Co-Expression of TLR2 and CCR5 on Human T Cells

Zoe Richmond

MSc by Research

University of York
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Abstract

Evidence has emerged for a subpopulation of human T cells that express TLR2, a pattern recognition receptor ordinarily found on innate immune cells. A cross-modulation pathway between TLR2 and CCR5, a chemokine receptor involved in cell migration, has been reported in human monocytes by the Signoret lab. CCR5 is expressed by several subsets of T cells and notably takes part in the pathogenesis of cancer and HIV-1 infection. Whilst a TLR2/CCR5 double-expressing T cell population has been previously reported, its function is unclear, and the possibility of a TLR2/CCR5 communication pathway is yet to be explored. Expansion of this population could allow functional characterisation, and elucidate the conditions favouring generation of these cells.

Frozen monocyte-depleted human PBMCs were expanded using 9-day PHA/IL2 stimulation. $TLR2^+$ T cells were identified by flow cytometry, and were characterised as a mixed population of $TLR2^+$ $CD25^+$ cells that can also express CCR5, CD4, CD8, CD45RO and FoxP3. This subpopulation was detected after three days of PHA stimulation, but could not be expanded in presence of IL2 and disappeared from culture by day 9, suggesting a transient cell phenotype. These experiments were repeated with a cell preparation from freshly isolated PBMCs; the same subpopulation could be identified, but the results suggest that the majority of $TLR2^+CD25^+$ cells are also $CD45RO^+$ and $CCR5^+$. Following a literature review, alternative stimulation conditions thought to favour TLR2 expression were tested. As with PHA/IL2, no significant expansion of the $TLR2^+$ $CCR5^+$ subpopulation was observed. A downmodulation experiment was carried out assessing the impact of TLR2 and CCR5 specific ligands on the cell surface expression of their receptors. TLR2 ligand stimulation did not affect CCR5 cell surface levels, indicating that receptor regulation on these cells may differ from that of monocytes. However, further experiments should be carried out before conclusions are drawn.

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Declaration

I declare that this thesis is a presentation of original work and I am the sole author. This work has not previously been presented for an award at this, or any other, University. All sources are acknowledged as References.

1. Introduction

1.1 The immune system: an overview

All vertebrates possess an immune system that allows the recognition and elimination of invading pathogens, microorganisms responsible for disease.[1] This immune system consists of specialist circulating blood cells (leukocytes), immune tissues and organs. Leukocytes are actively involved in the response to infection, while immune tissues (e.g. bone marrow) and organs (e.g. thymus) are primarily sites of leukocyte production, storage, maturation and activation.[2]

1.1.1 Leukocytes: the specialized cells of the immune system

Leukocytes are a diverse group of cells that take part in the inflammatory response, a process aiming to fight infection and prevent and/or repair tissue damage caused by injury or insult to the body.[3] Subsets of leukocytes play different roles in the inflammatory response; some are responsible for recognising and destroying pathogens, others repair tissue damage, and some play a regulatory or modulatory role.[4] Additionally, leukocytes secrete a wide range of molecules that target pathogens or serve communication-related functions. These molecules include antimicrobial peptides, which have direct pathogen-killing effects, as well as cytokines and chemokines, the chemical messengers responsible for leukocyte recruitment, activation and regulation. Cytokines and chemokines exert their effects by binding to specific cell-surface receptors, initiating intracellular signalling pathways that dictate cell behaviour.[5,6] This mechanism allows communication, regulating the different stages of the overall immune response.[7] Cells can be grouped according to the branch of the immune system that they belong to; either the innate (non-specific) immune system or the adaptive (antigen-specific) immune system. Leukocytes are then further divided into smaller subsets within the two groups based upon their function and specific markers they express. Innate leukocytes include

neutrophils, monocytes, macrophages and dendritic cells while adaptive leukocytes consist of B and T cells; lymphocytes.[2]

In broad terms, the innate and adaptive immune responses represent the non-specific initial response to infection/tissue damage, and then the pathogen-specific response that occurs several days afterwards. However, there is considerable overlap, and communication between the cells involved in the two different responses is required for effective pathogen clearance.[8] (see Figure 1 for an overview of the interactions between the innate and adaptive immune response)[9]

1.1.2 The innate immune response

The innate immune system provides the initial inflammatory response to infection or tissue damage. It allows a rapid response to occur to pathogens without the need for recognition of their specific type and without having encountered them before. In the event of infection, innate immune cells are able to quickly identify foreign cells by distinguishing between self and non-self.[10] This is achieved by the recognition of molecules that are produced by or expressed on the surface of pathogens, known as pathogen-associated molecular patterns (PAMPs). PAMPs are a group of small, highly conserved molecules expressed by pathogens but not mammalian cells. They are often essential for the survival of a pathogen and can also act as virulence factors, allowing effective colonisation of a host organism.[11] A notable example is lipotechoic acid (LTA), a molecule found on the surface of Gram-positive bacteria, that facilitates adhesion between host and bacterial cells.[12] As PAMPs are common to large groups of pathogens and are often required for cell survival, they allow innate immune cells to successfully distinguish between host and pathogen based on a relatively small selection of molecules.

In addition to foreign cells, damaged or dying cells are also recognised and targeted for destruction. Innate immune cells are sensitive to the presence of molecules released during tissue damage or cell death: damage-associated molecular patterns (DAMPs). Recognition of PAMPs/DAMPs results in a rapid, antigen-independent response, causing the clearance of dead and dying cells as well as pathogens.[13] This can be achieved

through phagocytosis, the process whereby leukocytes engulf and destroy unwanted material, ranging from cell fragments to entire pathogens. This process is enhanced by complement, a complex system whereby multiple plasma proteins in the blood assemble transmembrane channels (membrane attack complexes or MACs) on the surface of damaged or pathogenic cells, compromising their cell membrane.[14] As part of this cascade, cells can also be coated in complement proteins in a process known as opsonisation, marking them for destruction by phagocytes.[15]

In order to achieve these effects, innate immune cells utilize a variety of intracellular signalling pathways. Recognition of DAMPs and PAMPs is dependent on pattern recognition receptors (PRRs).[10] PRRs include the Toll-like receptor group (TLRs), a class of highly-conserved, single spanning transmembrane receptors present on the surface of certain leukocytes including neutrophils, macrophages, dendritic cells and monocytes, all of which are phagocytes.[16,17] Some TLRs are also found on the membrane of the early endosome, a compartment within phagocytes that holds ingested microbial components.[18] Binding of TLRs to DAMPs/PAMPs leads to activation of innate leukocytes, resulting in increased cytokine and chemokine production and secretion, which recruits both innate and adaptive immune cells into the area.[19]

The innate immune response is advantageous in the early stages of inflammation; it quickly recognizes a broad spectrum of pathogens, and inhibits their spread. However, it is a non-specific response, and relies upon a small group of molecules for pathogen recognition. Pathogens lacking certain PAMPs, or possessing mutated versions of the molecules are able to evade detection. They may also produce PRR inhibitor molecules that block binding of their ligands, preventing pathogen recognition.[20] In these cases, the innate immune response may be ineffective in entirely eradicating an infection. The adaptive immune system can then be employed to provide a pathogen-specific response to the infection. This is achieved through cytokine- or chemokine-mediated recruitment of adaptive immune cells, as well as through antigen presentation. Certain phagocytic cells (e.g. macrophages, dendritic cells) are classed as antigen-presenting cells (APCs). These are cells that possess major histocompatibility complex class II (MHCII) molecules, membrane receptors that display peptides from the surface of pathogens (antigens) to

adaptive immune cells. This allows recognition of a specific pathogen to occur, resulting in the activation of the adaptive immune response. [21] The toll-like receptors also play an active part in this process; for example, dendritic cells must first be activated by the binding of TLR ligands in order to mature into antigen presenting cells.[22]

1.1.3 The adaptive immune response

The adaptive immune response, otherwise known as the specific immune response, is the second phase of inflammation. It allows a specialized response to individual pathogens, targeting them directly via pathogen-specific antigens.[23] This response is mediated by B and T cells, which are collectively known as lymphocytes. They originate in the bone marrow as common lymphoid progenitor cells; B cells complete their maturation in the lymph nodes, whereas T cells mature in the thymus and tonsil. At this stage, the lymphocytes possess microbe-sensing mechanisms with affinity for a single pathogenic antigen. This is achieved through random gene recombination, allowing extensive diversity within B cell receptors (BCR) on the surface of B cells, and T cell receptors (TCR) on the surface of T cells. Inevitably, this process leads to the development of autoreactive cells; lymphocytes specific to the antigens expressed by host cells. To combat this, B and T cells are "tested" for autoreactivity within their respective maturation sites; cells that recognise self-antigen are stimulated to undergo apoptosis (a form of programmed cell death) to ensure they do not target host tissues.[24]

In terms of function, B cells are primarily responsible for antibody production, which allows them to immobilize pathogens as well as highlighting them for destruction by T cells. When the B cell is activated via antigen-binding to the BCR and/or stimulation by a helper T cell, it begins a process known as clonal expansion, rapid division of the B cell to produce large numbers of identical effector cells. These are released into the bloodstream, where they commence antibody production.[25] Antibodies, or immunoglobulins, are glycoprotein molecules with a hypervariable region at one end which is able to bind to antigens, as well as a conserved Fc region, that is able to bind to a certain class of Fc receptor on other immune cells.[26] These antibodies are secreted during

the immune response and bind to pathogens; this may neutralize the microbe by blocking surface receptors, cause agglutination, whereby antibody-coated microbes are attached together and immobilized, or simply mark the pathogen as a target for phagocytosis via stimulation of Fc receptors on phagocytic cells.[2]

T cells are divided into subsets, and play complex roles in the inflammatory response, which will be discussed further below.

A unique feature of the adaptive immune response is its ability to recognize previously encountered pathogens. A small subset of B and T cells specific to a certain pathogen remain in circulation beyond the resolution of an infection, and are able to expand if the individual is re-infected. These cells are memory cells, and their activation results in a rapid response in the event of a second infection by a pathogen.

1.1.4 T cells

T cells are a diverse group of lymphocytes, which are subdivided into many classes relating to their varied functions.[27] They mature in the thymus, a lymphoid organ located beneath the sternum in humans. [28] This is where T cells develop their T cell receptor, an antigen-sensing device that is assembled through rearrangement of the genes that code for its structure, analogous to the B cell receptor in B lymphocytes. The thymus also serves as the site where appropriate cells are selected; after TCR gene rearrangement, they undergo positive selection in the outer layer of the thymus, the thymic cortex, where they are offered a peptide bound to an APC. This ensures that they are indeed functional, and depending on the class of MHC (class I or class II), they are stimulated to express either the CD8 receptor or the CD4 receptor respectively (immature T cells express both). [29,30] This designates their functional role within the adaptive immune system; CD8 cells are classed as cytotoxic or killer T cells, whereas CD4 cells are helper T cells, which are able to recruit CD8 cells during infection. Once their purpose has been set, they can progress to the negative selection process in the thymic medulla. APCs within the medulla present the T cells with self-antigens, deleting cells which are found to be autoreactive, preventing their release into the peripheral tissues. [31]

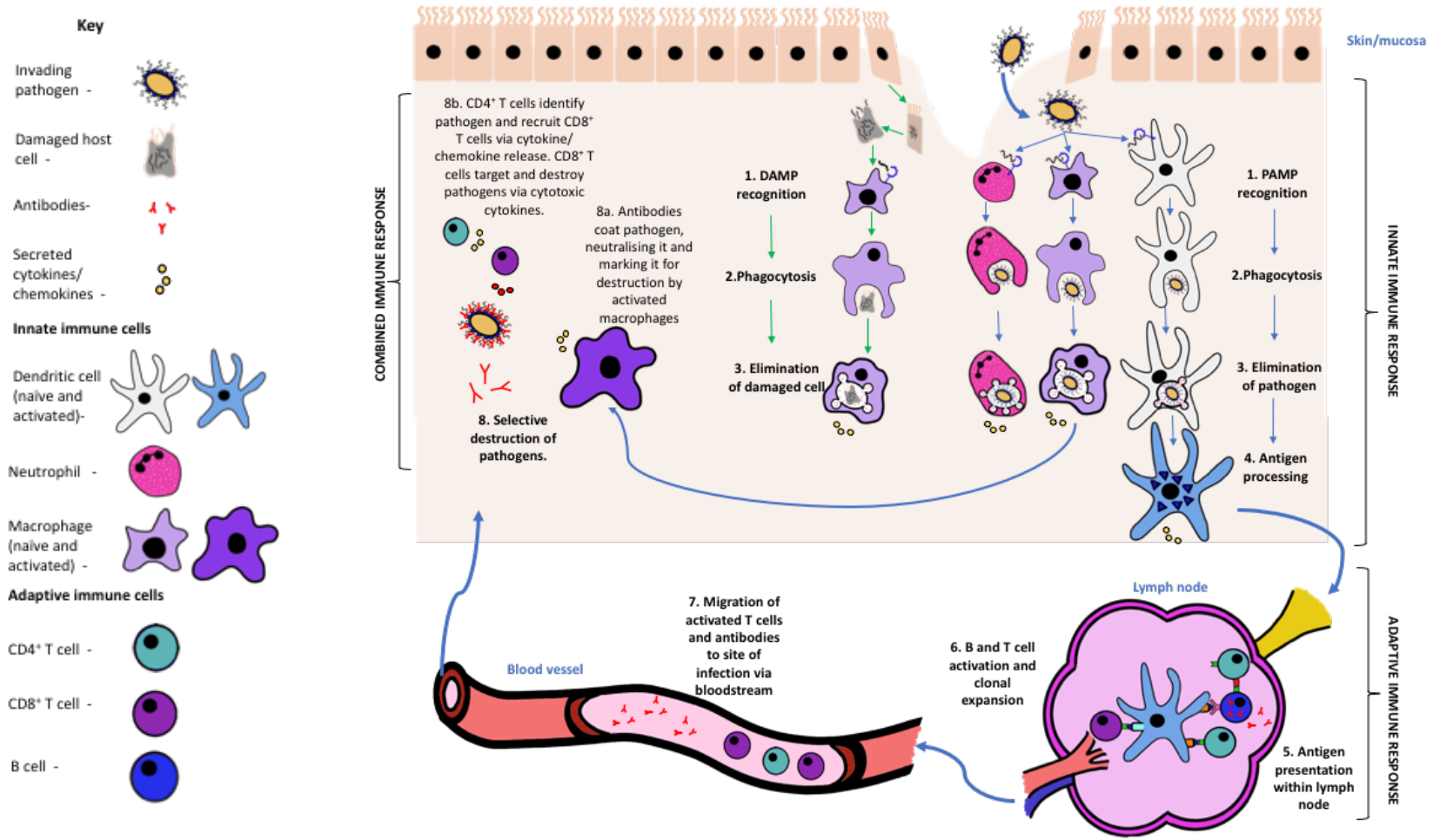

Figure 1: Interactions between the innate and adaptive phase of an immune response. Blue arrows represent steps in response. 1. DAMPs/PAMPs produced by pathogens or damaged cells, respectively, are recognised by antigen-presenting cells via TLRs. 2. These cells are phagocytosed by the APCs. 3. Pathogens are destroyed by the release of cytotoxic substances via lysosomes into the endosomal space. 4. Antigens on the pathogen cell surface are processed for presentation on the surface of dendritic cells. 5. Antigens are presented to B and T cells via MHCI (CD8 T cells) and MHC II (CD4 T cells and B cells) on the surface of the dendritic cells, activating them. 6. B and T cells are activated and expand in number. B cells secrete antibodies. 7. Activated T cells and antibodies are released into the bloodstream, and travel to the site of infection. 8. B cells secrete antibodies specific to the pathogen identified by the APCs, neutralising the pathogen and flagging it for destruction by $CD8^+$ T cells. $CD8^+$ cytotoxic T cells produce cytotoxic cytokines to destroy pathogens. Activated macrophages from step 3 are also recruited to the site of infection by chemokines released by CD4 T cells.

