at 3% oxygen eventually results in a depression in cytokine production. There are also many other environmental factors in the RA joint that may place additional pressures on the cytokine production of T lymphocytes. These include cytokine production from non-lymphoid cells, ROS production from neutrophils, lack of other nutrients, and a potential lack of other supporting structures/ factors that lymphocytes will find in other environments such as the lymph node for long-term cytokine production. This may provide some explanation for the increased cytokine production observed in this study at 3% oxygen compared to the chronic inflammatory environment of the joint.

4.3.3 Further cytokine plasticity at 3% and 21% oxygen

Not only Th17-Th1 plasticity was observed increased at 3% oxygen, as an increase in IFNγ+IL-10+ and IFNγ+IL-4+ double positive cells was also observed at both 3% and 21% oxygen. *In vitro* investigations of mouse T lymphocyte polarisation often provides very distinct populations of Th1 and Th2 T lymphocytes in response to stimuli [63, 142] although *in vivo* studies suggest that murine Th2-to-Th1 plasticity is

possible in previously antigen-exposed CD4+ T lymphocytes [154]. In humans, T lymphocyte plasticity is possibly more prevalent and re-expression of silenced genes may occur with respect to cytokines of opposing subsets [98]. Additionally, IL-4 production in effector memory T lymphocytes has previously been shown to fall into the IFNγ+ fraction after ten days stimulation [64]. Therefore, it is possible that these two oxygen levels promote increasing plasticity between the different Th1 subtypes.

As already alluded to, the data obtained from stimulation at 3% oxygen is interesting when considering physiological environments where this oxygen level will be experienced. Cytokine production needs to be maintained in the lymph node in response to antigen and the data obtained from stimulations at 3% oxygen suggest that this environmental oxygen level may promote the generation of pro-inflammatory T lymphocytes. CD4+ central memory lymphocytes, which are activated in the lymph node [63], do not maintain epigenetic regulation of their cytokine genes like effector memory T lymphocytes [98], and therefore may be easier to manipulate with regards to subsequent phenotype. As oxygen levels appear to have some regulation over phenotype and cytokine production, it would be interesting to take a closer look at the effects of environmental oxygen levels on this specific subset of memory T lymphocyte by investigating cytokine production either over a longer time period, or in response to specific antigenic stimulation.

4.3.4 Observations in reperfusion injury treatments

Observations made from reperfusion injury treatments gave an interesting insight into what may occur when oxygen levels fluctuate. The data still needs to be treated with some caution as the oxygen fluctuations were extreme and would not be experienced

by cells physiologically. That said, the data from this model reveals some subtle differences between the behaviour of cells stimulated at constant 1% oxygen and those that went on to experience reperfusion injury. Compared to constant 1% oxygen, increases in intracellular cytokine production of IFNγ, IL-17A and IL-10 were observed occurring alongside reductions in transcription factor expression. However, IL-4 intracellular cytokine staining was the lowest in reperfusion injury samples. As the number of replicates was low, further exploration of this would be beneficial.

4.3.5 A more resolving phenotype at 8.5% oxygen

Physiologically healthy oxygen levels of 8.5% oxygen revealed some differences compared to the other oxygen levels examined. In general, cytokine production was not as upregulated compared to stimulation at other oxygen levels. When closely examining the balance of cytokines in supernatants, IL-4, IL-5, IL-10 and IL-17A played a greater role at 8.5% oxygen than compared to the other oxygen levels, and that this may have been due to the down regulation of pro-inflammatory cytokines.

Intracellular cytokine staining for IL-10 production stained the highest percentage of cells after 48 hours stimulation at 8.5% oxygen, despite initial expression being lower at 24 hours. This suggested that CD4+ CD45RO+ T lymphocytes at 8.5% might have increased anti-inflammatory or regulatory capabilities. However, the percentage of CTLA-4+Foxp3+ cells was not the highest at 8.5% oxygen. IL-10 is commonly listed as one of the Th2-associated cytokines [47], and a reasonable percentage of the IL-10 observed at 8.5% may be associated with this subset. In addition, the percentage of CCR4+GATA3+ cells was high in 8.5% oxygen treatments after both 24 and 48 hours stimulation. Due to our initial population of CD4+ memory T lymphocytes, there

are several possibilities that could have caused this upregulation; either the resident Th2-like cells were preferably expanded at this oxygen level; or other non-Th2 cells (potentially including central memory) were converting to a more Th2-like phenotype. As IL-4 - a cytokine associated with Th2 immune responses - was not highly detected, confirmation of the presence of this subset would need further clarification. The increase in Th2-like phenotype was further suggested in experiments performed on naïve T lymphocytes, where stimulation at 8.5% resulted in the highest CCR4 MFI values, even in Th1 and Th0-polarising conditions. However, this work was limited by the lack of experimental repeats, and warrants future confirmation.

It is therefore possible that physiologically healthy oxygen levels of 8.5% oxygen result in more Th2-skewed phenotype. This may be a result of the environment lacking certain factors that cells normally respond to in inflammatory stimuli that may promote their pro-inflammatory phenotype. These factors could include ROS and the stabilisation of HIF-1α. HIF-1α is not expected to be stabilised in 8.5% oxygen [230], and physiologically healthy oxygen levels have previously been observed to limit the production of ROS [235, 244]. The increase in IL-10 as part of the cytokine population at this oxygen level will also promote the dampening down of the immune response, and promotion of a Th2-like inflammatory response will aid wound healing [113, 114].

4.3.6 IL-10 production

High IL-10 production was observed in 21% oxygen cultures. This was unexpected and highlights how *in vitro* work may detect more IL-10 than actually plays a role physiologically. As IL-10 is associated with anti-inflammatory and regulatory

processes, this is an important finding for *in vitro* research. For example, it will be important in the study of tumour immunology where regulatory cells are thought to play a role in dampening anti-tumour immune responses [363]. If IL-10 is over-represented in *in vitro* work, it may delay full understanding of the regulatory response occurring in the tumour environment. As tumour micro-environments are often severely hypoxic [206], the contrast in IL-10 production at 21% and 1% oxygen treatments revealed in this study highlights the importance of performing *in vitro* work at tissue-relevant oxygen levels.

Interestingly, IL-10 production did not appear to be more associated with either a more regulatory or Th2 phenotype. However, the double-positive gates applied did not allow for examination of IL-10 production in cells expressing a single marker. For example, there was a population of Foxp3 negative cells expressing both IL-10 and CTLA-4, and these cells could be Tr1s (as discussed in the main introduction) [139, 141, 364]. This further analysis was not performed as the number of IL-10 cells was low and it was difficult to draw any conclusions from this data. It may be of benefit to examine IL-10 production with respect to all different T helper subsets simultaneously in the future due, as IL-10 production has been observed from all subsets [47, 95, 128, 365].

5. RESULTS: POTENTIAL MECHANISMS OF HYPOXIC ALTERNATION OF MEMORY CD4+ T LYMPHOCYTE RESPONSE TO STIMULATION

5.1 Introduction

As we have shown already, hypoxia appears to have significant effects on the functions of CD4+ memory T lymphocytes. Dioxygen is a reactive molecule that plays a large role in cellular biochemistry, and its depletion therefore disrupts many cellular biochemical processes. On top of the basic biochemical changes that occur when a reactive molecule is depleted, regulatory mechanisms kick in due to the necessity of maintaining good oxygen levels. These processes are linked through the upregulation of hypoxia sensitive genes. Moreover hypoxia has been shown to affect biogenesis in T lymphocytes by altering membrane potential via the inhibition of Kv1.3 channels, resulting in a reduced calcium signal in response to TCR ligation [273, 275].

In this chapter two downstream effects of hypoxia on CD4+ memory T lymphocyte function have been investigated:

HIF-1α stabilisation. The stabilisation of HIF-1α is both a well-documented and well established response to hypoxia [224] and a full description of its regulation can be found in chapter 1. Once stabilised, HIF-1α subunits translocate to the nucleus, dimerise with HIF-β subunits, and initiate downstream transcription of hypoxia-responsive genes [224] which are able to regulate immune responses.

Intracellular reactive oxygen species (ROS) production increases in responses to lack of oxygen. As discussed in the main introduction, lack of oxygen can result in an increase in superoxide production from the mitochondrion [254-256], which

can act as a signalling molecule and result in an increased oxidative cellular environment [257].

Observations from these studies led to further study of proximal T lymphocyte signalling at different oxygen levels.

5.1.1 This chapter's aims

The aim of this chapter is to establish whether HIF-1α stabilisation and ROS provide a mechanism for the previous results observed at different oxygen levels. CD4+ CD45RO+ T lymphocytes were again isolated and frozen at -80˚C until required experimentally. HIF-1α was stabilised by the use of defoxamine, and the ROS environment of the joint mimicked using hydrogen peroxide. In contrast, the effects of the anti-oxidant *N*-acetyl cysteine were also examined. Flow cytometry was used to assess these different treatments.

5.2 Results

In chapter 3 an upregulation in CD69 expression at constant 1% oxygen was described. In this current chapter the effects of aspects of the low-oxygen environment on CD69 expression were initially investigated in more detail by examining the effects of ROS and HIF-1α stabilisation on CD4+ CD45RO+ T lymphocytes isolated and stored as in previous chapters. These effects were investigated at physiologically healthy oxygen levels of 8.5%, as investigation at 21% oxygen may have resulted in intrinsic increases in ROS production that would not have been controlled for, as previously culture at 21% oxygen was shown to decrease cellular levels of the anti-oxidant glutathione compared to the physiologically healthy oxygen level of 5% [244]. Furthermore, 21% oxygen has also been associated with increase oxidative damage [271].

Due to the nature of its regulation by Fe2+ dependent PHDs, HIF-1α can be stabilised using DFX treatment in higher oxygen levels by its action as an iron chelator [224, 225, 366]. DFX treatment was optimised in stimulated CD4+ memory T lymphocytes and 2.5μM DFX was found to be best in the stabilisation of HIF-1α treatment, as observed by brighter HIF-1α nuclear staining of CD4+ memory T lymphocytes compared to control (figure 5.1). Higher concentrations of DFX resulted in reduced HIF-1α stabilisation compared to 2.5μM treatments.

To investigate the role of redox at different oxygen levels, H_2O_2 was used at 8.5% oxygen, whereas NAC was used at 1% oxygen. The superoxide formed from mitochondrial ROS production is rapidly converted to H_2O_2 by MnSOD [211, 256]. H_2O_2 therefore is the most physiologically relevant ROS with respect to hypoxia, as it will be H_2O_2 that diffuses out of the mitochondria resulting in downstream signalling

and protein damage. Therefore, its effects in physiologically healthy oxygen levels were investigated. Contrasting experiments were conducted at 1% oxygen to determine the effects of antioxidants when oxygen is deficient. Glutathione is a major cellular anti-oxidant that scavenges ROS. NAC provides a cysteine for the generation of reduced glutathione and boosts antioxidant levels [367, 368], and was therefore used experimentally.

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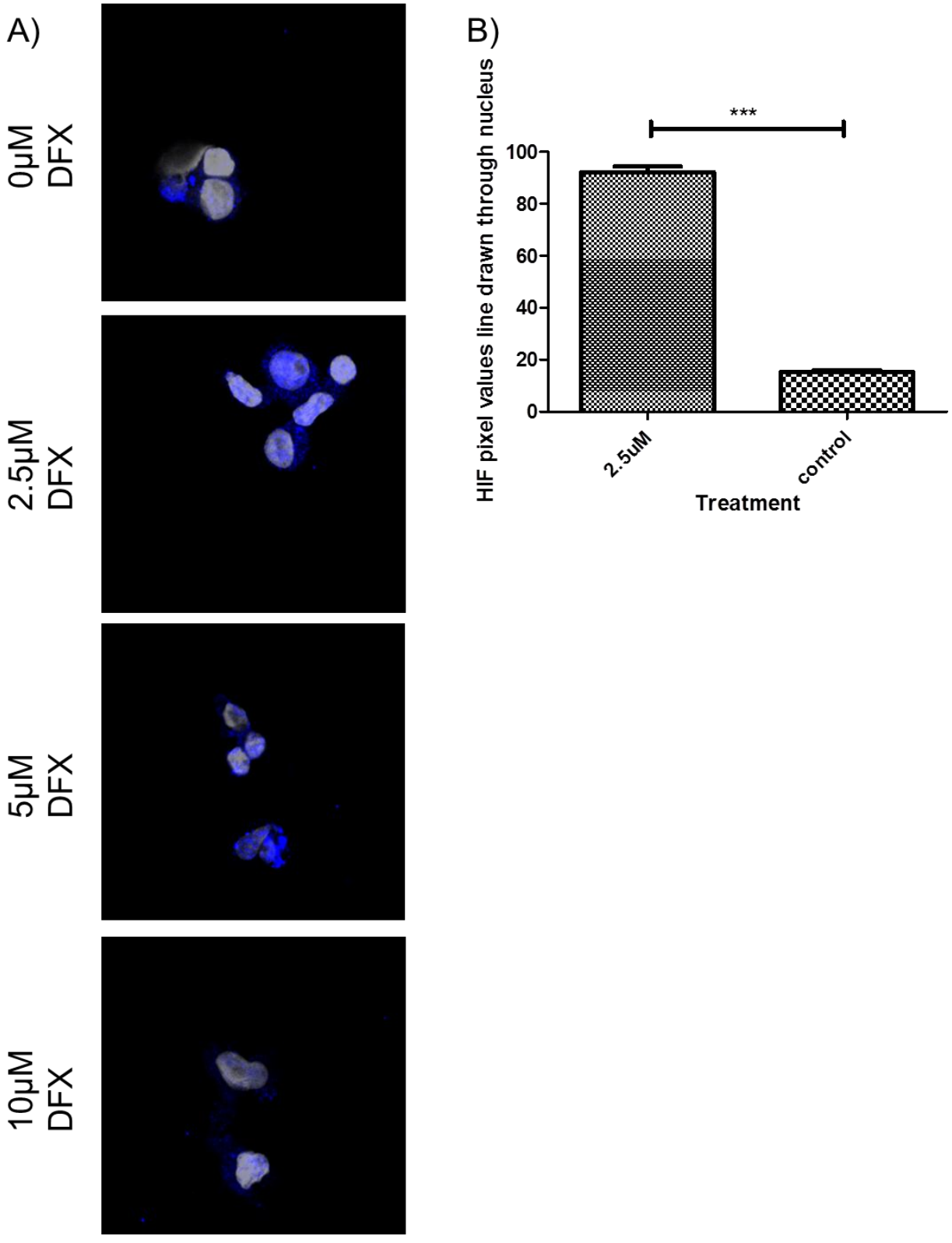
A)
0μM DFX
2.5μM DFX
5μM DFX
10μM DFX
B)
HIF pixel values line drawn through nucleus
***
100
80
60
40
20
0
2.5uM
control
Treatment

Figure 5.1

Figure 5.1. 2.5μM DFX is optimum for HIF-1α stabilisation by DFX treatment.