Once maturation is complete, the naïve T cells reside in lymphoid tissue reservoirs such as the lymph nodes, tonsil and spleen. Upon phagocytosis of a foreign pathogen, APCs process pathogenic antigens into smaller peptide fragments that are then bound to MHC I and II. These fragments are then presented to T cells, until a TCR is found that has affinity for the peptide. This activates the T cell, and it undergoes clonal expansion to produce large numbers of specific T cells that are then released into the bloodstream. They are recruited to the area of inflammation by chemokines released by other leukocytes.[32]

Many subsets of CD8 and CD4 T cells exist, each serving a specific function and possessing a signature combination of surface receptors (see Figure 2: T cell subsets and functions).[33] These groups include effector T cells, which are directly involved in the immune response, memory T cells, which provide a record of encountered pathogens, and modulatory T cells such as regulatory T cells (Tregs) that control the action of other T cells as well as other leukocytes.

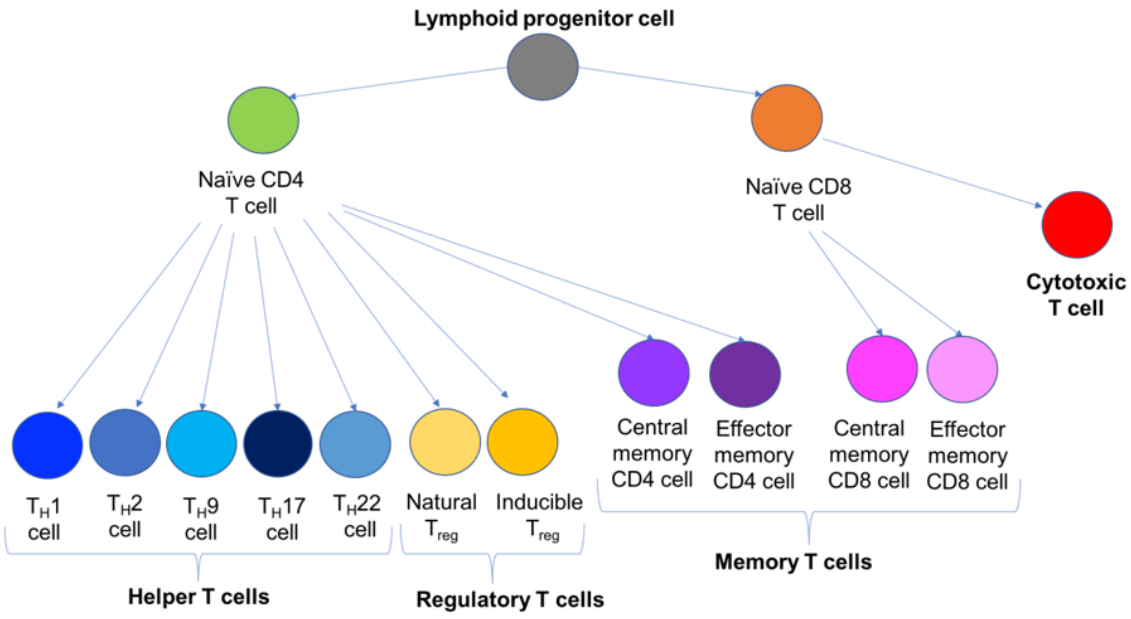

Figure 2: T cell subsets. T cells are derived from the same lymphoid progenitor cells as B cells in the bone marrow. Growth factors determine the terminal differentiation of T cells into their ultimate phenotypes.

1.1.5 Regulatory T cells

Regulatory T cells represent a modulatory subset of the T cell population. They are characterised by their expression of the nuclear transcription factor FoxP3 on human T cells; the majority of Tregs are CD4-expressing (although some subsets of CD8 Tregs are thought to exist); they also possess high surface levels of the activation marker CD25, the alpha-chain of the IL-2 receptor.[34,35] Their regulatory function allows tolerance of non-harmful foreign material and reduce tissue damage caused by overexuberant immune responses via their anti-inflammatory effect. Tregs are also able to recognise autoreactive T cells that have escaped deletion in the thymus, and can destroy aberrant cells via granzyme or perforin secretion, causing apoptosis.[36]

Regulatory T cells respond readily to the presence of IL-2, a pro-inflammatory cytokine produced by other T-cells, meaning that they are activated by the chemokine in areas where large numbers of conventional T cells are present.[37] This serves to prevent excessive numbers of T cells accumulating, and stops any excessive inflammation causing unnecessary tissue damage. Control of inflammation is crucial in situations such as viral lung infection, where inadvertent destruction of the respiratory membranes can have fatal effects.[38]

It also allows tolerance of self-antigens to occur, as well as to non-harmful foreign particles by suppressing inflammatory events.[39] There are many situations where it is not advantageous to eliminate non-harmful bacteria. For example, the microbiome in the gut is important for micronutrient metabolism as well as providing healthy competition to prevent bacteria, such as pathogenic strains of *Esterichia coli*, from forming harmful colonies.[40,41] In addition, other commensal organisms like *Bacteroides fragilis* often asymptomatically infect the GI tract.[42] An absence of Tregs in this circumstance has been found to cause inflammation, leading to the formation of lesions and the onset of colitis.[43]

Depletion or dysfunction of Tregs in human and mouse models leads to the development of inflammatory and autoimmune diseases such as systemic lupus erythematosus (SLE) and psoriasis vulgaris. Conversely, Tregs have been shown to be recruited by tumours in order to help them evade the immune response, with high numbers of Tregs in the tumour microenvironment associated with poor disease prognosis.[44]

1.2 T cells and TLR2

1.2.1 Function of TLR2

TLR2 is a member of the Toll-like receptor family, a series of receptors that serve to recognise PAMPs and initiate a pro-inflammatory response.[45,46] Toll-like receptors are a set of highly conserved pattern recognition receptors, specialised to recognise a wide range of pathogens including bacteria, fungi and viruses. There are ten different classes of TLR in humans, which are present on the cell membrane or the on the endosome, the space created in phagocytic cells when a pathogen is engulfed and contained before its degradation (see Figure 3).

TLR2 is typically found on cells of the innate immune system, such as monocytes and neutrophils, acting as an initial non-specific response to the presence of invading bacteria in the body. This leads to the release of inflammatory cytokines, which are responsible for numerous inflammatory effects, and are able to activate and regulate the function of T cells in the case of infection. These include TNF-α, and members of the interleukin (IL) family, including IL-2.[47,48]

Dysfunction of the TLR2-mediated recruitment pathway has profound effects on immune regulation. In the situation of bacterial presence in the bloodstream (bacteraemia), high concentrations of TLR2 ligands produced by bacteria such as LTA, derived from the Gram-positive bacterium *Staphylococcus aureus*, recruit large numbers of TLR2-expressing leukocytes. This leads to massive systemic inflammation, causing destruction of healthy tissues and potentially lethal sepsis.[49] In the case of antibiotic-resistant strains of bacteria, such as methicillin-resistant *S. aureus* (MRSA) prognosis is extremely poor, and leads to thousands of deaths every year, especially in the developing world where access to healthcare is difficult.[50,51]

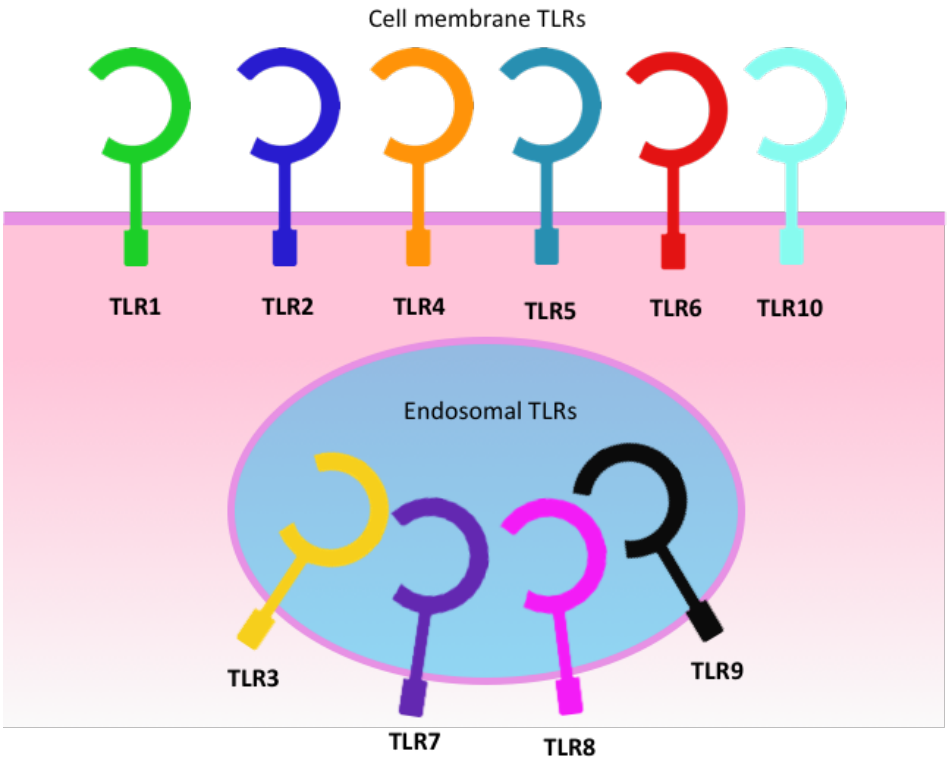

Figure 3: Human Toll-like receptors. Toll-like receptors are typically found on the cell or endosomal membrane of cells of the innate immune system. They serve to recognise pathogens and initiate chemokine signalling that recruits immune cells to sites of infection, or signals for the destruction of internalised pathogens.

1.2.2 Evidence for TLR2-expressing T cells

More recently, evidence has emerged that TLR2 is expressed by subpopulations of T cells, both in humans and mice. Regulatory and memory T cells have been reported to harbour varying levels of TLR2 on their cell surface. The function of TLR2 in this situation is unclear, given that it is primarily associated with the innate immune response.

One piece of evidence for the role of TLR2 on T cells relates to the receptor's endogenous ligands. TLR2 has many endogenous ligands, including DAMPs such as biglycan, human cardiac myosin and the heat shock proteins HSP60 and HSP70. The binding of these DAMPs to TLR2 signals that damage has occurred, recruiting immune cells into the area. This allows cells of both the innate and adaptive immune response to move into the area of inflammation.[52]

TLR2-expressing T cells are also known to play a crucial role in the development of disease. Wang et al observed an increase in the number of murine Th17 T cells that expressed TLR2 during Group A streptococcal infection; Bao et al found that

Pseudomonas aeruginosa-derived N-(3-oxododecanyl)-L-homoserine caused an increase in RNA and surface expression of TLR2 in human T cells.[53,54] Both of these studies discuss the role that TLR2 may play in the development of chronic infection and the presence of biofilms, suggesting that sepsis resulting from overproliferation of T cells in these diseases may be mediated by TLR2. Another role for TLR2 in chronic infection relates to its role in *S. aureus* infection, specifically in more virulent strains, including methicillin-resistant *S. aureus* (MRSA). Chronic inflammation during MRSA-related sepsis is thought to be mediated by the ligation of TLR2 by S. aureus-derived LTA in regulatory and Th17 T cells.[53]

1.2.3 Regulatory T cells and TLR2

Regulatory T cells are one of the subpopulations of T cells that have been reported to express TLR2 in humans and mice. In terms of functional pathways beyond cell recruitment, there is evidence to suggest that TLR2 acts to control expansion and proliferation of regulatory T cells in mice, with TLR2 knockout animals experiencing significantly reduced numbers of Tregs compared to their wild type counterparts.[55] Given that TLR2 is able to induce the release of chemotactic chemokines that recruit innate immune cells in response to DAMPs and PAMPs, it is probable that it serves the same purpose on regulatory T cells. This would result in the accumulation of Tregs in areas of inflammation to allow them to offset damage that may be caused to healthy cells. As Tregs are known to be activated by the release of the pro-inflammatory cytokine IL2, it is likely that signalling via TLR2 serves a similar purpose, allowing recruitment through TLR2-mediated chemokine release.[56] The fact that the addition of TLR2 ligands has been observed to maintain and expand the circulating Treg population adds further weight to this theory. Tregs are vital for the mediation of the inflammatory response, and so mechanisms for recruiting and retaining them at sites of inflammation must exist for Tregs to serve their purpose.

It is also possible that other organisms are able to hijack this pathway. Most successful pathogens have evolved to evade their host's immune response in order to enhance their

own survival. There is evidence to suggest that a range of TLR2 ligands, including bacterial lipoprotein (BLP) and the synthetic TLR2 ligand Pam_3CSK_4, are able to recruit and expand Treg numbers.[55,57] It is postulated that this induces an immunosuppressive effect that is advantageous in bacterial infection and aids pathogen survival. This phenomenon is also seen in *C. albicans* infection in mice, suggesting that TLR2-dependent Treg recruitment could play a significant role in the development of chronic disease.[58]

1.3 CCR5 and TLR2

1.3.1 Role of CCR5

CCR5 is a G-protein coupled chemokine receptor that ordinarily plays an important role in cell migration and activation in inflammation.[59] It possesses seven-transmembrane loops, associated with a G-protein, allowing downstream signalling in response to external stimulus (see Figure 4). However, it is known to act as a co-receptor for HIV-1 entry into cells.[60] This has effects on susceptibility to HIV infection in the population; for example, individuals homozygous for the Delta32 genetic mutation of the gene encoding CCR5 do not express the receptor, and can be exposed to HIV without becoming infected.[61] Additional to this, some CCR5 ligands have been shown to provide anti-HIV action by downmodulation of CCR5 on T cells, another area recently explored by the Signoret group.[62] It is also thought that CCR5 is an important factor in cancer pathogenesis, with evidence that it plays a part in recruiting regulatory T cells into tumours to assist in immune system evasion, notably in colorectal cancer and nasopharyngeal carcinoma.[63,64] CCR5 is known to be expressed on some memory and regulatory T cells; TLR2 expression has also been reported in these subsets.[65]

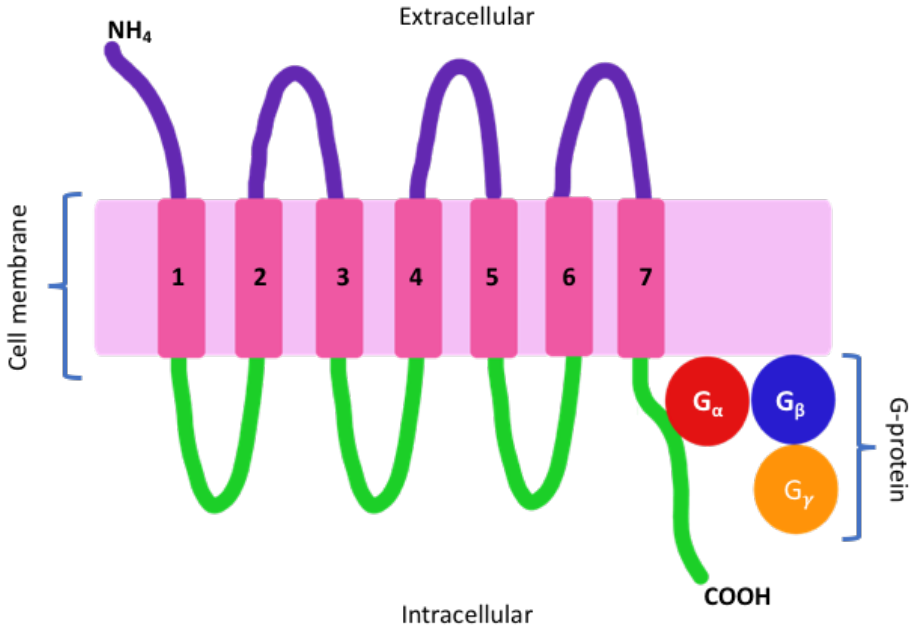

Figure 4: CCR5 structure and location. CCR5 is a G-protein-coupled receptor, located in the cell membrane of both innate and adaptive leukocytes. It consists of an extracellular N-terminal domain and an intracellular C-terminal domain connected by seven transmembrane loops, and associates with a G-protein at the C-terminus.

1.3.2 Possibility of a double-expressing T cell population

It is known that both TLR2 and CCR5 can be expressed by regulatory and memory T cells, meaning that a double-expressing population may well exist. However, this has not been demonstrated, and the role of a double-expressing population remains unclear.

A TLR2 and CCR5-expressing population would possess the ability to be recruited to areas of inflammation, both by the recognition of PAMPs/DAMPs through TLR2, and via CCR5-mediated chemotaxis driven by endogenously-produced chemokines. This would be advantageous in the case of Tregs, drawing them to sites of inflammation where they are needed to mediate damage caused by effector cells. However, it is more likely that the purpose of CCR5 is independent of TLR2 expression.

CCR5 serves as an essential receptor for regulatory T cells; it is known to be needed for recruitment of effector and memory Tregs to areas of inflammation. Additional to this, CCR5 is known to play a key role in the suppressive effect of Tregs; notably, Chang et al found that CCR5-/- murine Tregs were unable to suppress anti-tumour CD8+ T cells in vivo.[66] TLR2, however, is only expressed by a small subset of Tregs, and is associated with a memory phenotype. These cells patrol the body for an extended period of time,

providing a long-lasting anti-inflammatory effect, possibly to prevent states of chronic inflammation from occurring. Memory regulatory T cells exist in an activated state without antigenic stimulus for an extended period of time, originally defined by Loblay *et al* as being greater than 9 months. This allows peripheral tolerance to occur, preventing the onset of autoimmune disease. Memory regulatory T cells also exist that have been pathogen-stimulated, maintaining their antigen-specificity after the infection has been resolved. [67] They are thought to mediate the rapid killing response by $CD8^+$ cells during the enhanced secondary response to a previously-encountered pathogen. Further to this, it has been found that naive regulatory T cells in mice do not cause suppression of $CD8^+$ responses, only T cells that have been previously exposed to pathogenic antigens.[68] They also allow maternal tolerance of a foetus during pregnancy through sustained suppression of an immune response to the non-self-antigens expressed by foetal cells.[69]

However, it has been shown that memory Tregs are usually only a transient phenomenon, occurring during inflammatory events, and so isolating this population *in vitro* may present a challenge.[70] This could explain why a TLR2-expressing T cell population is dependent on TCR stimulation, as regulatory T cells only adopt a memory phenotype during periods of inflammation and the release of pro-inflammatory TCR ligands. This effect could be mimicked by the use of anti-CD3 antibody, a technique used in several papers to induce a TLR2-expressing population.

Evidence for the existence and role of a double-expressing population remains scant. It clear that further research is needed to determine whether such a subset of T cells exists. If so, it may be possible to elucidate the function of this population and the role it plays within the overall immune response.

1.3.3 Evidence for a TLR2- dependent downmodulation pathway for CCR5

Previous research by the Signoret lab has provided evidence that TLR2 downmodulates CC receptors 1,2 and 5 in human monocytes to inhibit their chemotaxis after pathogen recognition, allowing monocytes to accumulate at the site of pathogen invasion. However,

no such effect was seen with T cells, which were used as a control to further highlight the downmodulation seen in monocytes.[71]

In contrast to this, McKimmie *et al* found evidence that TLR2 ligation by bacterial lipoprotein does indeed lead to a downregulation of CCR5 mRNA in mouse CD4 T cells.[72] However, the CCR5 receptor differs both structurally and functionally between murine and human T cells, meaning that this result needs to be replicated in human T cells before conclusions can be drawn.[73]

It is also possible that the small numbers of TLR2 expressing T cells present in human blood mean that the presence of a downmodulation pathway has been overlooked. The large numbers of non-TLR2-expressing T cells present in fresh human blood mean that the methods used may not have been sensitive enough to show appreciable downmodulation of CCR5 on T cells. As the evidence for a $TLR2^{+}$ population focuses on a small subset of memory and regulatory T cells, looking at them in isolation may reveal that a downmodulation pathway does indeed exist. Issues with maintaining Tregs in culture also could also be a reason why this population has not been identified. Regulatory T cells normally exist in very small numbers in the blood of healthy individuals, and their survival is dependent on cell-cell contact in vitro. Another issue is that regulatory T cell numbers are known to decline with age, and vary dramatically between individuals, with numbers between 0.6-15% being reported.[74,75]

1.3.4 Significance of the downmodulation pathway in T cells

Given that CCR5 is known to be a HIV-1 co-receptor, the presence of a downmodulation pathway could potentially present an interesting therapeutic target. If TLR2 ligation does indeed lead to the downmodulation of CCR5 from the membrane on some T cells, then this could be used to preserve T cell numbers in infected individuals by preventing viral entry in Tregs, as recently shown by Hirsch et al.[76] Additional to this, evidence has emerged that TLR2 plays its own role in the pathogenesis of HIV. Recently, it has been reported that HIV-1 replication is enhanced by TLR2 ligation in human CD4+ CCR6+ T cells.[77] It has also been observed that TLR2 ligation by HIV-1 structural proteins increases

the expression of pro-viral RNA, and results in an increase in CCR5 expression in T cells.[78] This raises interesting questions about the interplay between the two receptors, and provides more compelling evidence that a cross-talk pathway indeed exists.

Drawing this evidence together presents a question: As CCR5 and TLR2 can be expressed on regulatory and memory T cells, as well as regulatory memory T cells, then we hypothesize that a subset of these cells exists that co-expresses both receptors. If this is the case, then does a similar downmodulation pathway to that found in monocytes exist in co-expressing T cell populations?

1.4 Project aims

The aims of this project were to determine 1) whether TLR2-expressing cells are found in human blood; 2) whether a subpopulation of double-expressing $TLR2^{+}CCR5^{+}$ cells can be identified; 3) determine the phenotype of such cells as well as the culture conditions that favour their growth and finally; 4) test the possibility of a cross-downmodulation between TLR2 and CCR5 on these cells.

2. Materials and methods

2.1 Reagents and antibodies

Tissue culture reagents and secondary antibodies were purchased from Invitrogen; other reagents were from Sigma-Aldrich, unless stated. Purified LTA from *S aureus* and ultra-pure lipopolysaccharide (LPS; *Escherichia coli* 0111:B4) were purchased from Invivogen. The CCR5 ligand MIP1β was sourced from Peprotech, and AOP-RANTES was a gift from Dr Oliver Hartley. Antibodies were sourced from multiple manufacturers, detailed in Table 1.

2.2 Primary cell isolation and culture

Fresh peripheral blood mononuclear cells (PBMCs) were obtained from healthy donors (from apheresis cones taken from single donors; supplied by the NHS Blood and Transplant Service, United Kingdom) by density gradient centrifugation with the use of Lymphoprep (Axis-Shield). Monocytes were separated from lymphocytes by adherence to gelatine-coated plates, and cultured in RPMI containing 20mM HEPES, 10% fetal bovine serum, FBS, (PAA Gold), 100 U/mL penicillin, 0.1 mg/mL streptomycin, and 2mM l-glutamine.

The monocyte-depleted supernatant removed from the adherence plates was cultured, as well as samples of non-monocyte-depleted PBMCs. Activated lymphoblasts were established from lymphocytes by 3 days of culture in RPMI containing 10% fetal bovine serum (PAA Gold), 100 U/mL penicillin, 0.1 mg/mL streptomycin, and 2mM l-glutamine. Phytohemagglutinin (PHA) was added to media at a concentration of 5 µg/ml for 3 days of culture followed by 9 days of culture in media containing 100 U/mL interleukin-2 (IL-2) (PeproTech), added at 3 day intervals (see Figure 5: Fresh blood preparation)

Frozen monocyte-depleted PBMCs were prepared using the protocol above and transferred on day 0 of culture into freezing medium (90% FBS, 10% dimethylsulfoxide) before storage in vapour-phase liquid nitrogen. Cells were defrosted at 37°C in a water bath, before transfer into complete RPMI for culture and establishment of lymphoblasts using methods detailed above.

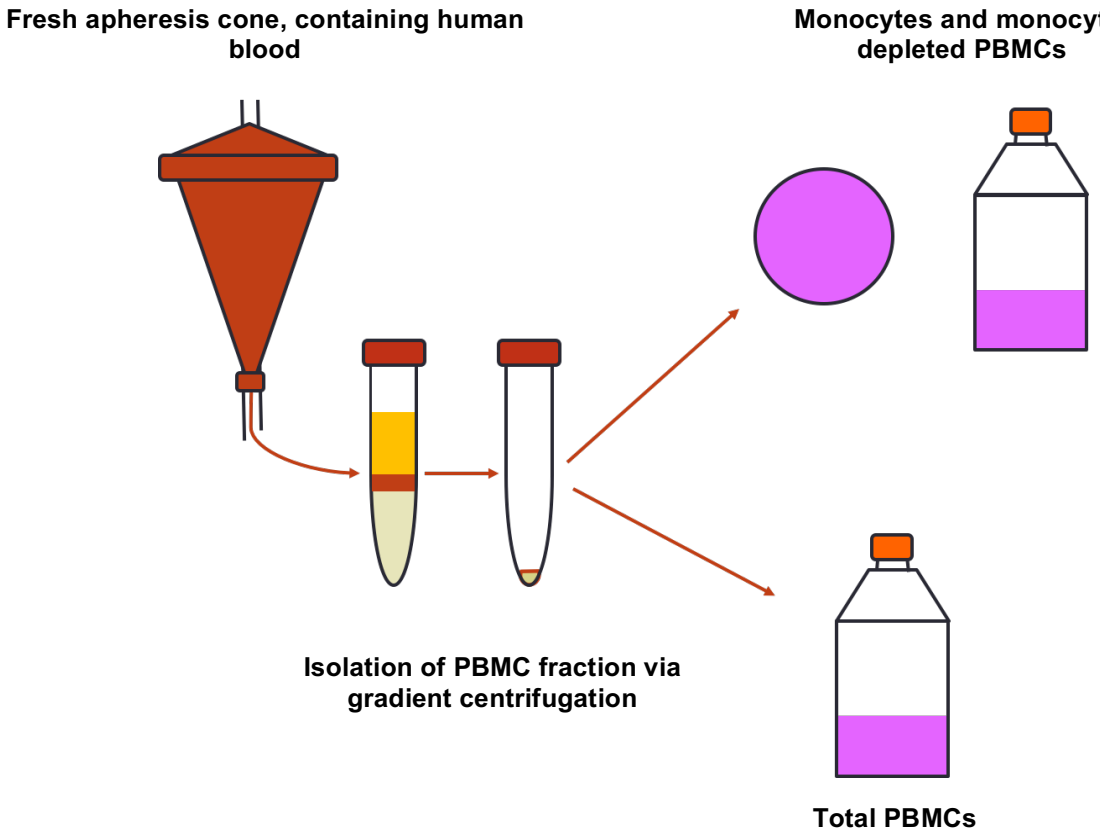

Figure 5: Fresh blood preparation. Fresh apheresis cones were obtained from the NHS Blood and Transplant service. Density gradient centrifugation with Lymphoprep (Axis Shield) was used to obtain PBMCs from the blood contained within the cone. The monocyte-depleted fraction was obtained from the supernatant of a gelatine adherence plate for use in experiments. Total PBMCs were also used in experiments.

Antibody clone	Specificity	Isotype	Type	Manufacturer
MC5	CCR5	IgG2a	Purified	(produced in house)
OKT3	CD3	IgG2a	Purified	Abcam
UCHT1	CD3	IgG1	Purified	eBioscience
T2.5	CD4	IgG2a	Purified/FITC conjugated	Abcam/Biolegend
RPA-T8	CD8	IgG1	Purified/FITC-conjugated	Biolegend
3.9	CD11c	IgG1	FITC-conjugated	eBioscience
BC96	CD25	IgG1	Purified/ PerCp/Cy5.5 conjugated	eBioscience/Caltag
HI100	CD45RA	IgG1	Purified	eBioscience
UCHL1	CD45RO	IgG2a	Purified	eBioscience
PCH101	FoxP3	IgG2a	Biotin-conjugated	BD Biosciences
TALB5	HLA-DR	IgG2a	Purified	eBioscience
Isotype control	Anti-mouse IgG1	IgG1	Purified/ biotin-conjugated	Cell Signalling/Novus
Isotype control	Anti-mouse IgG2a	IgG2a	Purified	eBioscience

Table 1 : List of antibodies used in experiments.

2.3 Cytokine/receptor ligand stimulation

Stimulation of cells was carried out between days 3-6 (after 3 days culture in PHA) or days 6-9 (after 3 days culture in PHA then 3 days culture in IL-2). 24-well tissue culture-treated plates (Dow Corning) were seeded with cells at a density of $1x10^6$ /ml and combinations of the following stimulants were added: 10ng/ml IFN-α, 10 ng/ml *S. aureus*-derived LTA, 10ng/ml *E. coli*-derived lipopolysaccharide (LPS) and 2mg/ml plate-bound anti-CD3. Anti-CD3 was bound to plates by addition of UCHT1 or OKT3 monoclonal antibodies (Abcam) in phosphate-buffered saline (PBS), followed by a 2 hour incubation at 37°C to allow the antibody to adhere to the plate. The supernatant was then aspirated away, and the plates were washed three times in sterile PBS at 20°C before being allowed to dry. Cells and other stimulants were added then directly to the wells, suspended in media. For representative combinations of stimulants, see Figure 6: Sample stimulation plate.

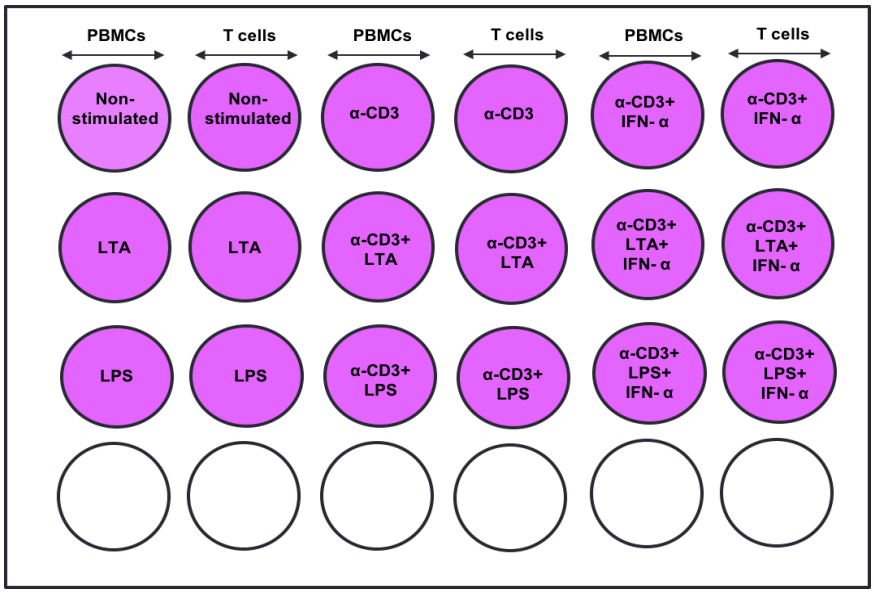

Figure 6: Sample stimulation plate. Anti-CD3 (clone OKT3 or UCHT1) was plate-bound by incubation of a 2mg/ml solution in non-tissue culture treated plates before removal and washing with PBS. Cells (fresh total PBMCs or fresh/defrosted monocyte-depleted PBMCs) were added to wells suspended in RPMI. Stimulants were added to directly wells at appropriate concentrations (see Materials and Methods: Cytokine/receptor ligand stimulation).