*CD4+ CD45RO+ T lymphocytes were isolated using a Miltenyi MACs CD4+ memory isolation kit and frozen at -80°C for later analysis. CD4+ CD45RO+ T lymphocytes were stimulated with 2μg/ml anti-CD3// 5μg/ml anti-CD28 at 21% oxygen and simultaneously treated with the stated concentrations of deferoxamine mesylate (DFX) in PBS for 18 hours. A) Cells were assessed by confocal microscopy for their expression of HIF-1α. B) Arbitrary analysis of HIF-1α pixel values in lines across nuclei revealed a significant increase in HIF-1α expression at 2.5μM DFX. Statistical analysis was performed using Paired T test and mean values are shown. ***= P>0.001. A minimum of ten cells were assessed across several slides. 2.5μM DFX resulted in a higher nuclear HIF-1a signal.*

5.2.1 Investigating the effects of ROS and HIF-1α stabilisation on CD69 expression

CD69 expression was examined at 8.5% oxygen in response to HIF-1α stabilisation and ROS treatment, or a combination. CD4+ memory T lymphocytes were treated for only 18 hours after equilibration for 24 hours at 8.5% oxygen. Previous studies have shown that longer treatment with DFX and H_2O_2 leads to cells death [[369]; Dr Steve Young, personal communication]. As a control, oxygen levels within the incubator were changed to 1% oxygen at the time of stimulation after equilibration to 8.5% oxygen.

The data presented in chapter 3 suggested an increase in CD69 expression occurred in low environmental oxygen. Therefore, it was hypothesised that an increase in CD69 would occur in response to DFX and H_2O_2 treatment alongside an increase in the 1% oxygen control. However, the opposite was observed, with the individual treatments, combination treatment and 1% oxygen control all reducing CD69 expression compared to untreated cells maintained at 8.5% oxygen. Therefore, DFX treatment and H_2O_2 did mimic 1% oxygen treatment in these experiments, but in a different manner than hypothesised (figure 5.2). The disparity in these observations suggested that the oxygen level during the non-stimulated equilibration period may influence later cellular responses to stimulation, such as CD69 expression (figure 5.2).

NAC treatment, given for the whole duration of the experiment including the equilibration period, did have the expected effect on CD69 expression. 1mM NAC treatment significantly reduced CD69 expression at constant 1% oxygen (figure 5.3). This reduction was on a par to CD69 expression seen at 8.5% oxygen in (figures 3.3

and 3.4). This suggested that redox played a role in the response of CD4+ memory T lymphocytes to hypoxia.

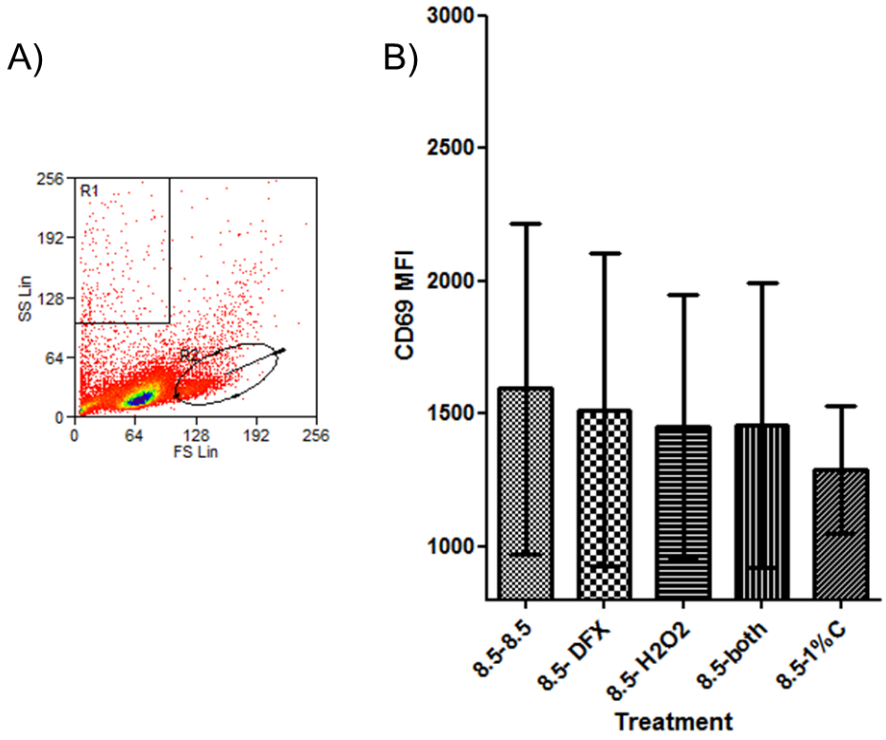

Figure 5.2. HIF-1α and ROS treatment mimic hypoxic treatment on the expression of CD69, and reveal the importance of hypoxic pre-exposure before stimulation*. CD4+ CD45RO+ T lymphocytes were isolated using a Miltenyi MACs CD4+ memory isolation kit and frozen at -80°C for later analysis. CD4+ CD45RO+ T lymphocytes were equilibrated for 24 hours to 8.5% oxygen before being treated with 2.5μM deferoxamine mesylate (DFX), 10μM hydrogen peroxide (H2O2) or both simultaneously with stimulation with anti-CD3//anti-CD28. After 18 hours stimulation, cells were stained for expression of CD69 and assessed by flow cytometry. A) show the gate applied to stimulated cells. B) CD69 expression was determined by investigating the mean fluorescence intensity (MFI) at the various treatments. Data is representative of at least 2500 cells in the stimulation gate. Statistical analysis was performed using Paired T test and mean values are shown in the data. Three matched donors were run at each treatment*

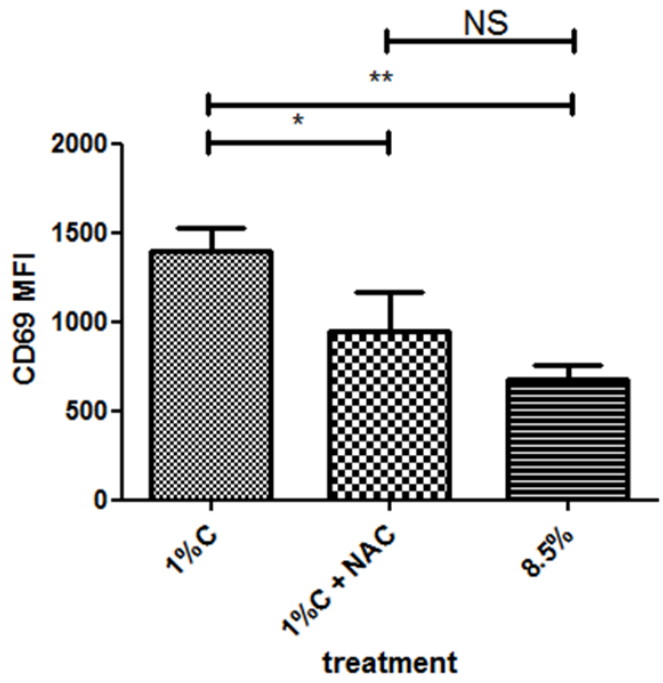

Figure 5.3. NAC treatment in hypoxia decreases CD69 expression on a par with physiologically healthy oxygen levels. *CD4+ CD45RO+ T lymphocytes were isolated using a Miltenyi MACs CD4+ memory isolation kit and frozen at -80°C for later analysis. CD4+ CD45RO+ T lymphocytes were treated with 1mM N-acetyl-L-cysteine (NAC) and simultaneously equilibrated to 1% oxygen for 24 hours. As a control, cells were equilibrated to 1% or 8.5% oxygen without NAC treatment. Cells were stimulated for 48 hours and then stained for expression of CD69 and assessed by flow cytometry. CD69 expression was determined by investigating the mean fluorescence intensity (MFI) at the various treatments. Statistical analysis was performed using the Paired Test and mean values are shown. * p< 0.05. **p< 0.005. NS non-significant. Data is representative of at least 2500 cells in the stimulation gate. Five donors were run at each treatment. NAC treatment resulted in a drop in CD69 expression at constant 1% oxygen. The same gating strategy was used as in figure 5.2*

5.2.2 Proximal T lymphocyte signalling is altered in hypoxia

The disparity in the results described above, compared to Chapter 3, suggested equilibration of resting cells at different oxygen levels may influence subsequent phenotype post-stimulation. Therefore, the effect of various oxygen levels on proximal T lymphocyte signalling machinery in resting cells was examined. The phosphorylation of the activatory residue – tyrosine 394 (Y394) - of Lck was chosen for investigation, as this is thought to be dominant over the inhibitory residue.

CD4+ memory T lymphocytes were equilibrated, resting, at a designated oxygen level for 24 hours. Paraformaldehyde solution was added to the cultures to a final concentration of 4% to fix them at the oxygen level they were maintained at to retain the level of Lck phosphorylation established over the equilibration period. Lck phosphorylation was then assessed in the fixed cells by staining and flow cytometry. A significant reduction in the percentage of positive Lck phosphorylated at Y394 in resting CD4+ memory T lymphocytes in 1% oxygen was observed compared to 8.5% oxygen (figure 5.4).

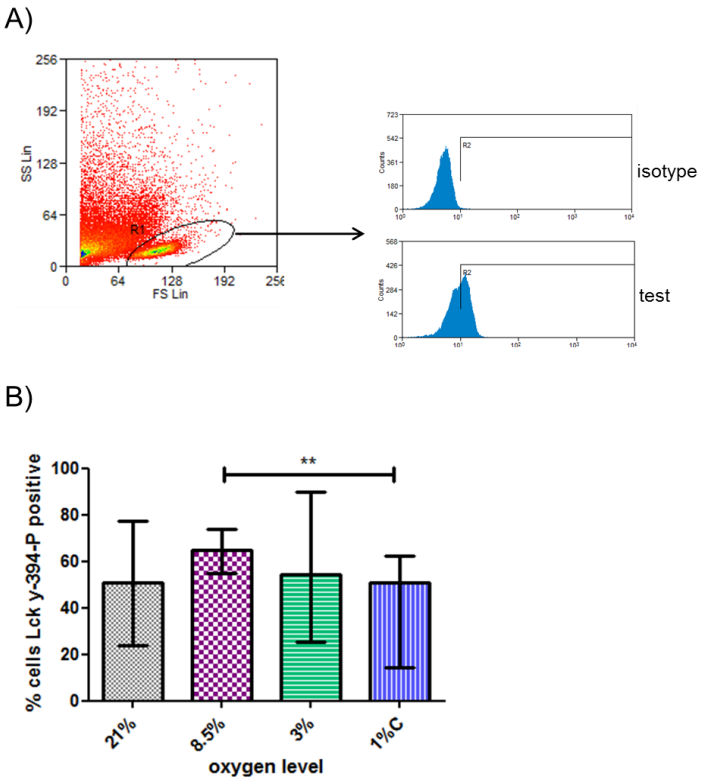

Figure 5.4. Lck phosphorylation at the activatory tyrosine residue 394 is reduced in hypoxia. *CD4+ CD45RO+ T lymphocytes were isolated using a Miltenyi MACs CD4+ memory isolation kit and frozen at -80°C for later analysis. CD4+ CD45RO+ T lymphocytes were equilibrated at 21%, 8.5%, 3% and 1% oxygen for 24 hours before being fixed with 4% paraformaldehyde directly in the hypoxystation or immediately after removal from 21% incubator. Cells were stained for phosphorylation at Tyr 394 on Lck and assessed by flow cytometry. Percentage positive cells were determined by comparison with isotype controls. A) Gating strategy for Lck Tyr Y394 phosphorylation assessment. B) Lck Tyr 394 phosphorylation at the oxygen levels stated. Mann Whitney statistical test was used for analysis and median values are shown in the data. **p< 0.005. 1%C = constant 1% oxygen. Data is representative of at least 2500 cells in the R1 gate. A minimum of 8 donors were investigated at each oxygen level.*

5.2.3 The effect of ROS and HIF-1α stabilisation on proximal T lymphocyte signalling

As DFX and H_2O_2 treatment had to some extent mimicked the effects of switching to 1% oxygen after equilibration at 8.5% oxygen on CD69 expression, the effect of these treatments on Lck activation was also assessed at 8.5% oxygen. Treatments were given from the beginning of the experiment for 18 hours, and cells were again resting. The pool of active Lck decreased in response to both treatments, albeit non-significantly (figure 5.5). H_2O_2 treatment again had the largest effect, but a combination of the treatments restored the level of active Lck within the cells. Interestingly, the pool of active Lck was reduced in this experiment at 8.5% oxygen controls compared to the 24 hour observations (figure 5.4), suggesting duration of exposure to different oxygen levels also influences subsequent readiness for priming.