2.4 Staining for flow cytometric analysis

Cells were added to non-tissue culture-treated 96 well plates (Dow Corning) at a density of 4-10x10^6 cells/ml suspended in 25μl FACS buffer (PBS with 10% FBS and 0.05% sodium azide). 25μl primary antibody solution (5-10μg/ml antibody in FACS/permeabilization buffer to give a final concentration of 2.5-5μg/ml) in FACS buffer was added per well, then the plate was incubated on ice in the dark for 1 hour. Three washes (centrifugation at 1200 RPM for 3 minutes) were carried out before resuspension in FACS buffer. Staining with secondary antibodies was carried out using the same methods and concentrations as the primary antibodies.

Cells were then fixed in FACS buffer with 3% paraformaldehyde for 20 minutes or 1% formaldehyde overnight before one wash in quenching solution (50mM ammonium chloride in PBS), then quenching by resuspension in 100μl of quenching solution overnight.

Intracellular staining for the nuclear transcription factor FoxP3 was carried out by first permeabilizing cells in permeabilization buffer (FACS buffer + 0.1% saponin from quillaja bark) for 15 minutes, then centrifugation and resuspension in 25μl of permeabilization buffer. Biotin-conjugated FoxP3 and biotin-conjugated IgG1 isotype control antibody solutions were added to appropriate wells, and incubated for on ice in the dark for 1 hour. Three washes were carried out, then cells resuspended in 200μl FACS buffer.

Flow cytometry was carried out on samples transferred into round-bottomed polystyrene tubes on a Becton Dickinson LSRFortessa X-20 flow cytometer (BD Biosciences) and results were analysed using FlowJo software (TreeStar).

2.5 Downmodulation experiment

A downmodulation experiment was carried out as previously described on day 3 of culture, after 96 hours of PHA stimulation but before addition of IL-2.[62] Briefly, 100μl cells at a density of 1x10^6/ml were added to round-bottomed polypropylene tubes (Falcon),

then centrifuged and resuspended in 1ml binding medium, BM, (100ml stock solution made up as 10ml of RPMI-10x (Sigma), 89 ml distilled water and sodium hydroxide solution added dropwise to achieve pH ≈ 7). 1ml were added to round-bottomed polystyrene tubes (100ml stock solution made up as 10ml RPMI, 1ml HEPES, CCR5-specific ligand (MIP-1β,100ng/ml or AOP-RANTES, 100ng/ml) and TLR2-specific ligands (LTA, 1ng/ml) were added. The tubes were transferred to a 37°C water bath, and incubated for 90 minutes with gentle shaking every 10-15 minutes. The tubes were then removed and placed on ice to halt binding, then transferred into a 96-well plate containing ice-cold BM and stained as normal for FACS analysis. This experiment was carried out on defrosted monocyte-depleted PBMCs as well as monocyte-depleted PBMCs and the isolated monocyte fraction for comparison.

3. Results

3.1 Rationale for use of PHA/IL-2 stimulated PBMCs

PHA/IL-2 stimulated PBMCs were used as a source of expanding T cells for use in our experiments. The rationale for this relates to the reduced proliferative capacity of T cells in isolation, and the requirement of accessory cells for expansion. The Signoret lab possesses an abundant stock of frozen monocyte-depleted PBMCs, which contain fewer monocytes than fresh total PBMCs, but still readily expand in response to PHA/IL2 stimulation.[62] The defrosted cell population that remained in culture consisted mainly of T cells, with other leukocytes such as monocytes, macrophages and B cells also present in smaller numbers. These cells were observed to decrease in culture over time, with very few non-T cells remaining after 6 days. This makes defrosted monocyte-depleted PBMCs a useful source of T cells for initial experiments, although fresh total PBMCs were also used for comparison. As the proliferative capacity and survival of T cells has been shown to be diminished by cryopreservation, use of fresh total PBMCs allows confirmation of the results seen with frozen cells, as well as allowing the influence of accessory cells such as monocytes, more accurately reproducing conditions found *in vivo*.[79,80]

The number of monocytes remaining in the monocyte-depleted fraction varies between samples, and depends upon experimental technique as well as variation in the number of circulating monocytes in the blood of individual donors. Fresh blood is also likely to contain other cell populations that do not survive the freezing process, including neutrophils, B cells and macrophages. It is possible that the influence that these accessory cells have upon T cells in culture could affect their proliferation and receptor expression, providing evidence that is more similar to the situation *in vivo*.

Regulatory T cells have been observed to exhibit reduced suppressive activity after freezing and thawing, and to express lower levels of L-selectin than non-frozen cells, suggesting that the freeze/thaw process can cause T cells to behave in a different manner than cells *in vivo*.[81] As this is the case, the strategy employed in our experiments was to use frozen monocyte-depleted PBMCs to provide initial evidence that could be further explored later through the use of fresh total PBMCs.

PHA/IL-2 stimulation was chosen as a method of expanding the cells without inhibiting CCR5 expression, as is the case when using other standard methods of cell expansion such as anti-CD3/anti-CD28, something that has also been observed in the Signoret lab.[82,83] PHA is a plant-derived lectin that acts as a mitogen, sensitising T cells to IL-2 stimulation by upregulating expression of the IL-2 receptor, leading to their proliferation in culture. It has been observed that monocytes act as accessory cells during PHA/IL2 stimulation, and are required to present PHA to effectively cross-link the TCR in order to expand cells.[84] As the populations of interest may consist of small numbers of T cells, it was desirable to be able to use either defrosted monocyte-depleted PBMCs or total PBMCs subjected to PHA/IL2 stimulation in order to expand the total T cell population.

The approach taken in our experiments was to compare defrosted monocyte-depleted PBMCs using standard expansion with PHA/IL2 to those grown in a variety of stimulation conditions that have been reported to enhance TLR2 or CCR5 expression taken from a review of literature. Studies reporting methods for increasing the population of $TLR2^+$ cells are listed in Table 2A; papers reporting methods of generating CCR5-expressing T cells as well as a paper describing a $CCR5^+$ $TLR2^+$ double-expressing subpopulation are detailed in Table 2B.

Flow cytometric analysis was used to define the phenotype of T cell populations of interest. These experiments were then repeated on a fresh sample of total PBMCs in order to compare the results obtained. A downmodulation experiment was then carried out with chemokines known to activate cells via CCR5 (AOP-RANTES and MIP1β) as well as the TLR2 ligand LTA in order to examine any differences in expression between the two receptors that could potentially be caused by a communication pathway.

Study	Receptor expressed by cells	T cells used and selection methods	Culture medium	Conditions of culture	Additions to culture medium	Timings	Comments
Komai-Koma et al, PNAS 2004[65]	TLR2	$CD45RA^+$ (naïve) (and $CD45RO^+$ (memory).) $CD4^+$ T cells isolated by negative selection using MACS microbeads (Miltenyi Biotech) and sorted by double positive selection for $CD4^+$ and $CD3^+$ by FACS.	RPMI 1640 +10% FCS.	37°C with 5% CO_2-cultured with anti-CD3 and IFN -α after selection in 24-well plates.	Cultured with plate-bound anti-CD3* (1-5μg/ml) and IFN-α (100-1000 units/ml). *Induction of TLR2 expression was found to be TCR activation-dependent.	Experiments were carried out 72hr after stimulation began.	IFN-α was found in this study to enhance TLR2 expression in $CD45RA^+$ T cells. (Memory cells were found to constitutively express TLR2.) Cell-surface TLR2 expression peaked between 12 and 72hr after activation and continued to remain at high levels up to 92hr.
Nyirenda et al, J Immunol 2011[85]	TLR2	$CD4^+$ T cells isolated from PBMCs using MACS microbeads T_{reg} cell isolation kit (Miltenyi Biotec); depletion of non-$CD4^+$ T cells then positive selection of $CD25^+$. Multiple separate subpopulations of T_{regs} (defined as being $FOXP3^+$) isolated from PBMCs by FACS.	RPMI 1640 +5% FCS.	37°C- 2.5x 10^3 cells/well (96 well U-bottom plate)	Cultured with plate-bound anti-CD3 (1μg/ml) and anti- CD28 (1μg/ml).	Experiments carried out 48 or 72 hrs after stimulation began.	TLR2 was found to be particularly highly expressed by $CD45RA^+CD25^{++}$ ("resting" T_{regs}) and $CD45RA^-CD25^{+++}$ ("activated" T_{regs})

Table 2A: Techniques for generating TLR2 positive T cells, taken from a review of available literature

Study	Receptor expressed by cells	T cells used and selection methods	Culture medium	Conditions of culture	Additions to culture medium	Timings	Comments
Abraham et al, J Autoimmun 2008[86]	Tregs (CCR5)	$CD4^+$ T cells positively selected from PBMCs (Dynabeads M-450 CD4 beads)- some were isolated from frozen PBMC samples	RPMI 1640 +10% FCS.	37°C with 5% CO_2.	Plate bound anti-CD3 (1µg/ml) , recombinant human IL-2 (10U/ml) .	Incubated with plate-bound anti-CD3 for 5 days, then moved to uncoated plates with the addition of human IL-2. Cultured for 10-13 days before analysis.	Stimulation of CD4 T cells with anti-CD3 induces regulatory phenotype that suppresses self-reactive PBMCs. Cells are anergic once regulatory phenotype established.
Schmidt et al, PLOS One 2016 [87]	Tregs (CCR5)	Naive $CD4^+$ T cells were positively selected using with MACS Naive $CD4^+$ T cell kit from fresh PBMCs isolated from buffy coats.	RPMI + 10% FCS for one night then serum-free X-Vivo medium with 1% Glutamax and 100U/ml IL-2 for rest of experiment.	37°C with 5% CO_2.	Plate bound anti-CD3 (5µg/ml) and 100U/ml IL2 throughout the stimulation period. 0.4 µg/ml anti-CD28 and 10ng/ml TNF- α1 added over 6 day incubation, plus10nM all-trans retinoic acid (ATRA).	1 night incubation, then 6 days with stimulation.	FoxP3 expression with 5ng/ml TNF- α1 and 0.4 µg/ml CD28 was comparable to expression with 5ng/ml TNF- α1 and 1µg/ml CD28. Overnight incubation with RPMI carried out to deplete adherent monocytes.
Thibault et al, J Immunol 2007 [88]	TLR2 and CCR5	CD45RA (naive) and CD45 RO (memory)- negative selection using immunomagnetic selection. Bulk $CD4^+$ T cells were then sorted into memory and naive cells by negative immunomagnetic selection.	RPMI +10% FCS	37°C with 5% CO_2.	IL-2 (30 U/ml), Pam_3Csk_4 (5 µg/ml) and crosslinked anti-CD3 (OKT-3 at 1 µg/ml with goat anti-mouse at 5 µg/ml) or control PHA-L+ IL-2	Exposure to Pam_3Csk_4 and/or anti-CD3 (clone OKT3) for 24 or 72 hrs after selection.	Stimulation of TLR2 with the agonist Pam_3Cysk_4 resulted in expression of CCR5 by naive and memory T cells- memory had the highest expression, with 10.0% expressing when stimulated with Pam_3Csk_4 alone, increasing to 15.5% of cells expressing with the addition of OKT3, compared to PHA + IL-2 which resulted in 13.7% of memory cells expressing CCR5.

Table 2B: Techniques for generating $CCR5^+$ and $CCR5^+$ $TLR2^+$ T cells taken from a review of available literature.

3.2 Monocyte-depleted PBMCs

In order to identify a TLR2-expressing population, defrosted monocyte-depleted PBMCs were stained for flow cytometric analysis, using different combinations of markers to confirm phenotypes of TLR2-expressing cells (see Tables 1 and 3). Cells were tested at day 0, directly after defrosting, but no TLR2-expression could be detected via flow cytometry (not shown). PHA/IL2 stimulation was then used to assess if TLR2 receptor expression could be seen after activation and expansion (see Materials and Methods: 2.2 Primary cell isolation and culture). Flow cytometric analysis was carried out at day 3 of culture, after stimulation with PHA but before IL2 was added. T cells were defined as $CD3^+$ cells. Live/dead staining was used in order to exclude dead cells from the analysis (results not shown). Figure 7 demonstrates the gating strategy for one representative set of results of n=12, and shows that $TLR2^+$ T cells could be identified as one subset based on TLR2 positive fluorescence signal and their unique forward vs side scatter (FSC vs SSC). These cells were classified as subpopulation A. A second $CD3^+$ subpopulation was identified, subpopulation B, which was found not to contain significant numbers of $TLR2^+$ cells. Both subpopulations were found to be present in all donor samples based on this gating strategy, with TLR2-expression limited to subpopulation A. This subpopulation was found to be larger in terms of FSC vs SSC than the rest of the T cell population. Combined with the fact that no $TLR2^+$ cells could be found at day 0 before addition of PHA, this suggests that these cells were activated lymphoblasts rather than naïve T cells.[89]

3.2.1 PHA/IL2 stimulation kinetics

At day 3, after stimulation with PHA, it was possible to identify $TLR2^+$ T cells in subpopulation A. Cells were subsequently expanded for a total of 12 days in PHA/IL2 in order to appraise changes in the population over time and the effect of IL-2. CCR5 expression has been observed to slowly increase over 12 days of stimulation with IL-2. [90] However, no substantial evidence currently exists for changes in TLR2 expression.

Antibody combination	Secondary antibodies	Purpose
TLR2+CCR5	Anti-mouse IgG1-488+ IgG2a-647.	Isolation of double-expressing population.
TLR2+CCR5+ LIVE/DEAD	Anti-mouse IgG1-488+ IgG2a-647.	Confirmation of viability of population.
CD3+TLR2	Anti-mouse IgG1-488+ IgG2a-647.	Allows gating on CD3+ cells to only consider T cell populations
CD4-FITC+CCR5	Anti-mouse IgG2a-647.	Assessment of proportion of expressing population that is CD4+/CD8+
CD4-FITC+TLR2	Anti-mouse IgG1-488.	
CD8-PE+CCR5	Anti-mouse IgG2a-647.	
CD8-PE+TLR2	Anti-mouse IgG1-488.	
CD11c-FITC+CCR5	Anti-mouse IgG2a-647.	Negative control- CD11c is a marker of non-T cells (e.g. monocytes).
CD11c-FITC+TLR2	Anti-mouse IgG1-488	
CD25 +CCR5	Anti-mouse IgG1-488 + IgG2a-647	Assessment of whether T cell population is activated.
CD45RO+TLR2	Anti-mouse IgG1-488+ IgG2a-647	Assessment of whether T cells are memory (CD45RO+) or naïve/effector T cells (CD45RO-)
Foxp3-biotin+CCR5	Anti-mouse IgG2a-647+ streptavidin-PE.	Assessment of whether T cells are regulatory phenotype (Foxp3+)
Foxp3-biotin+TLR2	Anti-mouse IgG1-488+ streptavidin-PE	

Table 3: Summary of antibody combinations used for flow cytometric analysis in the experiments presented in the results section.