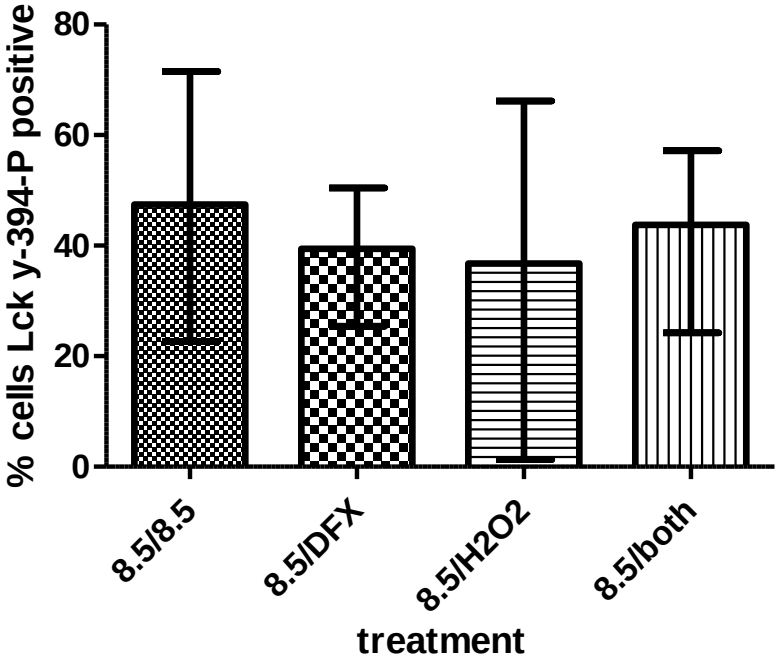

Figure 5.5. The effect of HIF-1α and ROS treatment on proximal T lymphocyte signalling. *CD4+ CD45RO+ T lymphocytes were isolated using a Miltenyi MACs CD4+ memory isolation kit and frozen at -80°C for later analysis. CD4+ CD45RO+ T lymphocytes were treated with 2.5μM deferoxamine mesylate (DFX), 10μM hydrogen peroxide (H2O2), or both, and equilibrated to 8.5% oxygen for 18 hours. As a control, no treatment was given. Cells were stained for phosphorylation at Tyr 394 on Lck and assessed by flow cytometry. Percentage positive cells were determined by comparison with isotype controls. Mann whitney statistical test was used for analysis but no statistical differences were found. Medians are shown in the data. Data is representative of at least 2500 cells in the stimulation gate. Five donors were investigated for each treatment. The same gating strategy was used as in figure 5.4.*

The effect of NAC on CD69 expression suggested cellular redox may play a role in the response of CD4+ memory T lymphocytes to hypoxia. As Lck had revealed differences in the level of activation at different oxygen levels and in response to H_2O_2/ DFX treatments, levels of the activatory phosphorylation at Y394 were determined in resting CD4+ memory T lymphocytes after 24 hours treatment with NAC at 1% oxygen. NAC treatment resulted in the significant increase in Lck Y394 phosphorylation at 1% oxygen compared to non-treated cells (figure 5.6). This mimics the effect of increasing oxygen levels, as at 8.5% oxygen a significant increase in active Lck was observed (figure 5.4).

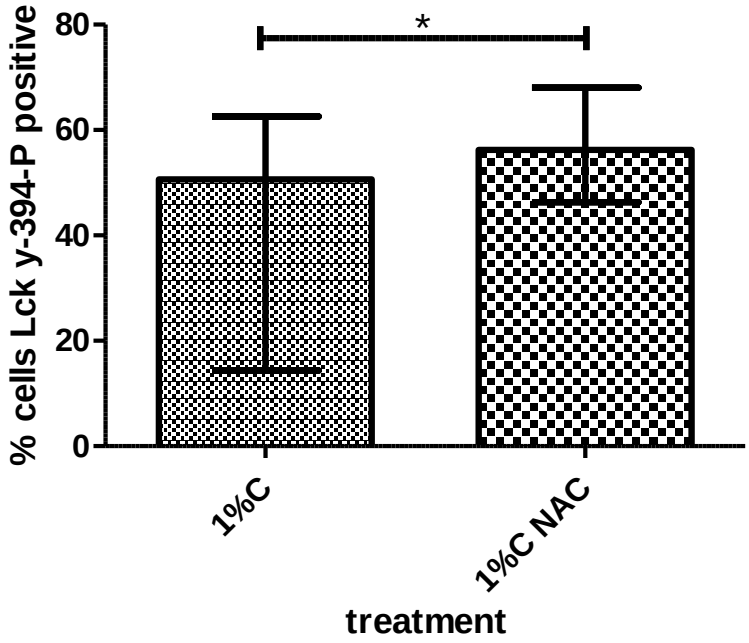

Figure 5.6. NAC treatment in hypoxia reveals the redox regulation of proximal T lymphocyte regulation. *CD4+ CD45RO+ T lymphocytes were isolated using a Miltenyi MACs CD4+ memory isolation kit and frozen at -80°C for later analysis. CD4+ CD45RO+ T lymphocytes were treated with 1mM N-acetyl-L-cysteine (NAC), or no treatment, and equilibrated for 24 hours to 1% oxygen. Cells were stained for phosphorylation at Tyr 394 on Lck and assessed by flow cytometry. Percentage positive cells were determined by comparison with isotype controls. Mann Whitney test was used for statistical analysis and median values are shown in the data. * $p<0.05$. Data is representative of at least 2500 cells in the T lymphocyte gate. A minimum of nine donors were investigated for each treatment. NAC treatment resulted in an increase in Lck Tyr 394 phosphorylation at 1% oxygen. The same gating strategy was used as in figure 5.4.*

5.2.4 The influence of HIF-1α stabilisation and ROS on T helper cell subset cytokines and markers

In addition to investigating the effect of DFX and H_2O_2 treatment on CD69 and Lck expression, an experiment was performed to examine their effects on the phenotypic markers investigated in chapter 4. Surface and intracellular staining was performed after 18 hours stimulation with the different treatments.

The combination of DFX and H_2O_2 treatment resulted in similar patterns in cytokine, transcription factor and surface marker expression compared to the patterns observed between 8.5% and constant 1% oxygen culture after 48 hours stimulation (as described in chapter 4). With regards to pro-inflammatory cytokines and markers, Tbet+ and IL-17A intracellular cytokine staining revealed reductions when cells were exposed to both DFX and H_2O_2, similar to what is see at constant 1% oxygen. CCR6 expression also appeared to be increased after exposure to H_2O_2. However, n numbers and experimental repeats were limited, and would require increasing in future.

With regards to the other panel investigated, CCR4 and CTLA-4 expression was reduced when H_2O_2 treatment was given alone or in combination with DFX, as did the percentage of CTLA-4+ Foxp3+ cells. IL-4 and IL-10 were barely detectable by intracellular cytokine staining after 18 hours stimulation, and therefore were not included in the analysis.

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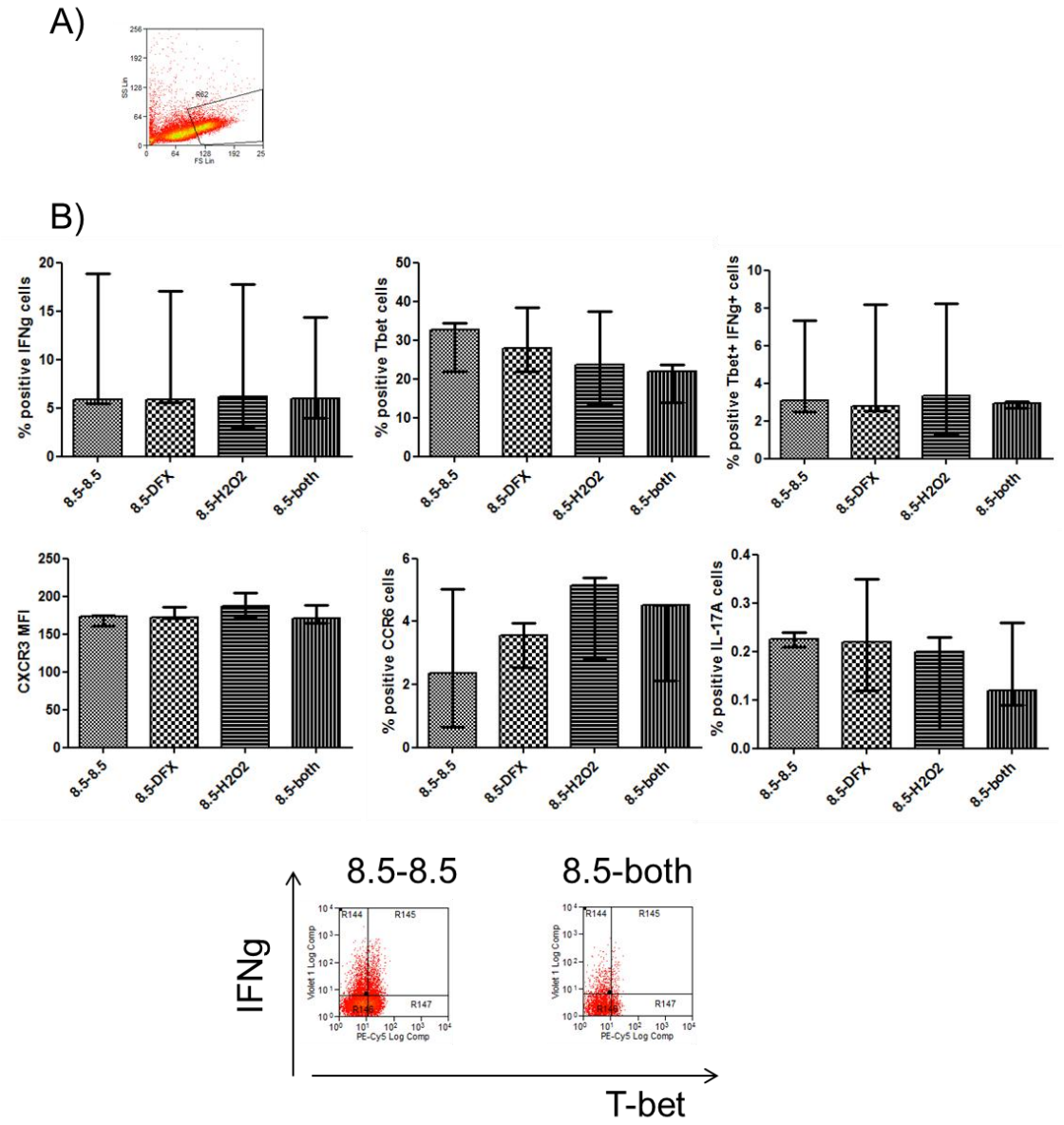

Figure 5.7. The effect of DFX and H_2O_2 treatment on markers associated with Th1 and Th17 pro-inflammatory T helper cells. *CD4+ CD45RO+ T lymphocytes were isolated using a Miltenyi MACs CD4+ memory isolation kit and frozen at -80°C for later analysis. A) shows the FS/SS plot with the selected gate on stimulated cells. B) Markers associated with pro-inflammatory T helper subsets were assessed by intracellular staining and flow cytometry at 8.5% oxygen. 2x10^5 cells were equilibrated to 8.5% oxygen for 24 hours, before being treated with 2.5μM deferoxamine mesylate (DFX) and/or 10μM hydrogen peroxide (H2O2) at the same*

time as being stimulated with 2µg/ml anti-CD3/ 5µg/ml anti-CD28 for 18 hours. 2µg/ml brefeldin A treatment was given for the last three hours of stimulation. Three donors were investigated for each treatment except for 8.5%-8.5% IL-17 where n=2. Experiments were performed on two different occasions. Example dot plots for T-bet vs IFNg are shown. Statistical analysis was performed using Mann Whitney test, but no significant differences were observed. Median values are shown in the data.

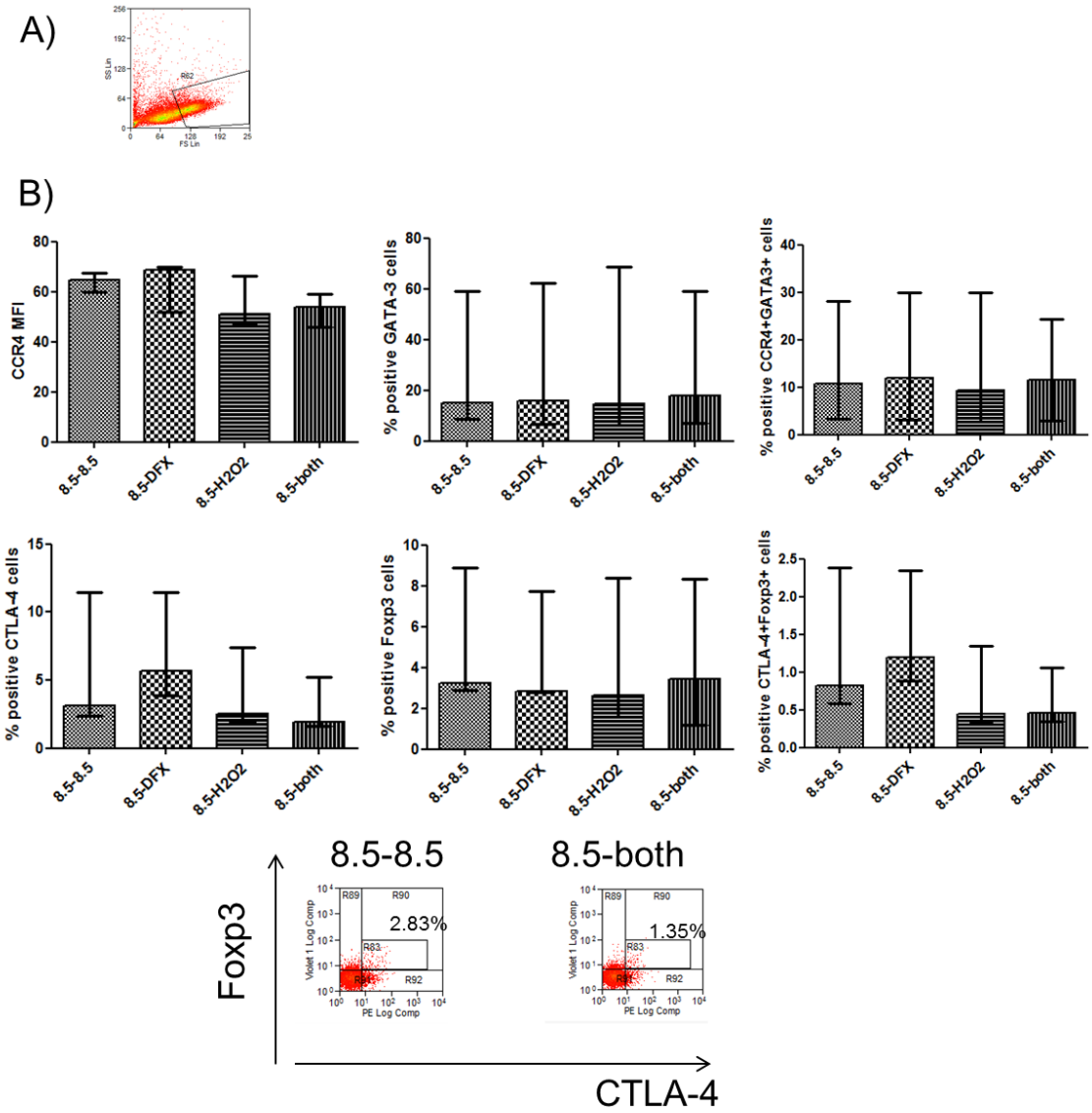

Figure 5.8. The effect of DFX and H_2O_2 treatment on markers associated with Th2 and regulatory T helper cells. *CD4+ CD45RO+ T lymphocytes were isolated using a Miltenyi MACs CD4+ memory isolation kit and frozen at -80°C for later analysis. A) shows the FS/SS plot with the selected gate on stimulated cells. B) Markers associated with anti-inflammatory T helper subsets were assessed by intracellular staining and flow cytometry at 8.5% oxygen. 2x10^5 cells were equilibrated to 8.5% oxygen for 24 hours, before being treated with 2.5μM deferoxamine mesylate (DFX) and/or 10μM hydrogen peroxide (H2O2) at the same time as being stimulated with 2μg/ml anti-CD3/ 5μg/ml anti-CD28 for 18 hours.*

2µg/ml brefeldin A treatment was given for the last three hours of stimulation. Three donors were investigated for each treatment. Example CTLA-4 vs Foxp3 dot plots are shown. Experiments were performed on two difference occasions. Statistical analysis was performed using Mann Whitney test, but no significant differences were observed. Median values are shown in the data.

5.3 Discussion

In this chapter the mechanisms behind the effects of hypoxia on CD4+ memory T lymphocytes have been explored by investigating both ROS exposure and the stabilisation of HIF-1α, two outcomes of cellular exposure to hypoxia reported in the literature [204, 224, 238, 255, 256]. ROS exposure was examined by treating cells with 10μM H_2O_2, and HIF-1α stabilisation was assessed by treating cells with 2.5μM DFX. DFX stabilises HIF-1α via its action as an iron chelator, as the PHDs that normally regulate HIF-1α protein stability are dependent on both dioxygen and iron as a co-factor [224, 225, 366]. H_2O_2 was found to have a slightly greater effect than HIF-1α stabilisation, suggesting that redox may influence CD4+ CD45RO+ T lymphocyte behaviour. In support of this, the addition of the anti-oxidant NAC in hypoxia reversed the pattern observed in hypoxia and with H_2O_2 treatment, confirming that this may be the case.

Initially, CD69 expression was assessed in response to different treatments due to the strong upregulation observed in response to constant 1% oxygen in Chapter 3 (figures 3.3 and 3.4). Responses, however, depended on when the treatments were given. Both H_2O_2 and DFX were given to cells at the time of stimulation, after a period of equilibration at 8.5% oxygen. A switch to 1% oxygen at stimulation was used as a control in these experiments. H_2O_2 and DFX mimicked 1% oxygen in their effect on CD69 with decreased expression. However, this was contrary to what shown in Chapter 3, where CD69 expression was increased at constant 1% oxygen. Two factors (other than the treatments) were altered in these experiments compared to those performed in previous chapters. Firstly, cells were equilibrated for 24 hours at 8.5% oxygen, but a switch in oxygen level to 1% oxygen at the time of stimulation was used as a control. Secondly, cells were only stimulated for 18 hours when

treated with H_2O_2 and DFX due to their toxicity, whereas CD69 expression was previously assessed after 24 hours stimulation. As only an additional 6 hours stimulation would be needed for the cells to upregulate CD69 at 1% oxygen, it was assumed that the latter difference in experimental procedure was not the cause for the difference in results observed. Instead, it is probable that the oxygen level during the equilibration period influenced subsequent cellular responses to stimulation. These results did show that under these equilibration conditions, H_2O_2 and DFX treatment mimicked the effect of oxygen levels of 1% oxygen, but also show that environmental oxygen levels have an effect even on resting cells. In contrast, cells treated with NAC were treated from the start of the experiment, as NAC is not toxic to the cells over time. The effect of NAC treatment on CD69 expression at 1% oxygen also highlight the potential role redox plays in determining T lymphocyte cell biology as it reduced the effect of 1% oxygen on CD69 to a level on a par with 8.5% oxygen.

Due to the observation that equilibration appeared to influence subsequent responses to hypoxia, proximal T lymphocyte signalling was investigated via the determination of phosphorylation of the activatory tyrosine residue on the kinase Lck. Phosphorylation at this residue is thought to be dominant over an alternative inhibitory residue [75, 84], and allows the kinase to phosphorylate downstream targets such as ITAMs in the TCR:CD3 complex, thus promoting downstream signalling [79]. Regulation of T lymphocyte signalling in response to hypoxia has already been touched upon within this thesis with the observation of differences in expression of CD3 and CD45RO at different oxygen levels in stimulated cells (chapter 3).

In this chapter, the population of active Lck in resting cells in 1% oxygen was decreased when compared to 8.5% oxygen. As the population of active Lck in resting cells is thought to determine TCR:CD3 ITAM phosphorylation post-stimulation and therefore subsequent signalling strength [75], this is a key finding. A smaller pool of active Lck may result in a lower signal being delivered to the lymphocyte [75]. Within a population of lymphocytes, this may result in a smaller number of cells passing the threshold for activation. Conversely, it may also protect cells from an overtly large activatory signal that could result in cellular apoptosis, and therefore may protect autoreactive cells from peripheral deletion [50, 52]. In environmental oxygen levels of around 1%, cells that are activated may be less activated than their 8.5%-cultured counterparts. This may have downstream effects on the lymphocytes function, such as reduced proliferation [81, 370].

The effect of the different treatments on Lck activatory phosphorylation were examined to see whether the redox environment and/ or HIF-1α stabilisation influenced proximal T lymphocyte signalling. As cells were resting in these experiments, all treatments were given from the start. Both H_2O_2 and DFX were again assessed at 8.5% oxygen culture conditions, and NAC at 1% oxygen. In these experiments, H_2O_2 and DFX resulted in a drop in Lck-394 phosphorylation when compared to 8.5% oxygen, which mimicked 1% oxygen treatments in earlier experiments (figure 5.4). H_2O_2 treatment had a greater influence than DFX, and NAC reversed the pattern observed at 1% oxygen. Therefore, redox appears to be an aspect of the hypoxic environment that can influence both proximal T lymphocyte signalling molecules, and the cellular response that occurs after stimulation has occurred. DFX can behave as an anti-oxidant [371], and therefore may have

alleviated some of the oxidative stress experienced by the cells in DFX plus H_2O_2 treatments. This may have reduced the effects of HIF-1α stabilisation on the cells. ROS has been suggested to be able to stabilise HIF-1α expression [256], and therefore, conversely, in the combination treatments DFX may have reduced any HIF-1α stabilisation induced by H_2O_2. Future work would need to be done to decipher the exact mechanism by which ROS influences both CD69 expression and proximal T lymphocyte signalling.

T helper subset-associated cytokines and markers were also assessed in response to DFX and H_2O_2 treatment, and to some extent mimicked the patterns of cytokine, transcription factor and surface marker expression observed at constant 1% oxygen compared 8.5% oxygen culture after 48 hours stimulation (as described in chapter 4). The combination of DFX and H_2O_2 induced subtle changes in T helper subset phenotypic markers, suggesting that both HIF-1α and ROS treatment in hypoxic conditions may be required to fully influence CD4+ T lymphocyte phenotypic preferences.

However, in this chapter all of the experiments performed with DFX and H_2O_2 revealed only trends, partly due to low numbers of repeats, and future work would need to be done for better understanding. The cytokines and marker data was similar to CD69 data in that not all the data agreed with previous results. For example, CTLA-4 expression was reduced when H_2O_2 was present. Therefore, there may be additional influences of these treatments occurring that need to be further investigated. Furthermore, DFX and H_2O_2 treatments had the greatest similarities to the 48 hour stimulations from chapter 4, not the 24 hour stimulations, but were only given for 18 hours. It is possible that HIF-1α stabilisation and ROS treatment

accumulate gradually in hypoxic cultures, in contrast to the DFX and H_2O_2 treatments which were administered that would have resulted in a more acute response, and therefore mimicked the 48 hour response.

6. GENERAL DISCUSSION

6.1 Introduction

CD4+ T lymphocytes are thought to play a role in the pathology of many chronic inflammatory environments [165], and therefore understanding their behaviour in response to the stresses present in these environments is important for successful future therapeutic intervention. CD4+ T lymphocytes experience many different oxygen levels physiologically, from the hypoxic thymus [245, 246] and lymph nodes [239, 245], to more oxygen-perfused blood [241]. With regards to CD4+ memory T lymphocytes; central memory are much more likely to experience antigen in the relatively low-oxygen environment of the lymph node; whereas effector memory will directly enter - and be stimulated in - affected tissues which may already be deprived of their normal oxygen levels. Therefore, both types of CD4+ memory T lymphocyte may normally function in physiologically low-oxygen environments.

Chronic inflammatory environment such as the rheumatoid joint are known to be lower in oxygen than their healthy-tissue counterparts, and this hypoxia is thought to persist [199]. Therefore, the infiltrating cells entering a chronic inflammatory environment may experience potentially very low oxygen levels. Evidence suggests that T lymphocytes in these environments behave differently than expected in acute inflammatory situations. In the rheumatoid joint, T lymphocytes appear to be in a ‘stuck’ phenotype, with delayed apoptosis [372], and depressed cytokine production [155, 156, 176, 180, 373] and proliferation [172, 173]. In this thesis the influence that changes in oxygen level can have on CD4+ CD45RO+ T lymphocytes has therefore been investigated.

6.2 Alterations in proliferation and proximal T lymphocyte signalling reveal fundamental influence of environmental oxygen on effector memory CD4+ T lymphocyte biology

In this study, at the physiologically healthy oxygen level of 8.5% oxygen, CD4+ CD45RO+ T lymphocyte proliferation was greatest compared to the other oxygen levels examined. This agrees with previous studies that observed increases in long-term proliferation at 5% oxygen, an oxygen level also deemed as physiologically healthy [235, 284, 285, 288]. Investigation at the slightly higher oxygen level of 8.5% is novel, and may further reflect the CD4+ T lymphocyte response to being stimulated at a reasonably perfused tissue. However, anatomic sites in which T lymphocytes are presented antigen are often more hypoxic in nature [9, 239, 245, 246], and therefore T lymphocytes may only be rarely stimulated in oxygen levels of around 8.5% oxygen. Proliferative responses were expected to be most robust in central memory T lymphocytes. Specifically, this particular memory subset would probably not be exposed to oxygen levels higher than 5% when presented with antigen, as revealed by studies detailing oxygen levels of between 0.5-4.5% [239], and 1.3-3.3% [245] in the spleen. Therefore, the physiological relevance of this result needs to be better assessed in the future by prior separation of central and effector memory T lymphocytes before exposure to antigenic stimulation at different oxygen levels.

Regarding the lower oxygen levels observed in lymph nodes; a reduction in proliferation was observed at 3% oxygen and constant 1% oxygen, and was also observed in 21% oxygen cultures, and suggests that the oxygen environment of the lymph node may limit T lymphocyte responses to antigen. A reduction in proliferation in hypoxia (1-3% oxygen) has been observed previously, although a comparison of

1% oxygen with 3% and 8.5% oxygen is novel [235, 240, 275, 289, 290]. Furthermore, RA T lymphocytes directly stimulated from the RA joint had reduced 72 hour proliferation in comparison to those rested at 21% oxygen levels for 5 days [316]. As we observed better proliferation in the reperfusion injury group compared to constant 1% oxygen treatments, it is possible the later addition of oxygen may enhance proliferation, despite the oxidative environment, through mechanisms as of yet unknown.