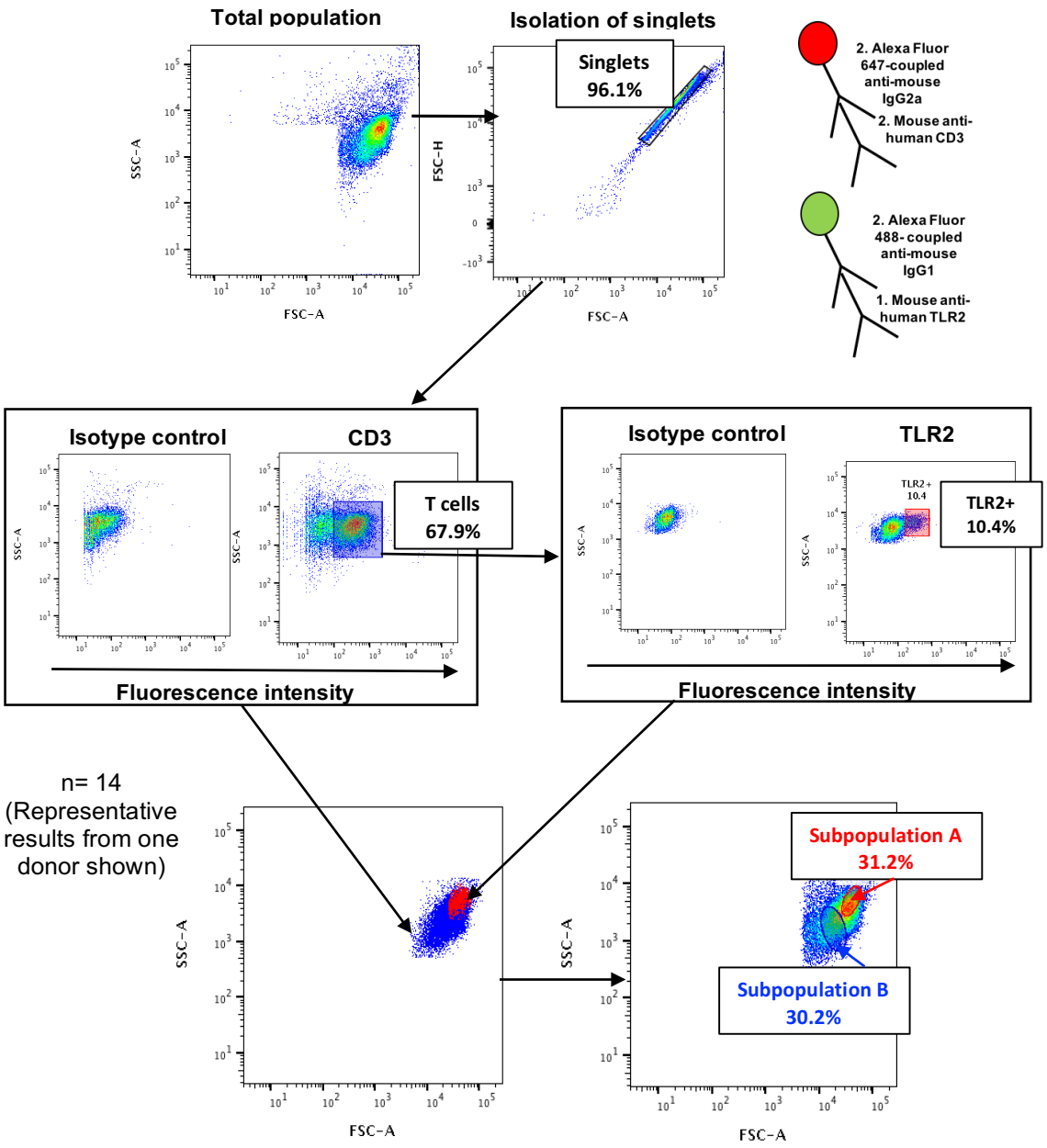

Figure 7: Gating strategy for TLR2-expressing T cell subpopulation. Cells were double-stained for CD3 to define T cell populations, and TLR2. FSC-A vs FSC-H was used to isolate singlet populations. A gate was set on $CD3^+$ cells by comparing CD3 fluorescence intensity vs SSC to an isotype control. TLR2 fluorescence intensity vs SSC was used to define a $TLR2^+$ T cell gate by comparison to an isotype control. The $TLR2^+$ T cell subpopulation was overlaid on a scatter plot of FSC vs SSC for the total T cell population in order to set gates for the $TLR2^+$ subpopulation (Subpopulation A) and a non-$TLR2^+$ T cell subpopulation (Subpopulation B).

Cells were taken from culture every 3 days in order to observe subpopulation size on days 3,6,9 and 12 of culture. However, through live/dead staining, it was found that the majority of the cells (>50%) had died in culture by day 12 (data not shown), and so only days 3, 6 and 9 were considered.

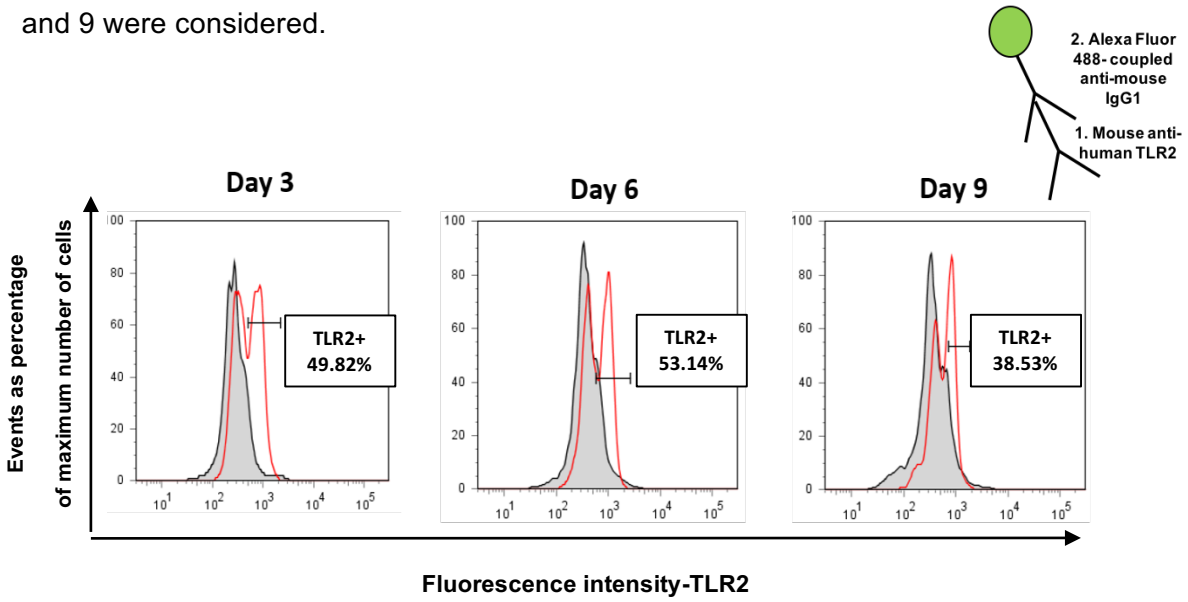

Figure 8:TLR2 expression in Subpopulation A over 9 days of stimulation. Cells were singly- stained for TLR2 expression and analysed using flow cytometry. Gating was carried out to define Subpopulation A, using the gating strategy outlined in Figure 7. Histograms of stained cells (red histograms) were overlaid on isotype controls (grey shaded histograms) and gated to indicate percentage TLR2-expressing cells in Subpopulation A. Experiments were carried out for n=12 donors; representative results shown from one donor.

Figure 8 shows variation in $TLR2^{+}$ cell percentages in the T cell Subpopulation A over time.

When comparing receptor expression kinetics for all the donors over the course of the 9-day stimulation, it appeared that the percentage of cells expressing TLR2 remains relatively stable, despite cell death within the overall population. Upon examination of data from 6 different donors, the percentage of TLR2 expression within the small subpopulation A follows a similar trend to the percentage represented by these cells in the overall population (see Figure 9A). However, Figure 9B demonstrates that the number of live cells in Subpopulation A decreases over time, suggesting that the cells are disappearing from culture. It appears that whilst the subpopulation itself changes in size, expression of the receptors on these cells is not affected by the number of live cells present in culture. n.b. As there is overlap between the error bars for the box plots in Figure 9B, no statistical significance can be inferred from this set of results in terms of mean numbers of live cells

for each day over four different experiments. However, it is possible that this downward trend may become statistically significant with further repeats of the experiment.

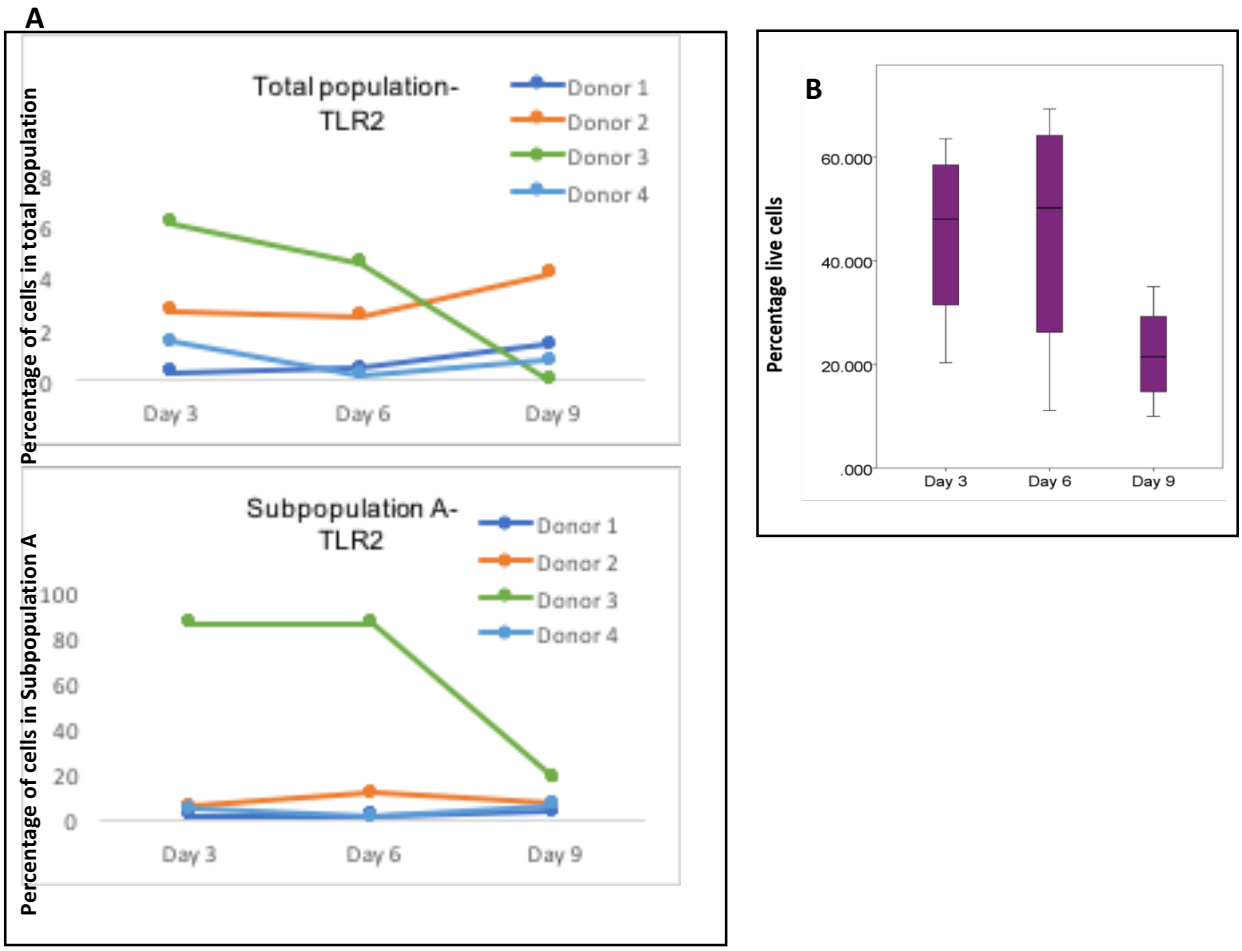

Figure 9: TLR2 expression in Subpopulation A over 9 days of stimulation, and percentage live cells in Subpopulation A. Cells were single- stained for TLR2 expression and fixable live/dead stain was added to assess viability. Subpopulation A was identified using the gating strategy described in Figure 7. A. TLR2 expression over a 9 day stimulation with PHA/IL2; each coloured line represents results for a single donor. B: Percentage live cells within Subpopulation A. This experiment was carried out on n=4 donors.

After confirming the presence of $TLR2^+$ cells in Subpopulation A, CCR5 expression was then examined (Figure 10); CCR5 is known to be widely expressed by $CD4^+$ T cells, so a subpopulation of $CCR5^+$cells was expected to be present within the sample.[91] Cells stained for CCR5 were analysed by flow cytometry and the presence of $CCR5^+$ cells in Subpopulation A was assessed using the same FSC/SSC gating strategy described in Figure 7 (see Figure 10).

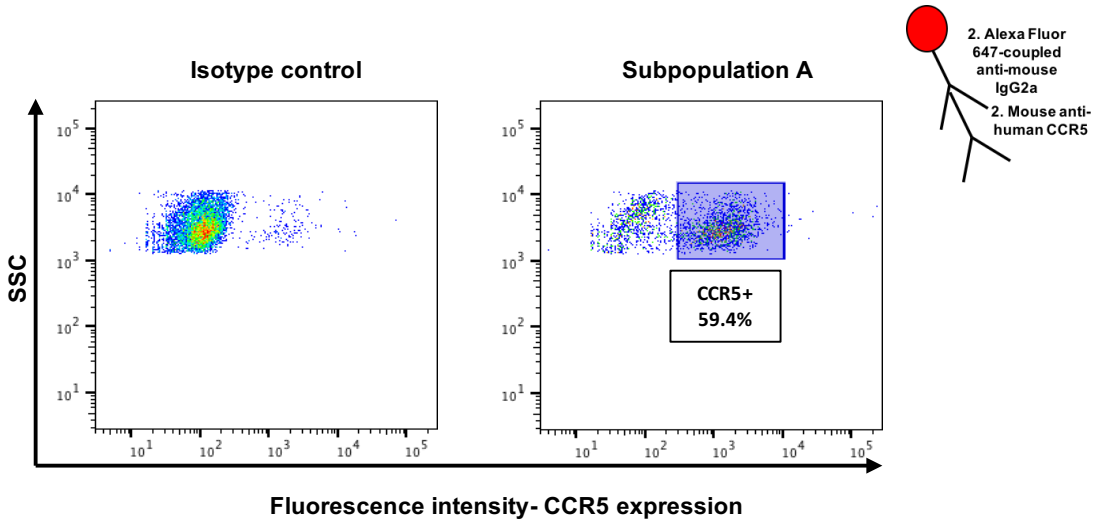

Figure 10: CCR5 expression in Subpopulation A. Cells were singly- stained for CCR5 expression and analysed using flow cytometry Subpopulation A was identified using gating strategy described in Figure 7. A scatter plot of CCR5 fluorescence intensity vs SSC was used to identify CCR5-expressing cells, using an isotype control for comparison. Representative results shown from a single donor; this experiment was carried out on n=12 donors.