The lack of proliferation at 21% oxygen observed is contrary to previous published results with mixed T lymphocyte populations [235, 244, 281]. In the current study using CD4+ memory T lymphocytes, there was an increase in the number of cells that did not undergo any rounds of proliferation at 21% oxygen. Due to their exposure to previous inflammatory responses, it is possible that CD4+ memory T lymphocytes are more adapted to hypoxic environments, and that the hyper-oxic environment of 21% oxygen levels is inappropriate for their culture. In support of this concept, CD4+ T lymphocytes lose mitochondrial mass as they differentiate [374]. Exposure to higher oxygen levels would result in overloading of memory T lymphocyte mitochondria, and may result in the production of ROS as previously observed after culture at 21% oxygen [244, 271]. Mixed T lymphocyte populations cultured at 21% oxygen have been shown to have reduced glutathione compared to physiologically healthy oxygen levels (5%) [244] and have increased ROS damage [240, 271].

Hypoxia also results in the increased release of ROS from both the mitochondrial electron transport chain [254-256] and extra-mitochondrial sources [375]. Hypoxia has therefore been associated with an increase in oxidative damage in cell culture specifically in stimulated CD4+ T lymphocytes [235, 240]. This phenomenon of ROS

production at both extremes of oxygen availability has also been described for skeletal muscle [376]. The redox environment has been previously implicated in negatively regulating T lymphocyte proliferation [173, 235, 377]. Therefore when a redox imbalance occurs in both hyper- or hypo- oxic conditions, it may result in an oxidative intracellular environment that leads to reduced long-term proliferation, and it is possible that the redox environment resulting from culture at these oxygen levels may play a role in inhibiting long term proliferation in CD4+ memory T lymphocytes. Furthermore, hypoxia has been previously found to inhibit membrane potential by acutely inhibiting Kv1.3 channel function and lowering its membrane expression over 24 hours exposure to 1% oxygen [275] and this additional mechanism may have resulted in the inhibition in proliferation observed in this study.

There could be alternative explanations for the differences in proliferative responses observed at different oxygen levels. The observation that the population of Lck in resting memory T lymphocytes differs in its activation status depending on which oxygen environment it has been exposed to for the last 24 hours suggests that proximal T cell signalling may be involved in the T lymphocyte response to environmental oxygen. T lymphocyte proliferation is dependent on a high number of TCR:CD3 ITAMs being phosphorylated post ligation [321], and it is therefore possible that the higher population of active Lck that would have been present after 24 hours equilibration at 8.5% oxygen would have allowed for better initiation of proliferative responses, whereas the lower population of active Lck at 1% oxygen may have reduced any subsequent proliferation. The decrease in the population of active Lck contradicts current literature as previously Lck Tyr 394 phosphorylation was found to be unaltered after 3 minutes exposure to 1% oxygen compared to 21% oxygen [233].

As 24 hours elapsed before Lck phosphorylation status was assessed in this study, it is possible that Lck activation status changes over time (and this was seen from comparisons of 18 hours and 24 hours equilibration). The reason for the decrease observed could be greater auto-phosphorylation, or decreased dephosphorylation by phosphatases such as LYP. The difference in Lck activation in resting cells at different oxygen levels also highlights how the physiological oxygen environment may influence T lymphocyte biology before stimulation. Hence, T lymphocytes stimulated rapidly upon entering a hypoxic tissue from a well-perfused blood vessel may respond differently than if a delay in stimulation occurs once the cell has entered the tissue.

As RA synovial T lymphocytes also have lowered phosphorylation of the TCR:CD3 complex [156, 172] it is possible that the effect of low oxygen levels on proximal T lymphocyte signalling observed in this *in vitro* study has some physiological relevance. Furthermore, a genetic alteration in the gene for LYP, *ptpn22*, is the second highest ranking single nucleotide polymorphism (SNP) associated with risk for RA, further suggesting that TCR proximal signalling machinery may be important in the development of the disease [157, 378, 379]. The outcome of this SNP is thought to be gain-of-function protein [159, 161, 162]. In this instance, the LYP molecule will be over-active, dephosphorylating the activatory residues of the Lck molecule more readily, thus mimicking what was observed in hypoxia. Many patients do not carry the *PTPN22* polymorphism, and hence it is interesting that environmental oxygen levels can mimic its outcome.

Potential mechanisms behind differences in the population of active Lck at different oxygen levels may also be due to the redox environment present. H_2O_2 was found to

non-significantly decrease Lck activatory residue phosphorylation at 8.5% oxygen, whereas NAC treatment significantly increased Lck activation at 1% oxygen. As NAC is also known to inhibit NFκB [367] it would need to be further confirmed that the redox environment was involved in T lymphocyte regulation. The stabilisation of HIF-1α at 8.5% oxygen via DFX treatment appeared to have less of an effect than H_2O_2, and when the treatments were combined a small increase in activated Lck was observed when compared with the H_2O_2-only treatment. This could be due to the fact that DFX has the ability to act as an antioxidant [371], and could therefore counteract the effects of ROS treatment. However, these data need repeating before more solid conclusions are made from them. Alternative hypoxia mimetics, such as cobalt chloride, could be utilised in the future to further assess the effect of HIF-1α on Lck activation status. H_2O_2 treatment may have been able to stabilise some HIF-1α expression, as ROS has previously been shown to inhibit PHD activity [196, 224]. This would need to be investigated more in the future to delineate the different effects that redox can have on hypoxic cell biology. However, as HIF-1α is not necessarily stabilised in resting T lymphocytes in hypoxia [204], it may be that it does not play much of a regulatory role in this situation.

Redox is known to influence T lymphocyte biology, and the TCR signalling machinery has been shown to be particularly susceptible to this [380]. Lck protein levels have been shown to drop in the presence of ROS overtime due to proteosomal targeting, and this was linked to a decrease in proliferation [380]. This is also very interesting when considering chronic autoimmune environments such as the rheumatoid joint, where many ROS-producing neutrophils can be observed. Direct culture with activated neutrophils was shown to reduce T lymphocyte proliferation [380], and

therefore the oxidative environment generated by neutrophils and low environmental oxygen in the RA tissue may give rise to the depressed phenotype associated with RA T lymphocytes.

6.3 Expression of surface markers and markers of activation are altered at different oxygen levels, implying different effector functions

The assessment of activation markers is often used to investigate cellular responses to particular stimuli or treatments. The expression of CD69, CD3, CD45RO and HLA-DR at different oxygen levels is discussed below. HLA-DR expression was not found to be significantly different in response to different oxygen levels. HLA-DR is a molecule normally associated with APCs. However, its expression is also upregulated on CD4+ T lymphocytes as a late marker of activation [308, 381]. HLA-DR expression has been observed in the RA joint associated with T lymphocytes [170, 316]. Due to its nature as a ‘late’ activation marker, it is possible HLA-DR expression needs to be assessed over a longer timescale.

6.3 1 The activation marker CD69 is upregulated at lower oxygen levels

The early activation marker CD69 was observed to be upregulated in response to 1% oxygen at both 24 and 48 hours compared with 8.5% oxygen. An upregulation in CD69 has previously been described at 5% oxygen compared with 21% oxygen culture in PBMCs [244, 288] but the description of a specific upregulation in CD4+ memory T lymphocytes at lower oxygen levels is novel. CD69 expression is rapid in response to stimulation, but can depend on the stimulation method used. After TCR

engagement, the method used within this current study, surface expression peaks between 18-24 hours [312].

The expression of CD69 on lymphocytes is important in the inhibition of thymic and lymph node egress via the internalisation of sphingosine-1-phosphate receptors, which normally bind tissue S1P gradients to promote migration [314, 315]. Both mature T lymphocytes and thymocytes express CD69 after stimulation [308, 313, 314], as it plays an important role in inhibiting lymphocyte migration out of lymphoid organs before they are fully developed and also allows them to provide support to B lymphocytes [314]. CD69 deficiency has previously been associated with a lack of B lymphocyte help from CD4+ T lymphocytes [382]. Therefore, the finding that the expression of CD69 is increased on the surface of CD4+ memory T lymphocytes in low oxygen suggests that environmental oxygen levels can further promote lymphocytes residence within a tissue or organ. For example, central memory T lymphocytes activated in a lymph node will upregulate CD69 more if environmental oxygen levels are low. This will therefore result in their longer duration resident in the lymph node, and may aid their effector development and function. On the other hand, the upregulation of CD69 in the inflammatory tissue may also have a similar effect. S1P is found on the surface of all cells [314], and therefore the upregulation of CD69 may also influence migration of a lymphocyte through the tissue via S1P receptor internalisation [314]. In a chronic inflammatory site, altered migration may be reduced, and the cells may spend weeks in the tissue, as determined by T lymphocyte CD45RB^{dull} status (a marker that is decreased over several rounds of proliferation and stimulation) [306]. It would be interesting to determine what role CD69 plays in this.

The upregulation of CD69 in hypoxia suggests it may play an important role in immunity. In a murine model of CIA CD69 deficiency alleviated symptoms [317, 320]. However, the protection from disease may have been due to a lack of CD69 expression on neutrophils, although this was not directly shown experimentally [320]. In contrast, CD69 deficiency has been suggested to result in a more pro-inflammatory phenotype [317, 319]. CD69 is readily detected in chronic inflammatory environments [170, 383] such as lupoid kidneys, tumours and the rheumatoid joint, where around 60% of CD4+ T lymphocytes from RA synovial tissue were CD69 positive [40, 170, 311, 316, 383, 384].

Despite the lack of an extracellular ligand, CD69 is thought to have signalling potential [309, 310, 313]. In T lymphocytes it co-precipitates with a 40kDa protein suspected to be involved in signal transduction [169, 310] and CD69 cross-linking can result in ERK phosphorylation [319] . CD69 is also constitutively serine phosphorylated, suggesting downstream signalling is potential in physiological settings [309, 310]. When the crystal structure of CD69 was determined, an unexpected electron density was observed at the site where ligand binding was predicted. This electron density suggested a carbohydrate may be a potential ligand for CD69, such as those on other surface molecules [311]. CD69 crosslinking results in a sustained influx of cellular calcium. This can induce proliferation and cytokine production alongside PKC activation (such as PMA treatment). CD69 therefore may act as a co-stimulatory molecule in immune cell signalling [309-311, 313], and its upregulation in low environmental oxygen conditions may result in as-of-yet unknown signals being sent into cells expressing it. Futhermore, failure of CD69 expression

has been associated with a lack of a specific memory T lymphocyte population in the bone marrow that is associated with the production of high affinity antibodies [382].

The higher and sustained expression of CD69 observed at lower oxygen levels may also be detrimental. CD69 has been implicated in the T lymphocyte cell-contact dependent upregulation of TNFα from macrophages, including synovial T lymphocyte mediated TNFα monocyte production [28, 40, 164, 169, 194, 195] via a yet-to-be identified receptor or ligand. This is particularly poignant when investigating chronic inflammatory conditions where TNFα is thought to play a pathological role, such as the rheumatoid joint. Anti-TNFα therapy has proved to be extremely beneficial in the treatment of RA [35, 194]. Macrophages are thought to be the main producers of TNFα in the RA joint, but T lymphocytes are thought to be heavily involved in promoting its production. Therefore, in hypoxic, chronically inflamed tissues, CD69 may play a role in perpetuating inflammation. However, it is possible that T lymphocytes are not in close proximity with macrophages in the RA joint [175], although some argue they are localised [194]. Further *ex vivo* studies are required to confirm whether CD4+ T lymphocyte CD69 upregulation is involved in human autoimmune disease.

Interestingly, CD69 expression is associated with a subset of tissue-resident T lymphocytes [55, 58, 61, 382]. Unlike CD4+ effector memory T lymphocytes, these T lymphocytes do not circulate through the blood and tissue, but remain in tissue after the resolution of inflammation, and CD69's associated suggests that it has a functional role in this cell population. The finding that CD69 expression is upregulated on CD4+ memory T lymphocytes in hypoxia is therefore interesting, as it suggests

that oxygen levels may influence the generation and maintenance of tissue resident CD4+ T lymphocytes. More longitudinal studies would have to be performed to confirm this. It is also interesting to note that CD69 is abnormally upregulated in the rheumatoid joint [40, 170, 316]. This could be linked to the development of T_{RM} in the rheumatoid joint. It may be the case that not all T lymphocytes infiltrate into the RA synovium, but some could have 'set up home' within the joint environment before chronic inflammation was initiated. It also suggests that T lymphocytes that have migrated into the joint may have differentiated to a T_{RM} -like phenotype. Future work in discerning whether this is in fact the case, and the effect this may have on pathology, may be of great benefit to the understanding of chronic inflammatory diseases. As CD69 expression may be directly involved in the persistence of T_{RM} populations [55, 382] it is possible that its upregulated expression also results in the maintenance of T lymphocytes in chronic inflammatory sites.

CD69 upregulation at 1% oxygen may be due to differences in proximal T lymphocyte signalling. CD69 expression under both DFX and H_2O_2 treatment mimicked that of the relevant 1% oxygen control, albeit non-significantly. However, the 1% oxygen control revealed a different pattern as to that previously obtained at stimulation at 1% oxygen, and this was probably due to the fact that before they were stimulated and switched to a 1% oxygen environment, they were equilibrated at 8.5% oxygen (whereas before they had been equilibrated at 1% oxygen). Therefore, the oxygen level resting cells experience may influence their subsequent biology, even if the oxygen level subsequently alters. This could have profound implications on cells circulating in the blood and then entering tissue and being activated, compared to those resident in tissue that are subsequently stimulated. Out of the treatments

investigated in this thesis, H_2O_2 treatment provided the closest parallel to the 1% oxygen control. In contrast, the anti-oxidant *N*-acetyl cysteine (NAC) reduced CD69 expression at 1% oxygen to levels close to those observed at 8.5% oxygen. Therefore, redox may play an important role in determining CD69 expression on CD4+ T lymphocytes. As environmental oxygen levels can cause alterations in cellular redox status, this may be one way in which differing environmental oxygen levels influence CD4+ memory T lymphocyte biology.

6.3.2 The key signalling molecule CD3ε reveals a different pattern of expression at different oxygen levels.

CD3ε surface expression was observed to alter over time depending on environmental oxygen level. After 24 hours, there was no significant difference between cells stimulated at 8.5% oxygen and those stimulated at constant 1% oxygen. However, by 48 hours, the expression of CD3ε in 1% oxygen had significantly increased, whereas cells at 8.5% oxygen continued to decrease their expression. A decrease in TCR:CD3 complex expression is expected after stimulation, and has previously been attributed as an intrinsic regulatory mechanism to limit further activation [322-325]. TCR:CD3 complex surface expression is not static, and normally cycles through constant recycling via exo- and endo- cytosis [323]. There is some disagreement as to whether decreased exocytosis [323] or increased endocytosis [325], results in the reduction of TCR:CD3 complexes at the surface, and there is some data suggesting they are released from the surface of the cell [72].

The observed increase in CD3ε expression in constant 1% oxygen over time post-stimulation suggests that this cycling is altered in different oxygen levels. As cellular granularity was also observed to be increased in constant 1% oxygen compared to 8.5% oxygen, it is possible that CD4+ memory T lymphocytes in 1% oxygen contain more intracellular vesicles involved in recycling of several different molecules. The TCR:CD3 complex could be one of these molecules and hence its expression may be upregulated. Physiologically, this increased surface TCR:CD3 expression may allow T lymphocytes to prolong exposure to antigen in more hypoxic tissues. In the inflamed lymph node, as cells proliferate, available antigen is thought to become reduced [54]. As cells are proliferating, it can also be presumed that a drop in available oxygen may occur within the lymph node. Therefore, if cells that migrate in to the lymph node later upregulate CD3ε in response to decreased oxygen levels, they stand a greater chance of sustained TCR:CD3 contact with an APC. Duration of exposure to antigen is thought to decide whether naïve T lymphocytes become effector or central memory T lymphocytes [54, 62], and reduced duration is also associated with the development of an anergic phenotype. Therefore, the hypoxic-induced surface upregulation of the TCR:CD3 complex may influence CD4+ T lymphocyte biology.

6.3.3 The memory T lymphocyte marker, CD45RO, is expressed at different levels at different oxygen levels.

In this thesis an increase in CD45RO expression was observed at 3% oxygen. A complete lack of CD45RO inhibits T lymphocyte signalling due to increased inhibitory phosphorylation of Lck in resting cells [88, 160]. Conversely, CD45RO

overexpression also results in a reduction in signalling [328], whereas intermediate expression levels (10-60% of wild-type) result in an overactive T lymphocyte response due to a lack of Lck Y394 dephosphorylation [88]. Hence a fine balance in CD45RO isotype expression needs to be maintained for proper T lymphocyte signalling, and to pass the signalling threshold required for proper activation of the cell as set by Lck activation status. Due to the alterations in cellular signalling that subtle changes in CD45RO expression can incur, it is difficult to predict what effect increase CD45RO expression at 3% oxygen may have on subsequent cellular responses and stimulation.

As the CD4+ T lymphocytes in this study expressed normal levels of CD45RO, it is possible that the small increase in CD45RO expression at 3% oxygen may have promoted further T lymphocyte signalling via the dephosphorylation of tyrosine 505 (Y505) on Lck. A correlation between CD45RO expression levels and Lck Y505 dephosphorylation has been previously observed in resting cells [88]. Indeed, the upregulation of CD45RO post-stimulation at 3% oxygen would probably only have its full effect on the Lck pool when cells return to a resting state, as this is when these two molecules are again exposed to each other [73, 88]. As the cells used experimentally in this study were mainly memory, it is possible that many of them would not survive the stimulation process. Future studies into the expression of CD45RO on memory cells generated from naïve T lymphocytes stimulated at different oxygen levels could be of benefit in further deciphering whether this observation has any functional applications.

In the context of the lymph node, in which 3% oxygen may be the local environmental oxygen level [239, 245], increased activation may be beneficial as it will improve the

chances of full activation. However, in the context of autoimmunity, this reduction in the signalling threshold required to activate a cell may be detrimental. As the average oxygen levels of the RA joint are also 3% [199-201], it is possible that CD45RO expression is increased in autoreactive CD4+ T lymphocytes migrating into this environment. If they are subsequently stimulated in the RA tissue, the increase in CD45RO expression may result in a greater number of T lymphocytes successfully becoming re-activated over the course of exposure to several antigen-bearing cells. The opposite is also possible, however, as CD45RO is also thought to be able to dephosphorylate the activatory residue on Lck, Y394, especially at higher CD45RO membrane concentrations [88, 160]. Therefore, as CD45RO levels increase, it is possible that the Lck molecules will also be inactivated, and downstream signalling will be inhibited.

6.4 Cytokine production at different oxygen levels is altered

Cytokine production is a cardinal response of CD4+ T lymphocytes to stimulation. In this thesis, differences were observed in the balance of pro- to Th2/anti-inflammatory cytokine production at different oxygen levels. An increase in pro-inflammatory cytokines was observed at lower oxygen levels, whereas an increase in the ratios of IL-4, IL-5 and IL-10 were observed at 8.5% oxygen. Additionally, an upregulation in the expression of IL-10 was observed at 21% oxygen. Despite the differences observed being subtle, in a chronic inflammatory environment where successive cohorts of lymphocytes continually migrate into the tissue, subtle variances may have cumulative effects on local inflammatory responses and consequently influence

pathology. In an environment where cells are in close contact, the overall balance of cytokine production will be important in either an autocrine or paracrine manner.

6.4.1 Constant 1% oxygen is more pro-inflammatory in nature

Despite the reduction in cytokine production in more severe hypoxia, the results in this study do suggest that in lower environmental oxygen levels a more pro-inflammatory phenotype may be favoured. This agrees with the pro-inflammatory nature of the chronically inflamed environment of established RA [30, 35]. Increasing ratios of pro-inflammatory cytokines at 1% oxygen compared to 8.5% oxygen were observed and intracellular cytokine staining after 24 hours stimulation suggested that IFNγ expression was greatest at 21% oxygen, constant 1% oxygen and in reperfusion injury treatments, whereas the least was detected at 8.5% oxygen. Hypoxia has been found to promote IFNγ production over 72 hours in mixed CD4+ T lymphocytes populations compared to 21% oxygen [237], and it is therefore possible that the oxygen environment of the joint influences cytokine production from CD4+ T lymphocytes. However, this does not explain the upregulation of IFNγ expression in 21% oxygen cultures. Increased CXCR3 expression was also observed at 1% oxygen, although this did correlate with cell size. However, the increase in the number of CXCR3 molecules on larger cells could play a functional role physiologically. As increased CXCR3 was also observed on naïve T lymphocytes polarised to a Th1 phenotype in 1% oxygen, it is possible that lower oxygen levels do promote a more Th1-like phenotype in both naïve and memory T lymphocytes.

The increase in a pro-inflammatory phenotype at constant 1% oxygen was also accompanied by a lack of Th2 and Treg-associated cytokines and markers. In

supernatants, only low levels of IL-4, IL-5 and IL-10 were detected, and limited IL-10 was detected by intracellular staining at both 24 and 48 hours post-stimulation. The number of CCR4+GATA3+ positive cells was also decreased at constant 1% oxygen after 24 hours, but did increase again after 48 hours stimulation. Culturing at 1% oxygen has previously been found to result in the upregulation of IL-4 [237]. However, the study that reported this also simultaneously investigated IFNγ production, and found that the upregulation in the latter cytokine was greatly increased compared to IL-4 in 1% oxygen [237]. However, the data from this thesis needs to be treated with some caution, as IFNγ (and IL-5 to some extent) appeared to be less affected by freezing than the other cytokines investigated, and therefore using cells that had been frozen may have skewed the response to appear more pro-inflammatory than it was. Future work using fresh cells would be of benefit to confirm the patterns observed.

6.4.2 Changes from 24 to 48 hours reveal the unsustainable nature of hypoxia

Despite the pro-inflammatory phenotype of CD4+ memory T lymphocytes after 24 hours stimulation at constant 1% oxygen, intracellular cytokine staining suggested that this phenotype was not maintained to 48 hours stimulation compared to 21%, 8.5% and 3% oxygen treatments. However, according to the cell viability data in Chapter 3, this is not due to increased cell death. This may not have been fully reflected in the analysis of supernatants from 48 hour stimulation due to the detection of cytokines produced in the earlier stages of stimulation. The percentage of T-bet+IFNγ+ cells decreased over time at constant 1% oxygen and reperfusion injury, whereas it increased in every other oxygen level assessed. In addition, the

percentage of both IL-17A and IL-10 positive cells at constant 1% oxygen did not increase to similar levels compared to other oxygen treatments (excepting IL-10 at 3% oxygen), further suggesting a general depression in cytokine production in constant hypoxia.

Previously the deletion of HIF-1α has been observed to result in the upregulation of cytokine production [234]. This may explain the drop in cytokines observed at 1% oxygen, when HIF-1α is expected to be stabilised. However, at 3% oxygen HIF-1α is also thought to be stabilised [230], and yet IFNγ and IL-17A production appeared to be well maintained to 48 hours stimulation. Therefore, factors other than HIF-1α stabilisation may cause the depression in cytokine production at 1% oxygen. Specifically focussing on IFNγ production over time, the three oxygen levels thought to be associated with the most ROS production - 21% oxygen, constant1% oxygen and reperfusion injury treatments [235, 244, 254-256, 271] - revealed the worst maintenance in this cytokine's production. Therefore, the oxidative environment in which CD4+ memory T lymphocytes are present may influence their ability to produce cytokines over time. The observation that the combination of DFX and H_2O_2 treatment slightly reduced the percentage of T-bet+IFNγ+ cells suggests that the combination of HIF-1α stabilisation and ROS may be responsible for the unsustainability of the hypoxic environment with regards to T helper cell function. T-bet is known to be able to bind the *ifnγ* locus and initiate transcription [99], although some redundancy in its action is possible [110]. Even if it does not initiate IFNγ transcription itself, its downstream effects result in this outcome [98, 345, 348]. Therefore, the drop in T-bet expression at constant 1% oxygen over time may be associated with the simultaneous drop in IFNγ production. Furthermore, cytokine

production is a cellular process that, like many other cellular processes, utilises cellular energy. Hypoxia results in the reduction of cellular ATP levels [262], and it is possible that the observed limit of cytokine production over time in hypoxia was a simple result of lack of cellular energy.

The reduction in cytokine production at constant 1% oxygen may be due to STAT signalling. STATs are phosphorylated by cytokine receptor-associated JAKs, and then dimerise and translocate to the nucleus where they promote the expression of cytokine genes. However in addition to cytokine binding, cellular stress is also able to activate some STAT transcription factors, and this has been especially reported in response to reperfusion injury [385]. Hypoxia mimetics were found to downregulate the expression of STAT1 protein [386] and STAT1 and STAT4 mRNA was reduced in 1% oxygen compared to 21% oxygen [237]. STAT1 is the immediate signal mediator for IFNγ signalling during Th1 differentiation, and therefore its downregulation may inhibit further Th1 processes [47]. In effector memory cells the downregulation of STAT1 may decrease the autocrine effect of initial IFNγ production, and therefore may explain the subsequent drop in cytokine production and lack of Th1-associated markers such as T-bet. Hypoxia mimetics were found to downregulate the expression of IFNγR, potentially resulting in a lack of autocrine positive feedback (which again would involve STAT signalling) and a subsequent lack of cytokine production over time [386]. Phosphorylation of STAT5a, which is associated with the promotion of a Th2-like phenotype, was found to increase in 1% oxygen compared to 5% oxygen [235], which may provide an explanation for the increase in GATA-3 expression observed over time. However, it still does not fully explain the lack of IL-4 expression. Therefore, it is possible environmental lack of oxygen influences the

expression of various STAT proteins, and this may result in the observed drop in pro-inflammatory cytokine production over time. The effect hypoxia has on STAT transcription factors' expression, phosphorylation and function in memory T lymphocytes would be a useful avenue for future research.

GATA-3 expression, however, increased over time in constant 1% oxygen (as indicated by both intracellular staining and the limited microarray) suggesting a phenotypic switch may occur albeit delayed. T-bet and GATA-3 mRNA expression has previously shown to be reduced in CD4+ T lymphocytes after 18 hours stimulation compared to 21% oxygen [237]. It was also interesting to observe a slight trend in increased GATA-3 expression after treatment with H_2O_2 at 8.5% oxygen (figure 5.8). It would be of benefit in the future to try to extend these observations over time to see whether this effect was amplified. GATA-3 is important in the maintenance of the CD4+ single positive phenotype in thymocytes [351], and GATA-3 expression has been observed in Th1 cells [99]. It therefore may be expressed at a basal level in CD4+ T lymphocytes, and thus the increase in its protein and mRNA levels may be due to a lack of regulatory proteins that normally suppress its expression in non-Th2 cells. As T-bet itself antagonises the activity of GATA-3 [110], the lower level of T-bet expression observed over time in constant 1% oxygen may have resulted in the upregulation of GATA-3 expression. Alternatively, a delayed promotion of anti-inflammatory mechanisms may occur in constant 1% oxygen, resulting in a depression in Th1-associated cytokines and markers, and instead an increase in GATA-3 expression.

Despite the increase in GATA-3 expression over time, IL-4 production did not appear to increase compared to other oxygen levels after 48 hours stimulation. As IL-4 could

not be detected at 24 hours, future investigations of IL-4 mRNA expression may be of benefit to fully elucidate what is occurring with this cytokine in hypoxia. As GATA-3 does not bind directly to the *il4* promoter [47, 117, 387], it is possible that other factors, such as c-MAF and STAT5, are more important in the regulation of this Th2 associated cytokine in low oxygen [47, 111, 112]. IL-5 levels were also somewhat diminished in supernatants from constant 1% oxygen stimulations (but not as diminished as at 3% oxygen). GATA-3 does bind this cytokine's gene locus directly, and therefore some questions do arise as to whether its expression determines the expression of downstream cytokines. As GATA-3 expression was initially depressed in constant 1% oxygen, it is possible that the upregulation of GATA-3 at 48 hours had not yet resulted in increased IL-4 and IL-5 expression. Intracellular cytokine staining of IL-5, and perhaps IL-13 (another Th2-associated cytokine), at various time points may provide more clarity with respect to this.