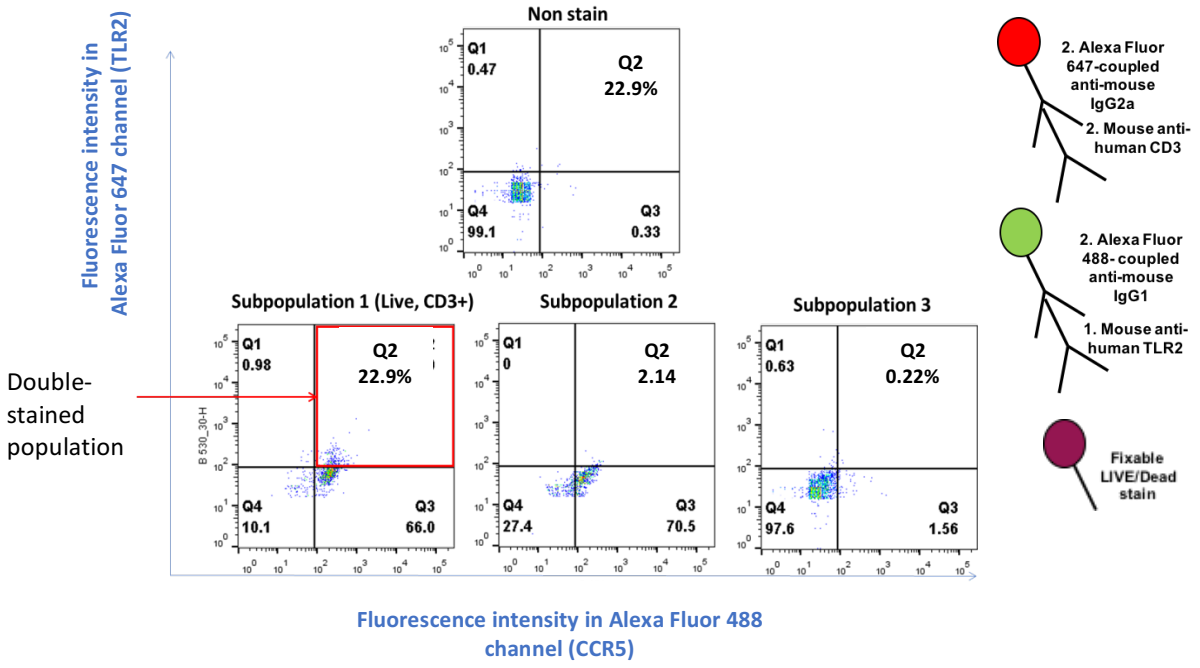

Figure 11: Evidence of double-expressing subpopulation. Cells were single- stained for TLR2 expression and analysed using flow cytometry. Subpopulation A was identified using gating strategy described in Figure 7. Quad gates were set on appropriate isotype controls; double-stained cells appear in Q2. Representative results shown from a single donor; this experiment was carried out on n=12 donors.

A distinct CCR5-expressing fraction could be seen in Subpopulation A, which raised the possibility of a subset of cells expressing both TLR2 and CCR5 being present in the

subpopulation. Co-staining for CCR5 and TLR2 was used in combination with live/dead staining to try and identify a possible double-expressing subpopulation (see Figure 11). After identifying Subpopulation A using our gating strategy (see Figure 7), quad gates were applied to a CCR5 and TLR2 double-stained sample, based on appropriate isotype controls. This allowed observation of $TLR2^+$, $CCR5^+$ and $CCR5^+$ $TLR2^+$ cells within Subpopulation A. While variations in the size of each subset of cells were seen with different donors, a small number of live double-positive cells could consistently be found, and makes up 22.9% of Subpopulation A in the example shown in Figure 11 .

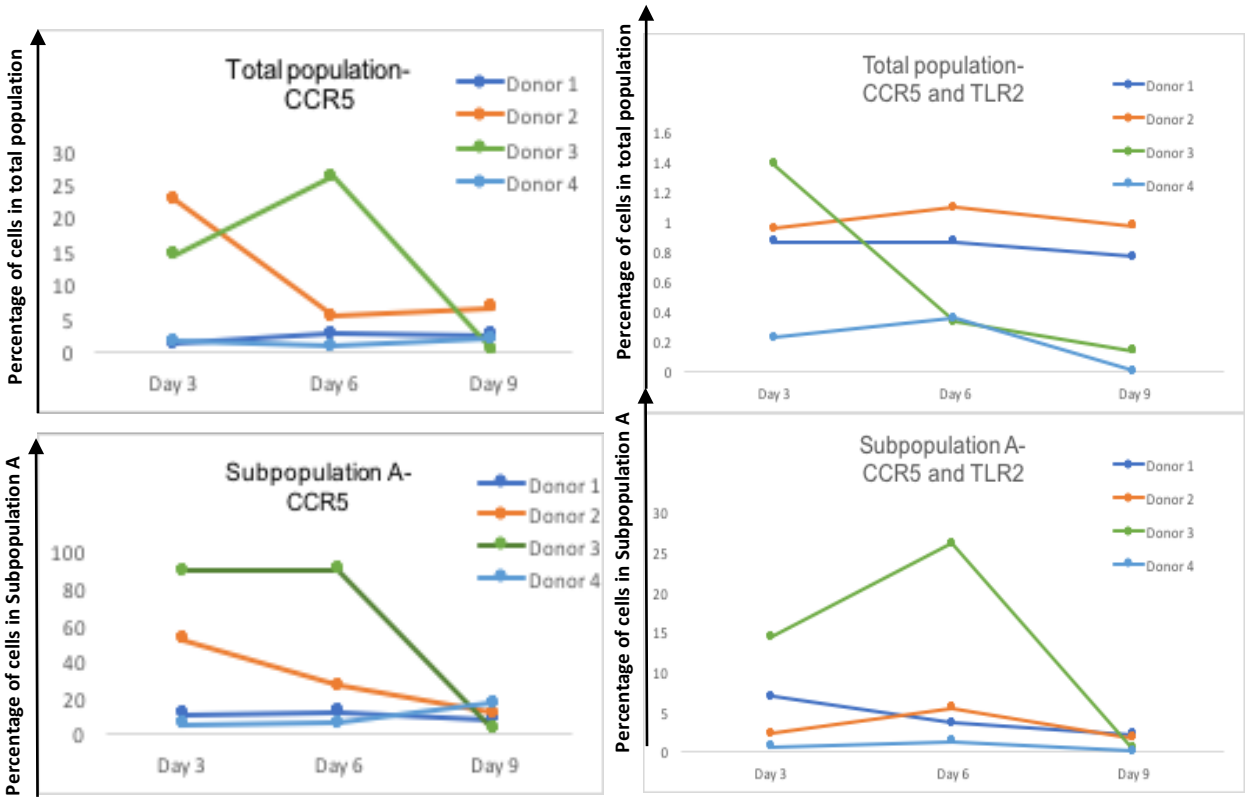

Figure 12: Percentage CCR5 expression and percentage of a double-expressing subpopulation in Subpopulation A over 9 days of stimulation. Cells were double- stained for CCR5 and TLR2 expression and analysed using flow cytometry., Quad gates were set as detailed in Figure 11, and percentage expression was recorded over 9 days of stimulation. This experiment was carried out on n=4 donors.

Once this double-expressing population had been identified, kinetics experiments were carried out to compare numbers of $CCR5^+$ and $CCR5^+$ $TLR2^+$ cells over time. As with the TLR2 kinetics experiments, cells were expanded for 9 days in PHA/IL2, with staining

carried out on days 3, 6 and 9. Flow cytometric analysis was used to ascertain the percentage of expressing cells present in Subpopulation A compared to the total cell population (see Figure 12)

Despite donor variability, we see that there is no expansion of the $CCR5^+$ cell population over time, with numbers remaining relatively constant in most cases. The double-expressing population also appears to remain relatively stable over the 9-day period. Whilst a small expansion of the $TLR2^+$ T cell population can be observed, it appears that no significant expansion of the $CCR5^+$ $TLR2^+$ T cell population occurs using defrosted monocyte-depleted PBMCs. However, as the $TLR2^+$ population is so small, variability between donors would have a large effect on cell numbers. It is possible that use of a larger number of cells to begin with could lead to expansion of the $TLR2^+$ fraction, allowing it to be characterised more effectively. Since Subpopulation A does not appear to expand in culture, the implication is that these cells are anergic, and cannot be expanded.[92] In either case, the use of fresh PBMCs could confirm whether a larger starting population is needed. As regulatory T cells have been observed by Abraham et al and Schmidt et al to express CCR5, it is possible that they make up some of the subpopulation of interest.[86,87] However, Tregs are thought to expand poorly and exhibit reduced suppressive function after defrosting, so the use of fresh cells could elucidate whether this is causing them to fail to expand.[81]

Day 3 was subsequently chosen as an appropriate day to use for future experiments, as the largest CCR5/TLR2 double- positive population could be identified within Subpopulation A on this day of culture.

3.2.2 Characterisation of Subpopulation A

Further characterisation was carried out to ascertain the phenotypes of cells present within the subpopulation. Firstly, CD4 and CD8 staining was carried out to identify whether the cells belonged to a cytotoxic or helper T cell lineage. Most evidence relating to a $TLR2^+$ population has focused on $CD4^+$ populations, so a majority of $CD4^+$ cells was expected.[93]

The T cell fraction of Subpopulation A was identified by gating on a $CD3^+$ population, which identified a distinct population based on FSC vs SSC. This population was then examined to appraise CD4 vs CD8 expression within the T cells, then was compared to the non-$TLR2^+$ Subpopulation B, as well as to the overall cell population (see Figure 13).

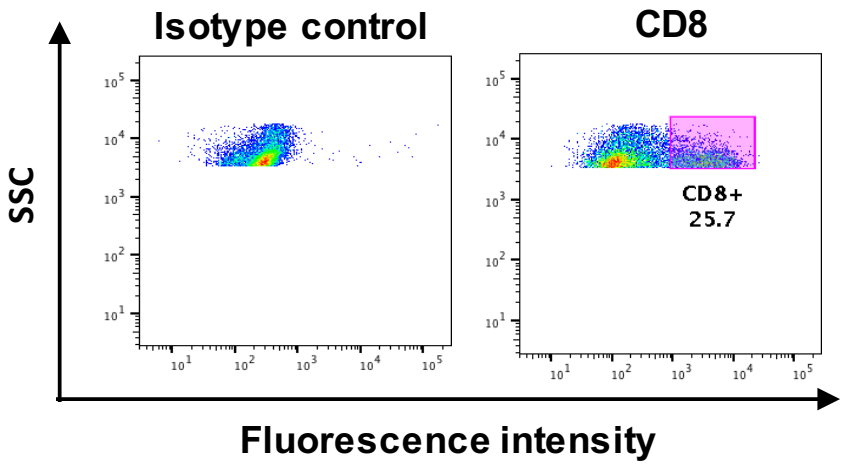

Figure 13: CD4/CD8 characterisation of Subpopulation A. Cells were single-stained for CD4 or CD8. Subpopulation A was identified using the gating strategy detailed in Figure 7, and a gate was set using FSC vs SSC. $CD4^+$ and $CD8^+$ cells were identified using fluorescence intensity vs SSC, compared to isotype controls. This experiment was carried out on n= 6 donors (representative results from one donor shown).

Subpopulation A was found to be a mixed population, composed of mostly $CD4^+$ T cells, (defined as either $CD4^+$ or $CD3^+$ $CD8^-$ depending upon antibody combinations available at the time of the experiment) with the mean expressing fraction on day 3 = 71.9%. CD8 expression in these populations was substantially lower, with a mean of 25.6% of expressing cells in Subpopulation A, and a small number of non-T cells making up the remainder (n=6). The proportions of $CD4^+$ and $CD8^+$ cells in Subpopulation A did not differ from the CD4/CD8 percentages in the total population (results not shown).

Further characterisation was carried out based on this information. TLR2 and CCR5-expressing cells have been observed to be of regulatory and memory phenotypes; as this

is the case, co-staining was carried out to evaluate the expression of the nuclear transcription factor FoxP3, a marker of human T cells (see Figure 11), and the surface receptor CD25, a component of the IL2 receptor and therefore an activation marker (see Figure 12).

CD45RO was found to be expressed by a large proportion of cells in Subpopulation A on day 3 (30.3- 60.1%), suggesting a sizeable memory T cell subpopulation. Furthermore, 19.6-50.1% of $TLR2^+$ cells were found to co- express CD45RO. Greater variation could be seen in FoxP3 expression, ranging from 6.33-73.0% of $TLR2^+$ cells, possibly due to the very small numbers of FoxP3 cells present, which caused problems with accurate gating. Despite this, a distinct $TLR2^+$ subpopulation can be seen in the $FoxP3^+$ subpopulation, although results were still variable, with $TLR2^+$ cells making up 7.55-53.7% of FoxP3 expressing cells.

Whilst $FoxP3^+CCR5^+$ T cells can be identified (results not shown), the numbers of $FoxP3^+$ $TLR2^+$ and $TLR2^+CCR5^+$ cells are so small that it was impossible to identify a convincing subpopulation that expressed all three receptors through triple staining experiments; the number of identifiable triple-positive cells was <50 in all cases (results not shown). This meant it was impossible to distinguish between actual triple stained cells and artefact.

CD25 and TLR2 co-staining was also carried out to assess the activation status of the $TLR2^+$ subpopulation (see Figure 12). CD25 was found to be expressed by 72.7-93.5% of $TLR2^+$ cells, suggesting that the majority of the subpopulation is made up of activated T cells.

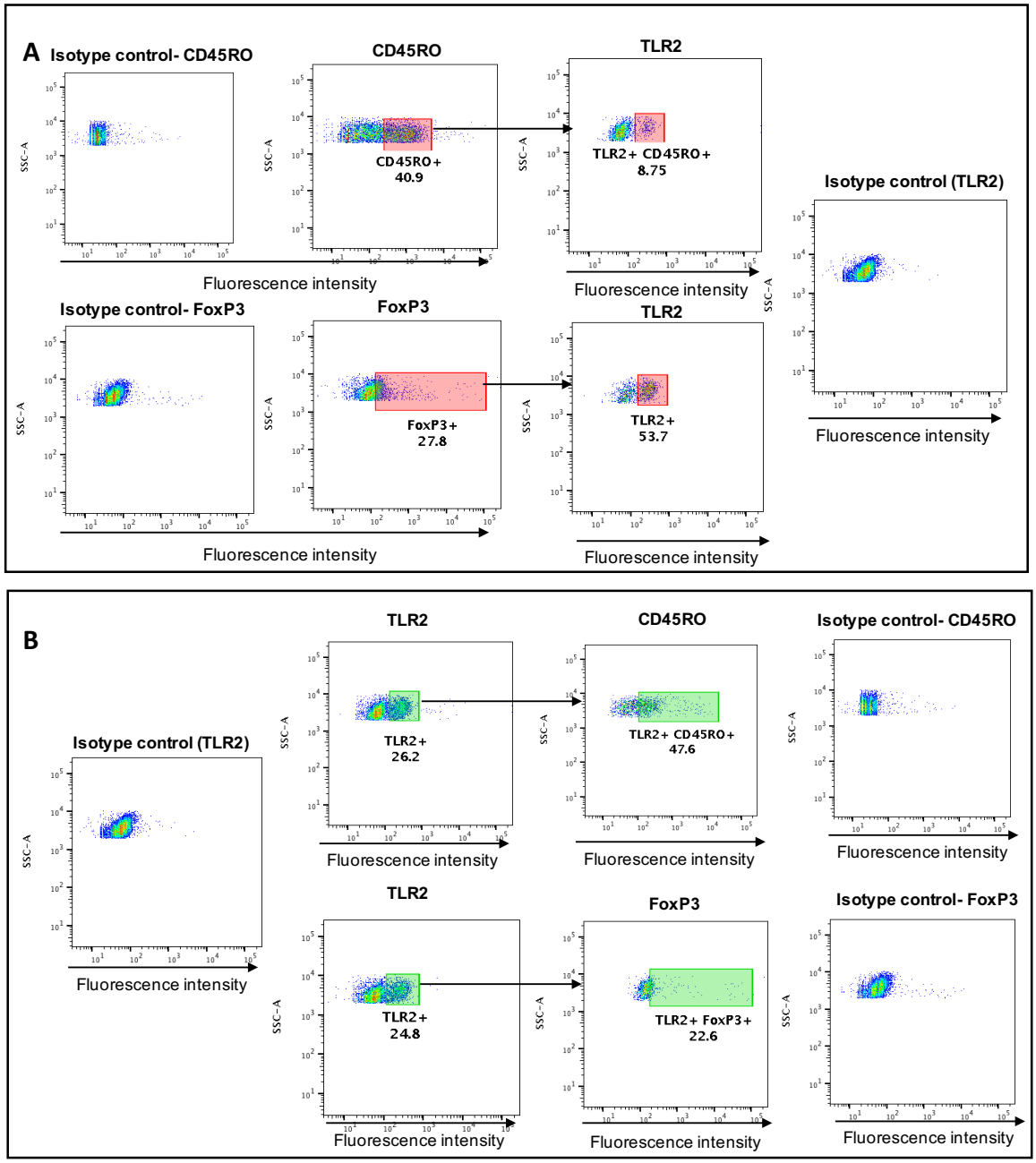

Figure 14: CD45RO and FoxP3 expression within Subpopulation A. Cells were double-stained for FoxP3/TLR2 and CD45RO on Day 3. The FSC/SSC gating strategy described in Figure 4 was used to define Subpopulation A. Fluorescence intensity vs SSC was used to sequentially gate on expressing populations compared to isotype controls. Sequential gating was used to define subsets of cells. A. TLR2 expression on $CD45RO^+$ and $FoxP3^+$ cells. CD45RO and FoxP3 expressing subpopulations represent 40.9% and 27.8% of Subpopulation A respectively. 8.75% of $CD45RO^+$ cells express TLR2; 53.7% of $FoxP3^+$ cells express TLR2. B: Expression of CD45RO or FoxP3 on $TLR2^+$ cells. $TLR2^+$ expressing cells represent ~ 25% of Subpopulation A. 47.6% of $TLR2^+$ cells express CD45RO; 22.6% of $TLR2^+$ cells express FoxP3. Experiments were carried out on n=5 donors; representative results from one donor shown.