These results suggest that in hypoxic inflammatory settings CD4+ memory T lymphocytes provide a rapid Th1 response, but that this response does not last as it may do in higher oxygen levels, even when compared to a modest increase to 3% oxygen. In tissues where an inflammatory response is required due to a new pathogenic insult, oxygen levels may initially be quite reasonable and physiologically healthier. Therefore the maintenance of cytokine production over time at higher oxygen levels allows for the full initiation of an immune response. Later in the inflammatory process, low environmental oxygen may have taken precedence due to the infiltration of inflammatory cells. The culling of cytokine production in this situation may alleviate further cell infiltration and detrimental inflammatory processes, and therefore may be a regulatory response to inhibit ongoing pro-inflammatory

processes that can cause tissue damage. The results presented in this thesis are also suggestive as to why there is a lack of T lymphocyte-associated cytokines in the rheumatoid joint [175-177, 180].

6.4.3 Cytokine production at 3% is both pro-inflammatory and maintained overtime

In contrast to stimulation at constant 1% oxygen, 3% oxygen cytokine production appeared to be more sustained over time and an increase in the number of double positive cells was observed. Both IFNγ and IL-17A expression increased over time in response to stimulation at 3% oxygen, becoming the most prominent cytokines after 48 hours stimulation at this oxygen level. An increase in T-bet expression was observed alongside this, suggesting that pro-inflammatory processes may be more promoted at this oxygen level. Furthermore, a greater percentage of cells co-expressed T-bet with IFNγ at 3% oxygen, suggesting the T-bet expression observed was functional. As previously mentioned, T-bet antagonises GATA-3 expression and function [110], and therefore the increase in T-bet expression observed at 3% may functionally suppress Th2-associated markers. However, intracellular cytokine staining also revealed the highest IL-4 cytokine production at 3% oxygen, which does question this assumption. As GATA-3 does not directly bind the *il4* locus [112, 117, 352, 387], the increase in IL-4 may be because 3% oxygen provides a better environment for general cytokine production than more hypoxic oxygen levels.

The data obtained from stimulations at 3% oxygen is interesting when considering physiological environments where this oxygen level will be relevant. An environmental oxygen level of 3% is regularly defined as hypoxic, partly because at

around this oxygen level many cell types will stabilise the expression of HIF-α subunits [230], therefore suggesting that the cells deem this environmental oxygen level as deficient. Despite this, several physiologically normal tissues that are regularly frequented by lymphocytes contain oxygen levels of around 3% oxygen, including the lymph nodes [239]. Additionally, 3% oxygen is the average oxygen level of the rheumatoid joint [199-201], and therefore this environmental oxygen level may be an important influence in not only healthy immune responses, but also aberrant ones. The sustained cytokine production in 3% oxygen observed in this study is somewhat contradictory to what would be expected of CD4+ T lymphocytes in the rheumatoid joint, as CD4+ T lymphocyte cytokine production is normally depressed in this environment [175-177, 180]. However, maximal cytokine production at 3% oxygen in the lymph node may be of benefit.

It is possible that gradients of oxygen may exist within the rheumatoid joint, as observed in the spleen. T lymphocytes found in more avascular synovial tissue may experience more severe hypoxia and diminish cytokine production over time in these niches. In contrast, cells experiencing slightly higher oxygen perfused tissue may acquire the ability to express other cytokines, and maintain cytokine production better. Tertiary lymphoid structures may also provide islands of different oxygen levels.

6.4.4 Increased plasticity at 3% oxygen, but reduced IL-17 production at constant 1% oxygen

In 3% oxygen, CD4+ memory T lymphocytes acquired the ability to express more than one cytokine at a greater rate than lower oxygen levels, suggesting that this oxygen level both supports cytokine production more, and may promote plasticity

between the different T helper subsets. An increase in the number of IFNγ+IL-4+, IFNγ+IL-17A+ and IFNγ+IL-10+ cells was observed at 3% oxygen. This could be due to Th1 cells in the population acquiring alternative phenotypes, or could be due to Th2 and Th17 cells upregulating Th1-associated cytokines.

When Th17 cells were first described they were thought to be distinct in nature but increasing plasticity has been highlighted in recent years. It is now thought that they are less terminally differentiated than Th1 or Th2 cells, and there is a growing amount of evidence suggesting a Th17 to Th1 switch occurs as part of the natural process of Th17 differentiation *in vivo* [93, 95, 128, 144]. Therefore, the relatively low levels of IL-17A production observed at all of the oxygen levels investigated in this study may be due to the original cell population chosen for investigation. Any Th17 cells isolated and subsequently used in the experiments may have initiated the next step in their differentiation program, thus switching to a more Th1-like phenotype after stimulation.

With respect to physiological environments where 3% oxygen is thought to be prevalent; double positive IFNγ+IL-17+ cells have been observed in the inflamed joint [145, 358]. The increase in T-bet expression observed after 48 hours stimulation at 3% oxygen may have maintained IFNγ production and potentially contributed to the plasticity of Th17 cells to a Th1-like phenotype, but would need further investigation as not much T-bet was found associated with IL-17 production at this time point. T-bet expression is observed in Th17 cells that have switched to a more Th1-like phenotype [147]. In inflammatory murine models such as EAE, diabetes and colitis, Th17 cells that co-express Th1-markers, or undergo a switch to a more Th1-like phenotype, are more pathological [93, 128, 147, 150]. Therefore their upregulation in

both the RA joint, and general 3% oxygen environments, could contribute to inflammatory disease.

A lack of IL-17A production both singularly and alongside less detection of both IFNγ and T-bet at constant 1% oxygen suggests that Th17 memory populations are not supported in low oxygen levels, whereas at 3% oxygen this phenotype may be more promoted. A small, non-significant drop in CCR6 was observed in constant 1% oxygen, suggesting that perhaps the original Th17 population had not expanded as much at this oxygen level compared to other oxygen levels. This was not expected as hypoxia promotes naïve T lymphocyte Th17 differentiation through the stabilisation of HIF-1α [243, 277]. The drop in IL-17A production at constant 1% oxygen may be a result of increased terminal differentiation (and therefore more of a Th1-like phenotype) of the Th17 CD4+ effector memory cells present, or may be a reflection of the depression in pro-inflammatory cytokine production observed overtime. Additionally, it could suggest that Th17 cells present in the memory population do not respond to stimulation in low oxygen levels.

However, a mild increase in IL-17 production has been observed at the still relatively-hypoxic 3% oxygen. This was potentially due to an increasing number of IFNγ+IL-17+ cells, as already discussed. A reduction in IL-17A production has previously been observed in naïve T lymphocytes under Th17 polarising conditions in a model of 1% oxygen reperfusion injury, whereas 3% and 5% oxygen conditions were optimum for IL-17 production [236], supporting the results observed in this thesis. As HIF-1α is thought to be stabilised in 3% oxygen [230], it is possible that it was able to induce IL-17A production at this oxygen level. HIF-1α inhibitors have been previously observed to limit Th17 persistance in an *in vivo* model [120], and therefore it is

possible hypoxia does have an effect on Th17 cells. Additional factors in constant 1% oxygen may have negated the effect of HIF-1α on IL-17A expression, thus generating the contrast between these two subtlety different hypoxic oxygen levels. However, as ROS have previously been found to upregulate IL-17 [300], the oxidative environment in hypoxia may not contribute to the depression in IL-17 production, although initial experiments in this thesis (figure 5.7) did suggest that redox may depress IL-17A production by CD4+ memory T lymphocytes. IL-17A has also been recently implicated in pathology of several autoimmune diseases including RA, which - as discussed - is thought to have oxygen levels of around 3% oxygen [130, 131].

6.4.5 Reperfusion injury at 1% oxygen

An increase in the number of cytokine double positive IFNγ+IL-17A and IFNγ+IL-10+ cells was also observed in reperfusion treatments compared to constant 1% oxygen, again supporting the notion that cytokine production is more supported over time when oxygen is re-introduced into a system. This occurred despite the cells again being exposed to 1% oxygen between 24 and 48 hours post-stimulation, suggesting the first 24 hours of increased oxygen exposure post-stimulation influenced later cytokine production despite a concurrent drop in oxygen.

In spite of the slight increase in cytokine production compared to constant 1% oxygen, reperfusion injury did induce a reduction in expression of markers associated with the different T helper cell subsets investigated. T-bet, GATA-3 and Foxp3 expression were reduced in reperfusion injury environments, suggesting that transcription factor expression is not sustained when oxygen levels fluctuate. This observation is also interesting as it suggests that later cytokine production is not

necessarily associated with transcription factor expression, as at constant 1% oxygen. Despite maintaining a higher level of transcription factor expression, cytokine production dropped over time in comparison. Physiologically, these results suggest reperfusion injury could have far-reaching effects. For example, cytokine production may be better supported when oxygen is re-introduced into an ischemic environment. Reperfusion injury is associated with the activation of local inflammatory cells and the upregulation of cytokine production [211-214]. It has previously been found that endothelial cells' activation in response to reperfusion injury induce cytokine production from inflammatory cells, but the observed increase that a burst of oxygen into a previously hypoxic site can result in better T lymphocyte cytokine production suggests that the oxygen environment itself may influence immune cell behaviour in a previously ischemic tissue.

6.5 General conclusions

In this thesis, the effect of different environmental oxygen levels on CD4+ memory T lymphocytes was assessed. Differences were found, such as in increased proliferation at 8.5% oxygen, and a more pro-inflammatory phenotype at lower oxygen levels. Therefore, it is possible that environmental oxygen levels have some influence over the behaviour and function of T lymphocytes physiologically. Recently it has been postulated that the strict definitions of different T helper subsets may be physiologically incorrect, and that tissue-specific instructions actually regulate the effector population of effector CD4+ T lymphocytes that are generated [361]. It was suggested that Th1 or Th17 responses are only used when absolutely necessary, but that tissues generally instructed more gentle responses – such as a Th2 response -

to initially deal with low level antigenic exposure. It was also suggested that pro-inflammatory mechanisms would be switched off rapidly to limit the tissue damage that could occur, and listed hypoxia as a limiter of pro-inflammatory immune cells [361]. The data presented in this thesis certainly agrees with this idea, as a Th2 response was observed in higher oxygen levels, but a more pro-inflammatory response was observed when oxygen was more deficient, but was also switched off more rapidly at 1% oxygen. It is perhaps possible that hypoxia acts as the 'help' signal that pushes an immune response into more drastic action (figure 6.1) but also acts to limit it. At 8.5% oxygen, proliferation was increased compared to the other oxygen levels, and this was associated with a potential increase in proximal T lymphocyte signalling. The lack of this, in contrast, at lower oxygen levels, may reveal one way in which these more dangerous inflammatory responses are regulated, as cytokine production was cut-off more rapidly at 1% oxygen, and proliferation limited (figure 6.1). Therefore, environmental oxygen levels are able to influence CD4+ memory T lymphocyte responses to stimulation, and need to be considered, in light of the disease or organ model being investigated, for *in vitro* work. Oxygen levels of 21% appear to be inappropriate for the study of physiological responses. Certain aspects of the hypoxic environment, such as oxidative stress, may play an important role in determining CD4+ memory T lymphocyte behaviour.