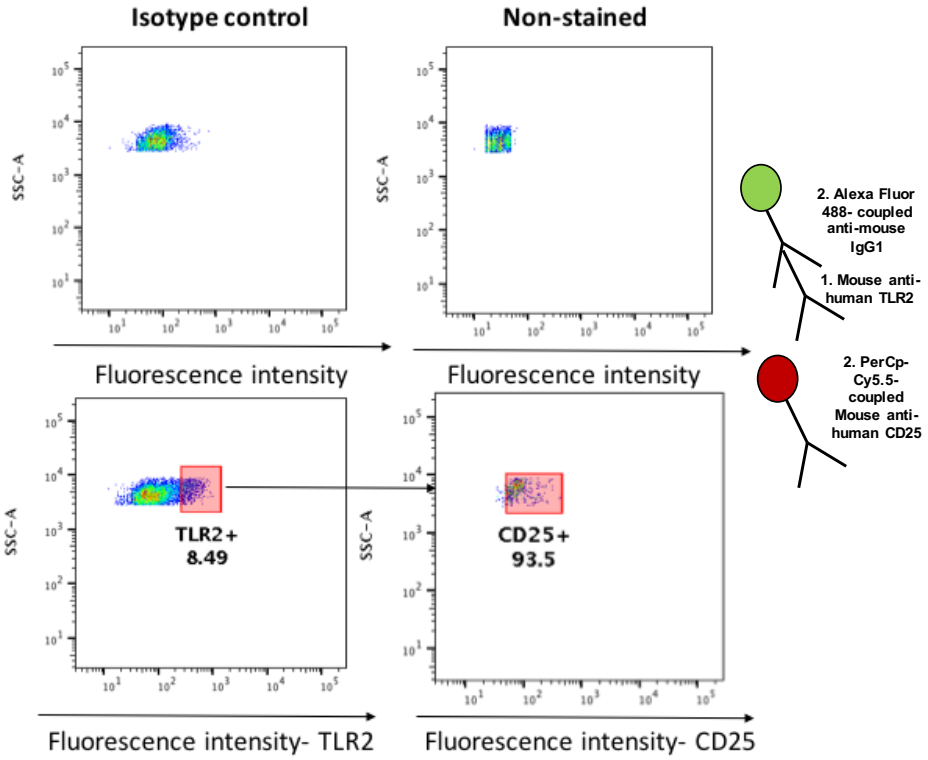

Figure 15:CD25 expression within Subpopulation A. Cells were double-stained for CD25 and TLR2. Subpopulation A was identified using the gating strategy described in Figure 7. A scatter plot of fluorescence intensity vs SSC was used to set gates for $TLR2^+$ and $CD25^+$ cells, by comparison to isotype controls. Percentage $TLR2^+$ cells in Subpopulation A= 8.49%; percentage $CD25^+$ cells in TLR2-expressing subpopulation= 93.5%. n.b Non-stained control used for comparison to CD25 as no appropriate isotype control was available at the time of experiment. Experiments were carried out on n=3 donors; representative results from one donor shown.

3.3 Fresh total PBMCs

A major problem with the previous experiments related to the size of the subpopulation of interest. Additionally, questions were raised about the viability of regulatory T cells after freezing and thawing. As this was the case, fresh blood samples were used in order to ascertain whether a greater number of $TLR2^+/CCR5^+$ cells could be obtained and a discrete subpopulation identified. These cells were stained with primary antibodies for the same markers as in previous experiments in order to examine whether they were in a different state of activation. This was especially relevant due to the monocytes now present in the blood; most monocytes did not survive after being defrosted.

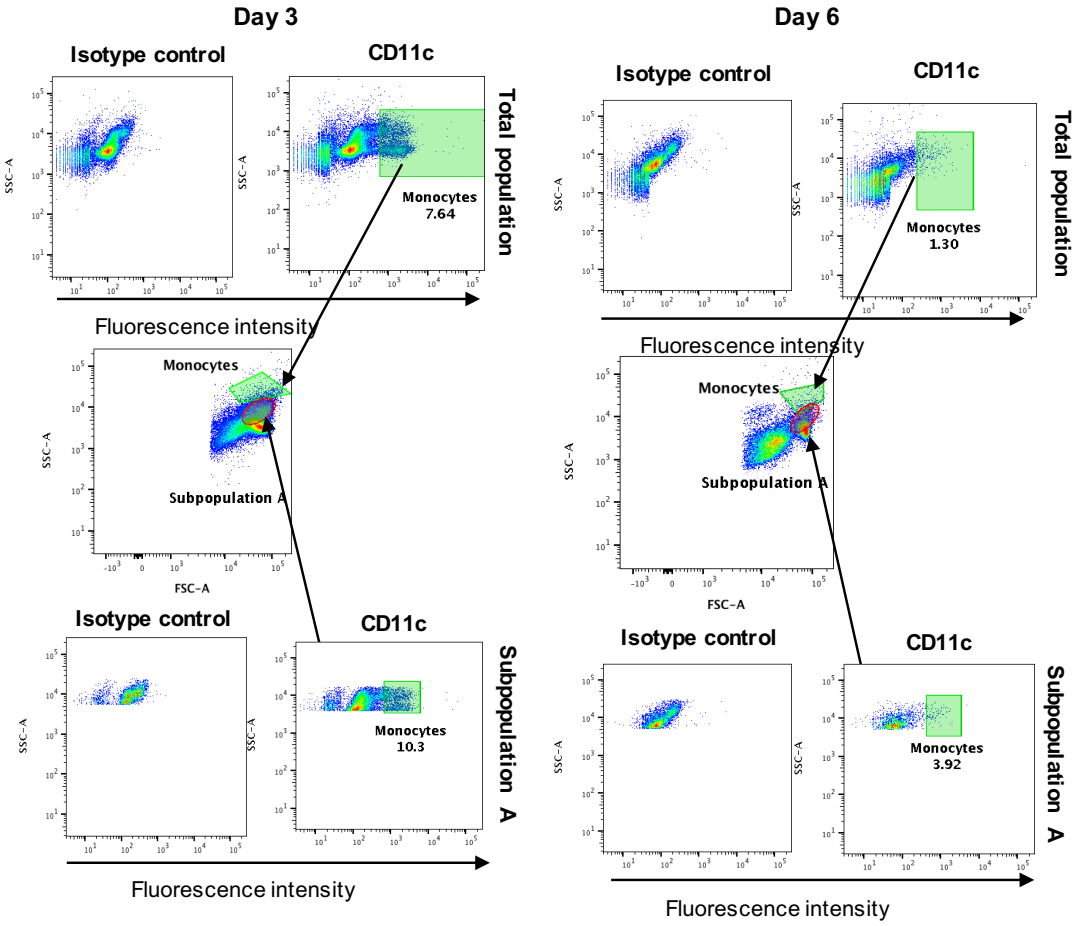

Figure 16 Identification of monocytes in fresh blood samples. Cells were singly stained for CD11c. FSC-A vs FSC-H was used to isolate singlet populations (not shown). Fluorescence intensity vs SSC was used to gate on expressing populations compared to an isotype control within Subpopulation A and the total cell population. Numbers represent percentage expression of Subpopulation A or total cell population, to demonstrate the percentage of monocytes present in each. A gate was applied for the main monocyte population and its location based on FSC vs SSC was determined. This was overlaid on a scatter plot of the overall cell population, demonstrating its position relative to Subpopulation A.

In the fresh blood samples, a large monocyte population was easily identifiable through examining CD11c fluorescence intensity vs SSC, with monocytes making up 10.3% of cells in Subpopulation A, compared to 7.64% in the total cell population (see Figure 16). This number decreased on Day 6, with 3.92% monocytes remaining in Subpopulation A, and 1.1% in the total population. Gating of Subpopulation A was carried out to avoid the main monocyte population, based on the distinct FSC vs SSC associated with the T cell population. Co-staining with CD3 and/or CD11c were used in order to confirm only T cells were being appraised for receptor expression.

3.3.1: PHA/IL2 stimulation kinetics

PHA/IL2 stimulation was used to expand fresh total PBMCs and monocyte-depleted PBMCs, using the same methods used in expansion of defrosted monocyte-depleted PBMCs (see Materials and Methods: Primary cell culture and isolation). The gating strategy described in Figure 7 was used to identify Subpopulation A, which was found to contain $TLR2^+$, $CCR5^+$ and $TLR2^+$ $CCR5^+$ cells within the $CD3^+$ subpopulation (see Figure 11). We observed that the percentage of live cells in the sample increased over the 9 days of culture, suggesting that after a recovery period post-isolation, the cells were actively expanding in culture (see Figure 17). As with frozen cells, the $TLR2^+$ $CCR5^+$ subpopulation was largest on Day 3, with double-expressing cells making up 14.0% of the total cell population, notably higher than in the frozen monocyte-depleted PBMCs where the numbers rarely exceeded 1% of the total cell population on day 3 (see Figure 9A). This is possibly due to better survival of cells in culture than with defrosted PBMCs, influence of accessory cells, or the effect of the freeze/thaw cycle on regulatory T cells. However, this subpopulation decreased markedly in size over the 9 day stimulation, representing 3.71% and 1.21% of the total cell population on days 6 and 9 respectively. Total CCR5 expression in Subpopulation A increased considerably by day 9, in agreement with previously published data regarding T cells stimulated with PHA/IL2.[62] This suggests that the decreased size of the double-expressing subpopulation is not due to cells dying in culture, as the overall number of live cells within Subpopulation A increased over the nine days, unlike in the defrosted samples.

It appears that the double-expressing subpopulation does not expand with PHA/IL2 stimulation with fresh or defrosted PBMCs. However,it was not possible to carry out a triple co-stain with live/dead dye, anti-TLR2 antibody and anti-CCR5 for this particular experiment, and so dead cells could not be excluded from the analysis of the double-stained cells as they had been in the defrosted blood samples. Additionally, due to time constraints, this experiment was only carried out on one fresh blood sample. As this is the case, this experiment needs to be repeated before conclusions can be drawn.

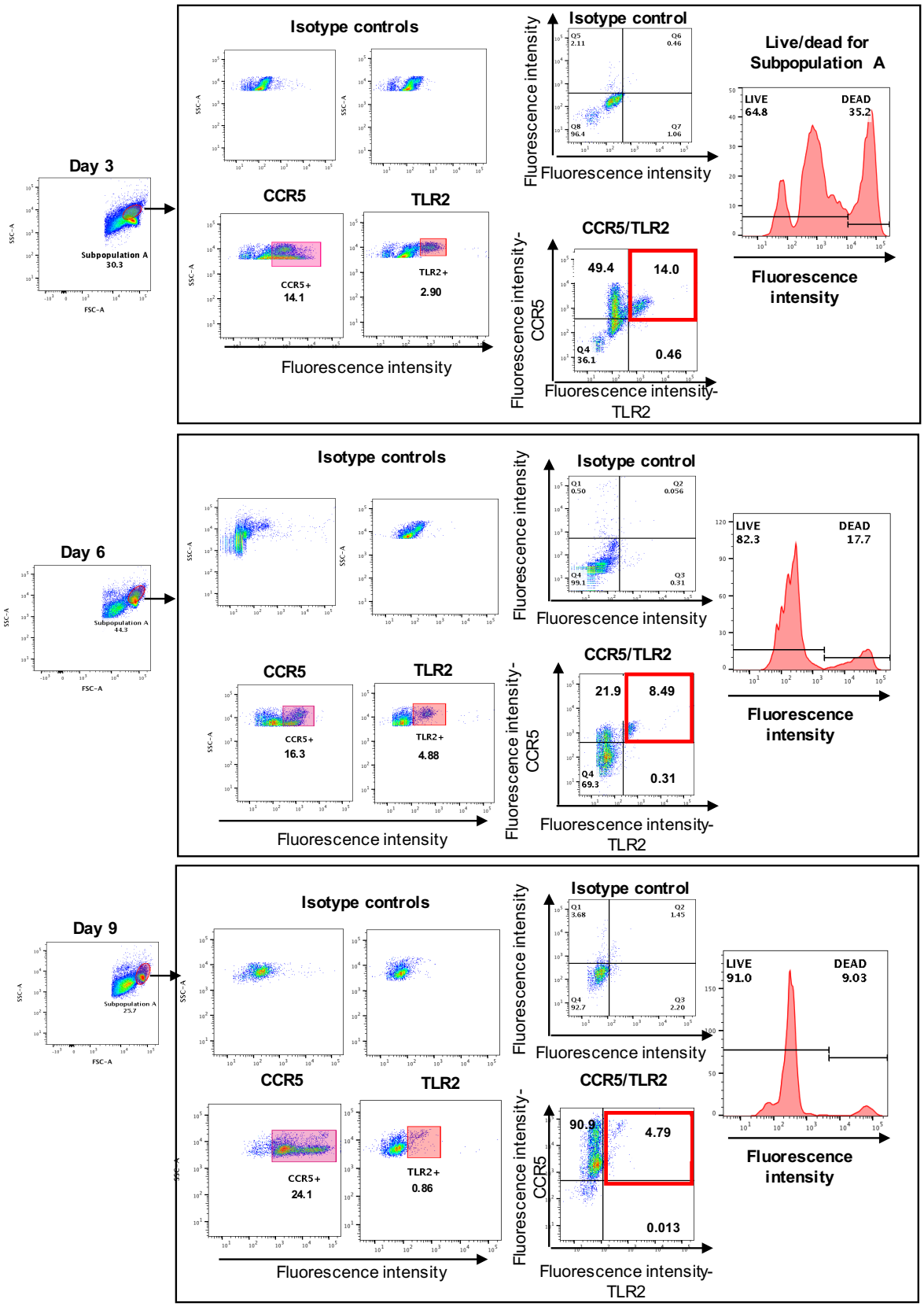

Figure 17: TLR2 and CCR5 expression in a fresh blood sample Cells were double-stained for CCR5 and TLR2. Fixable LIVE/DEAD stain was used to assess cell viability; only live cells were considered in the analysis. Subpopulation A was identified using the gating strategy described in Figure 7. Staining was undertaken at days 3, 6 and 9, before addition of mitogens. Fluorescence intensity vs SSC scatter plots were used to identify $CCR5^+$ and $TLR2^+$ cells in Subpopulation A, compared to isotype controls; numbers represent expressing cells as percentage of total cell population. Quad gates were set on appropriate isotype controls to identify double-positive CCR5/TLR2 cells (red-outlined quadrants); numbers represent expressing cells as percentage of Subpopulation A.