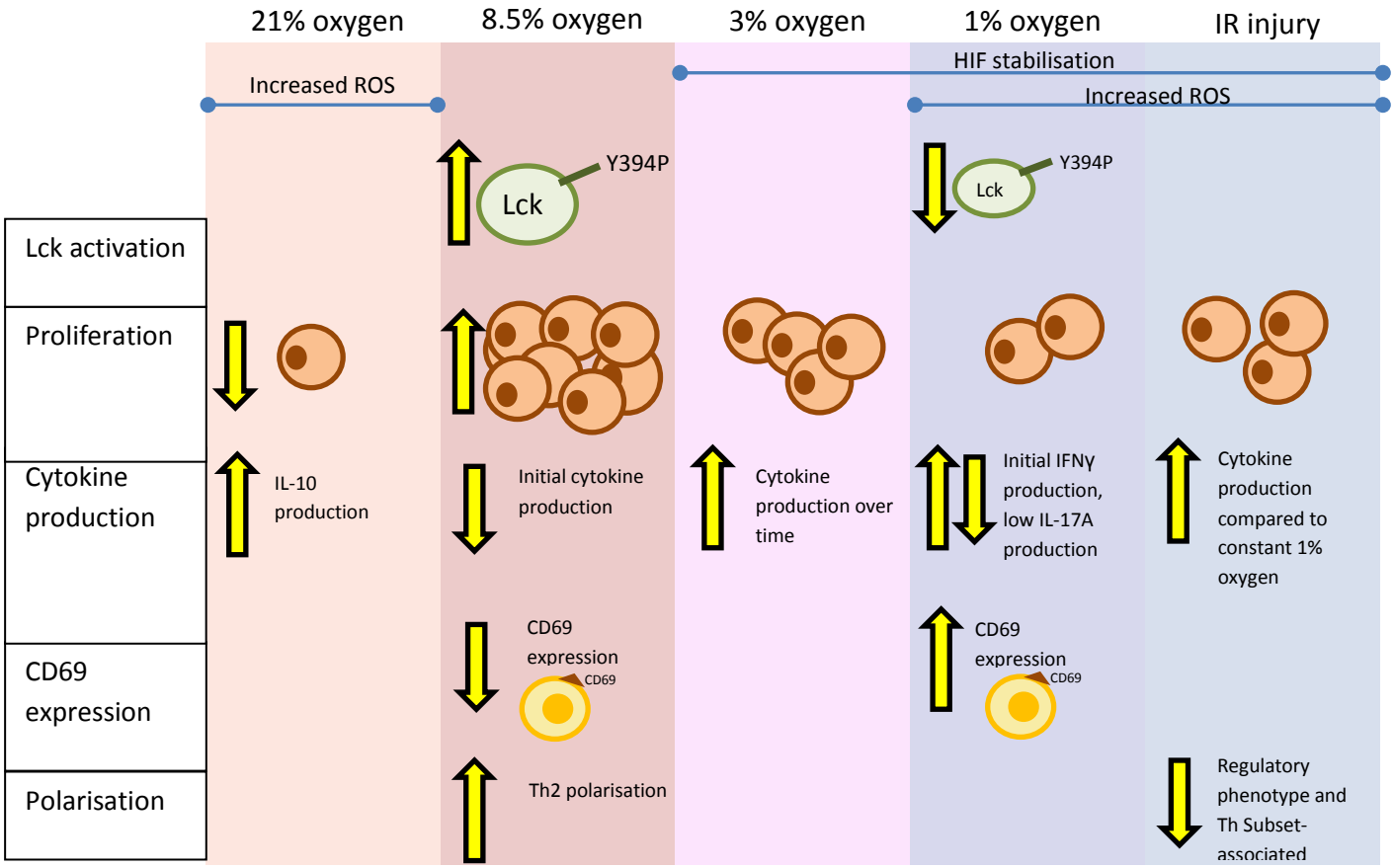

Figure 6.1. A summary of CD4+ memory T lymphocyte responses to different environment oxygen levels*. The five oxygen treatments chosen for assessment in this thesis are shown with the various aspects, such as reactive oxygen species (ROS) production and hypoxia-inducible factor-1α (HIF-1α) stabilisation, shown at the relevant oxygen levels. The main results are highlighted in this figure, such as the variation in Lck tyrosine 394 phosphorylation (Y394P), proliferation, cytokine production, CD69 expression and polarisation at different oxygen levels.*

7. Limitations and Future work

Limitations to the data presented within this thesis

Firstly, this data is limited by the small number of cytokines investigated. Only 3 cytokines deemed as ‘Th1/Th17-like’; 2 cytokines deemed as ‘Th2-like’; and one associated with both Th2 and regulatory T lymphocytes were investigated. Future work, perhaps using an array platform, would enhance understanding of cytokine balance at different oxygen levels. Additionally, we only assessed cytokine secretion at one time point (45 hours). The patterns of cytokine production can vary depending on the cytokine being investigated. Examining the balance of cytokines more shortly after stimulation may enable further understanding into the cytokine response of CD4+ T lymphocytes at various oxygen levels. For example, examining cytokine mRNA levels 6 hours post stimulation may provide initial clues as to the preferred balance of cytokines in different oxygen levels. Assessing cytokine production beyond 45 hours would also be of benefit. CD4+ T lymphocyte cytokine production can extend over several days [138] and as CD4+ T lymphocytes are believed to inhabit the RA joint environment for longer than 2 days, their cytokine production will continue to contribute to the overall cytokine population present in the inflamed joint. The effect of different oxygen levels of maintaining cytokine production would also be of interest.

Secondly, the cells used experimentally were frozen before assessment. Supernatant measurements of IFNγ (and IL-5 to some extent) appeared to be less affected by freezing than the other cytokines investigated, and intracellular staining of IFNγ was also less affected by freezing than the IL-4 and IL-10 after 24 hours stimulation.

Therefore using cells that had been frozen may have skewed the response to appear more pro-inflammatory than it was.

Future work

Making the environment of the cell more physiological and improving culture conditions.

Future work using fresh cells would be of benefit to confirm the patterns observed and would be of more physiological relevance. Furthermore, more physiological stimulation methods, such as using peptide-loaded APCs, may give more relevant results regarding inflammation.

Furthermore, it has been previously reported that a longer stimulation period results in a more delayed re-expression of the TCR:CD3 complex [322] and in this study cells were not removed from stimulation plates. Future investigations where cells are removed from stimulation plates after a shorter amount of time may provide more clarity as to the effect of different oxygen levels on TCR:CD3 expression.Studies where cell medium was replenished mid-experiment may help to determine whether this was the reason for decreased cell viability at physiologically healthy oxygen levels.

Longitudinal studies

In the chronic inflammatory environment, cells may be part of the tissue for much longer than the 48-72 hours investigated in this thesis. Therefore, cytokine production and CD69 expression over a longer duration would be of benefit.

Studies probing the activation status of Lck over time would be beneficial in determining whether fluctuations in activation status occur, as this may lead to better understanding of acute and more chronic responses to hypoxia.

Furthermore, due to its nature as a ‘late’ activation marker, it is possible HLA-DR expression needs to be assessed over a longer timescale.

It is possible that the upregulation of GATA3 at 48 hours had not yet resulted in increased IL-4 and IL-5 expression. Intracellular cytokine staining of IL-5, and perhaps IL-13 (another Th2-associated cytokine), at various time points may provide more clarity with respect to this.

More detailed studies of subsets

A better investigation of RORγ may give more details about the effect of different oxygen levels on Th17 cells. Furthermore, the analysis of STAT protein function at different oxygen levels would also allow for better understanding of the different signals involved. MAF, a possible transcriptional regulator of IL-10 [362], also needs to be assessed.

The relationship of T-bet with IL-17 and IL-10 production would also be of benefit for future investigation.

Furthermore, the different iTreg populations could be better examined in future, including, perhaps, a panel investigating IL-10 and TGFβ production alongside surface markers associated with Tregs and Foxp3 expression. Suppressive assays would also be very interesting to perform at oxygen levels where an increase in regulatory phenotype was observed.

Distinct cell populations

Prior separation of central and effector memory T lymphocytes before exposure to antigenic stimulation at different oxygen levels may allow for better delineation of what occurs in the tissue compared to what occurs in the lymph node. Additionally, it would allow for better understanding of proliferative and cytokine responses at different oxygen levels.

It would also be interesting to investigate naïve T lymphocytes over a long time frame in future, including assessment of the effect of re-stimulating at various oxygen levels.

Better investigation of mechanisms

Alternative hypoxia mimetics, such as cobalt chloride, could be utilised in the future to further assess the effect of HIF-1α on Lck activation status. This may negate the anti-oxidant effect of DFX. Furthermore, as NAC is also known to inhibit NFκB [367] it would need to be further confirmed that the redox environment was involved in the effect of low environmental oxygen on CD4+ memory T lymphocytes.

Increasing cell repeats.

The increase in Th2-like phenotype was further suggested in experiments performed on naïve T lymphocytes, where stimulation at 8.5% resulted in the highest CCR4 MFI values, even in Th1 and Th0-polarising conditions. However, this work was limited by the lack of experimental repeats, and warrants future confirmation. Experiments

looking at the effects of DFX and H_2O_2 were also limited in cell number, and need to be repeated in future.

8. Appendices

Panel design of pro-inflammatory and anti-inflammatory markers

Antigen	Colour/ filter	Surface/ intracellular
CXCR3 (CD183)	APC/ FL8	surface
CCR6 (CD196)	PECy7/ FL5	surface
IFNγ	Pacific Blue/ Fl6	intracellular
IL-17A	PE/ FL2	intracellular
T-bet	PerCP/ Cy5.5// FL4	intracellular
RORγt	FITC/ FL1	intracellular

Table 8.1 Details of the pro-inflammatory staining panel for flow cytometry

Antigen	Colour/ filter	Surface/ intracellular
CCR4 (CD194)	PerCP/ Cy5.5// FL4	surface
CTLA-4 (CD152)	PE/ FL2	surface
IL-4	FITC/ FL1	intracellular
IL-10	PECy7/ FL5	intracellular
GATA-3	Alexa Fluor® 647/ FL8	intracellular
Foxp3	eFluor® 450/ FL6	intracellular

Table 8.2 Details of the anti-inflammatory staining panel for flow cytometry

Details of the Human Th17 for autoimmunity and Inflammation microarray

Well	Symbol	Description
A1	CACYBP	Calcyclin binding protein
A2	CCL1	Chemokine (C-C motif) ligand 1
A3	CCL2	Chemokine (C-C motif) ligand 2
A4	CCL20	Chemokine (C-C motif) ligand 20
A5	CCL22	Chemokine (C-C motif) ligand 22
A6	CCL7	Chemokine (C-C motif) ligand 7
A7	CD247	CD247 molecule
A8	CD28	CD28 molecule
A9	CD34	CD34 molecule
A10	CD3D	CD3d molecule, delta (CD3-TCR complex)
A11	CD3E	CD3e molecule, epsilon (CD3-TCR complex)
A12	CD3G	CD3g molecule, gamma (CD3-TCR complex)
B1	CD4	CD4 molecule
B2	CD40LG	CD40 ligand
B3	CD8A	CD8a molecule
B4	CEBPB	CCAAT/enhancer binding protein (C/EBP), beta
B5	CLEC7A	C-type lectin domain family 7, member A
B6	CSF2	Colony stimulating factor 2 (granulocyte-macrophage)

B7	CSF3	Colony stimulating factor 3 (granulocyte)
B8	CX3CL1	Chemokine (C-X3-C motif) ligand 1
B9	CXCL1	Chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)
B10	CXCL12	Chemokine (C-X-C motif) ligand 12
B11	CXCL2	Chemokine (C-X-C motif) ligand 2
B12	CXCL5	Chemokine (C-X-C motif) ligand 5
C1	CXCL6	Chemokine (C-X-C motif) ligand 6 (granulocyte chemotactic protein 2)
C2	S1PR1	Sphingosine-1-phosphate receptor 1
C3	FOXP3	Forkhead box P3
C4	GATA3	GATA binding protein 3
C5	ICAM1	Intercellular adhesion molecule 1
C6	ICOS	Inducible T-cell co-stimulator
C7	IFNG	Interferon, gamma
C8	IL10	Interleukin 10
C9	IL12B	Interleukin 12B (natural killer cell stimulatory factor 2, cytotoxic lymphocyte maturation factor 2, p40)
C10	IL12RB1	Interleukin 12 receptor, beta 1
C11	IL12RB2	Interleukin 12 receptor, beta 2

C12	IL13	Interleukin 13
D1	IL15	Interleukin 15
D2	IL17A	Interleukin 17A
D3	IL17C	Interleukin 17C
D4	IL17D	Interleukin 17D
D5	IL17F	Interleukin 17F
D6	IL17RB	Interleukin 17 receptor B
D7	IL17RC	Interleukin 17 receptor C
D8	IL17RD	Interleukin 17 receptor D
D9	IL17RE	Interleukin 17 receptor E
D10	IL18	Interleukin 18 (interferon-gamma-inducing factor)
D11	IL1B	Interleukin 1, beta
D12	IL2	Interleukin 2
E1	IL21	Interleukin 21
E2	IL22	Interleukin 22
E3	IL23A	Interleukin 23, alpha subunit p19
E4	IL23R	Interleukin 23 receptor
E5	IL25	Interleukin 25
E6	IL27	Interleukin 27
E7	IL3	Interleukin 3 (colony-stimulating factor, multiple)
E8	IL4	Interleukin 4
E9	IL5	Interleukin 5 (colony-stimulating factor, eosinophil)
E10	IL6	Interleukin 6 (interferon, beta 2)
E11	IL6R	Interleukin 6 receptor

E12	IL7R	Interleukin 7 receptor
F1	IL8	Interleukin 8
F2	ISG20	Interferon stimulated exonuclease gene 20kDa
F3	JAK1	Janus kinase 1
F4	JAK2	Janus kinase 2
F5	MMP13	Matrix metallopeptidase 13 (collagenase 3)
F6	MMP3	Matrix metallopeptidase 3 (stromelysin 1, progelatinase)
F7	MMP9	Matrix metallopeptidase 9 (gelatinase B, 92kDa gelatinase, 92kDa type IV collagenase)
F8	NFATC2	Nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 2
F9	NFKB1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1
F10	RORC	RAR-related orphan receptor C
F11	SOCS1	Suppressor of cytokine signaling 1
F12	SOCS3	Suppressor of cytokine signaling 3
G1	STAT3	Signal transducer and activator of transcription 3 (acute-phase response factor)
G2	STAT4	Signal transducer and activator of transcription 4
G3	STAT5A	Signal transducer and activator of transcription 5A
G4	STAT6	Signal transducer and

		activator of transcription 6, interleukin-4 induced
G5	SYK	Spleen tyrosine kinase
G6	TBX21	T-box 21
G7	TGFB1	Transforming growth factor, beta 1
G8	TIRAP	Toll-interleukin 1 receptor (TIR) domain containing adaptor protein
G9	TLR4	Toll-like receptor 4
G10	TNF	Tumor necrosis factor
G11	TRAF6	TNF receptor-associated factor 6
G12	YY1	YY1 transcription factor
H1	B2M	Beta-2-microglobulin
H2	HPRT1	Hypoxanthine phosphoribosyltransferase 1
H3	RPL13A	Ribosomal protein L13a
H4	GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
H5	ACTB	Actin, beta
H6	HGDC	Human Genomic DNA Contamination
H7	RTC	Reverse Transcription Control
H8	RTC	Reverse Transcription Control
H9	RTC	Reverse Transcription Control
H10	PPC	Positive PCR Control
H11	PPC	Positive PCR Control

H12	PPC	Positive PCR Control

Table 8.3. Details of the Human Th17 for autoimmunity and inflammation microarray used (Qiagen Cat. No. 330231). Well position in the plate is listed alongside the symbol for the gene being investigated and full-name descriptions.

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