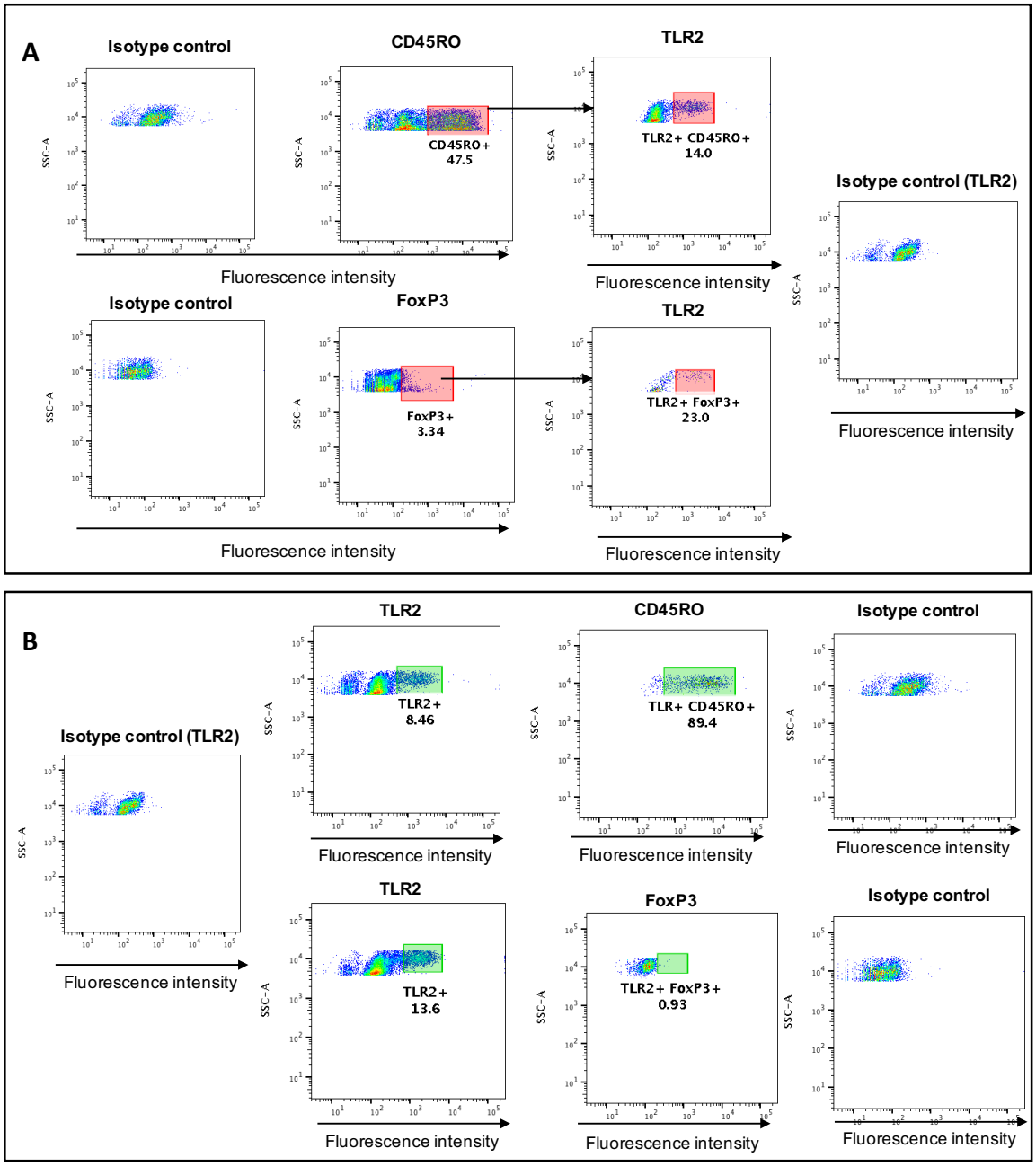

Figure 18: CD45RO and FoxP3 expression within Subpopulation A within a fresh blood sample. Cells were double-stained for FoxP3/TLR2 and CD45RO on Day 3. The FSC/SSC gating strategy described in Figure 4 was used to define Subpopulation A. Fluorescence intensity vs SSC was used to sequentially gate on expressing populations compared to isotype controls. Sequential gating was used to define subsets of cells. A. TLR2 expression on $CD45RO^+$ and $FoxP3^+$ cells. CD45RO and FoxP3 expressing subpopulations represent 47.5% and 3.34% of Subpopulation A respectively. 14.0% of $CD45RO^+$ cells express TLR2; 23.0% of $FoxP3^+$ cells express TLR2. B: Expression of CD45RO or FoxP3 on $TLR2^+$ cells. $TLR2^+$ expressing cells represent ~ 10% of Subpopulation A. 89.4% of $TLR2^+$ cells express CD45RO; 0.93% of $TLR2^+$ cells express FoxP3.

After examining the kinetics of TLR2 and CCR5-expressing populations, characterisation of the cells was carried out to compare with the results from defrosted cells. Cells were double-stained for CD45RO and TLR2, and FoxP3 and TLR2. Flow cytometric analysis was then undertaken, using the same gating strategy as with defrosted PBMCs (see Figure 18).

Similar results were found to the frozen samples; $TLR2^+$ cells were present in the $CD45RO^+$ and $FoxP3^+$ subpopulations, although the number of double-expressing cells as a percentage of Subpopulation A was smaller (2.19% $CD45RO^+$ $TLR2^+$ and 0.25% $FoxP3^+$ $TLR2^+$). This is most likely due to the higher proportion of other cell types (e.g. monocytes) in the fresh sample compared to the frozen cells (see Figure 16).

CD25 expression was also assessed; again, cells were single stained for CD25 and gated after flow cytometric analysis in the same manner as the defrosted samples (see Figure 19).

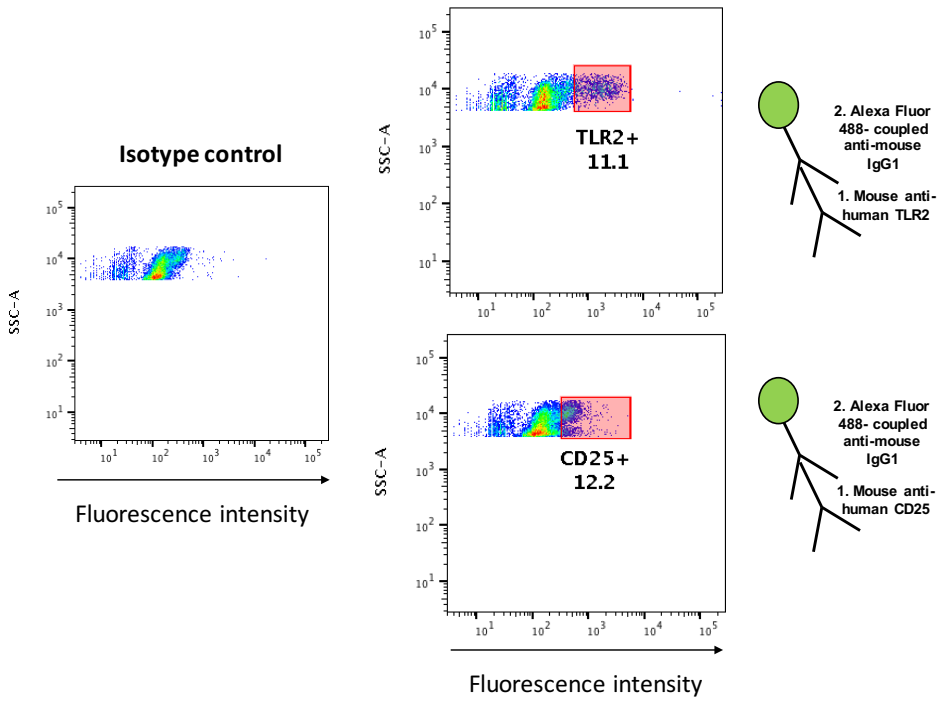

Figure 19: CD25 expression within Subpopulation A within a fresh blood sample. Cells were single-stained for CD25 and TLR2. Subpopulation A was identified using the gating strategy described in Figure 7. A scatter plot of fluorescence intensity vs SSC was used to set gates for $TLR2^+$ and $CD25^+$ cells, by comparison to isotype controls. Numbers represent the percentage of expressing cells within Subpopulation A.

Due to the antibodies available in the lab at the time of staining, it was not possible to co-stain for CD25 and TLR2 with the fresh PBMCs. However, it was found that a similar percentage of $CD25^+$ cells could be found in Subpopulation A as in the defrosted monocyte-depleted PBMCs. Additionally, a similar percentage of $TLR2^+$ cells and $CD25^+$ cells were present in Subpopulation A (11.1% and 12.2% respectively). It is therefore possible that the majority of $TLR2^+$ cells in Subpopulation A are $CD25^+$ in both fresh and frozen samples. This means that CD25 could be a significant marker of this subpopulation.

3.4 Stimulation experiments

After the identification of a double-expressing subset of cells within Subpopulation 1, stimulation conditions were taken from papers that had reported $TLR2^+$ T cell subpopulations in order to determine if these cells could be expanded through addition of stimulants to cells cultured in 24-well plates (see Tables 2A and 2B). LTA and LPS were added as they are known TLR2 ligands; IFN-α was added as it has been observed to expand the $TLR2^+$ T cell population. Plate-bound anti-CD3 was added as there is evidence that TLR2-expression is TCR stimulation-dependent. Addition of anti-CD3 and anti-CD28 is a standard method of expanding PBMCs; however, anti-CD28 has been shown to downmodulate CCR5 expression.[94] However, anti-CD3 stimulation alone has been shown to enhance the T cell population, although it is not known whether this affects cell survival in culture due to the lack of a second mitogenic signal.

Using frozen samples, no significant change in the number of TLR2 expressing cells was seen using combinations of these stimulants compared to a non-stimulated control (see Figure 20). It appears that the TLR2-expressing population cannot be expanded by these methods in a monocyte-depleted PBMC population.

In this experiment, it appears that addition of a combination of anti-CD3 and IFNα caused a reduction in CCR5 expression, resulting in lower fluorescence intensity than the isotype control. There is evidence that IFNα affects the expression of CCR5 on $CD4^+$ and $CD8^+$ T cells, and it has been observed to both upregulate receptor expression, which is at odds with the evidence from this experiment.[95] However, this phenomenon has only been observed in one set of experiments; further investigation is needed before conclusions can be drawn.

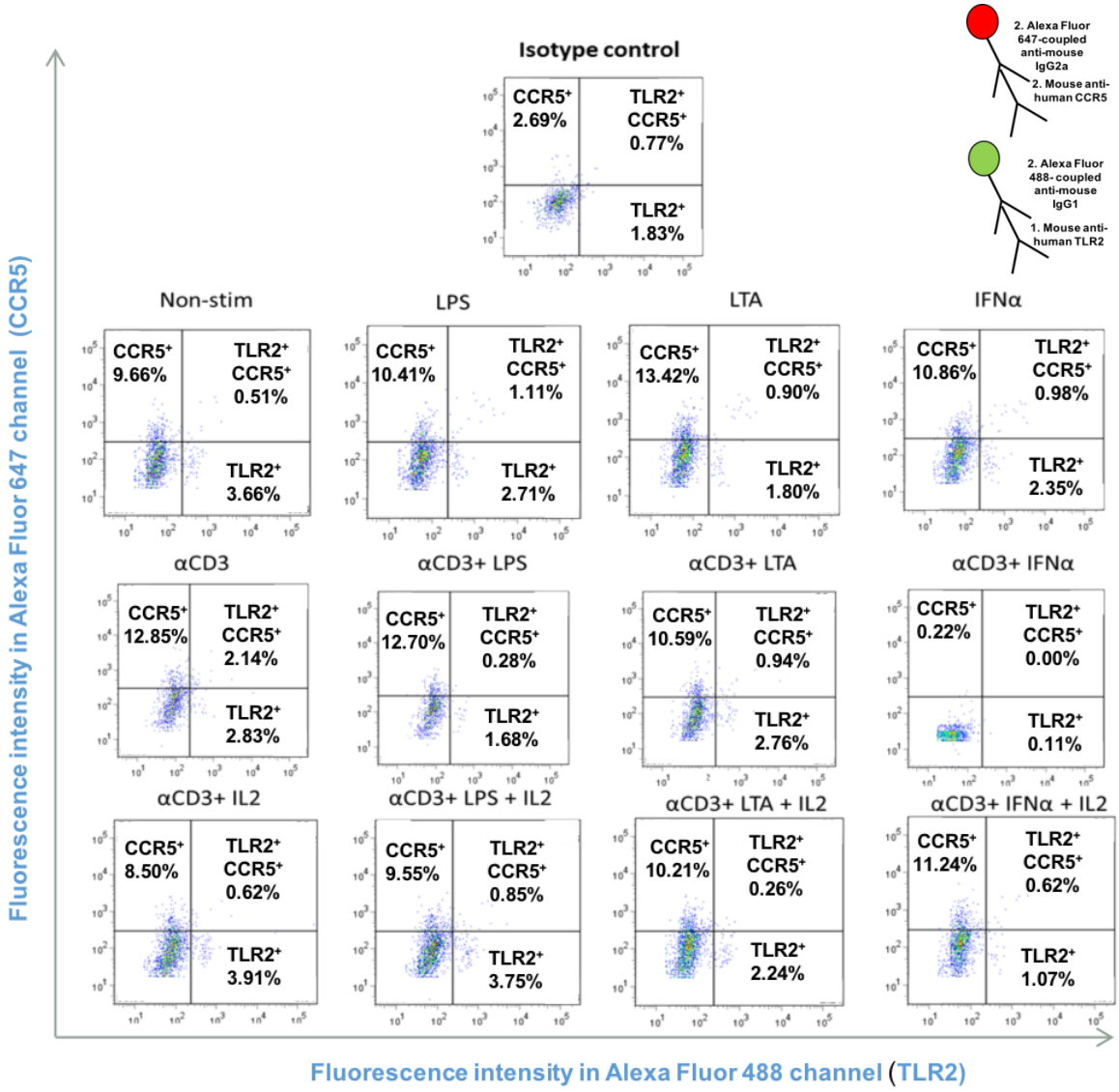

Figure 20: Stimulation experiments using frozen monocyte-depleted PBMCs. Cells were stimulated using conditions from a review of the literature, Table 2. Cell were then double-stained for TLR2 and CCR5. Subpopulation A was identified using the gating strategy described in Figure 4. Quad gates were set on an isotype control and applied to scatter plots of CCR5 fluorescence intensity vs TLR2 fluorescence intensity.

3.5 Downmodulation experiments

A very small subpopulation of $TLR2^+$ $CCR5^+$ cells could be identified in our experiments, which could not be expanded with the stimulation methods that we tried. As we were unable to increase the number of these cells, we decided to carry out a downmodulation experiment on Day 3 with a fresh blood sample to investigate whether any evidence of cross-talk could be observed between TLR2 and CCR5. TLR2- and CCR5-specific ligands were added in binding medium before incubation for 2 hours to allow receptor binding to occur. Cells were placed on ice to halt binding, and staining for flow cytometric analysis was carried out (see Materials and methods: Downmodulation experiment).

CCR5 exhibited a decrease in surface expression upon addition of the CCR5-ligands AOP-RANTES and MIP-1β, but no change was seen with the TLR2- ligands LTA or LPS (see Figure 21). TLR2 expression was not affected at all by addition of any ligands. This means that no evidence could be found in this case for crosstalk between the two receptors. However, this experiment was only carried out once, so these results should be verified through further investigation.

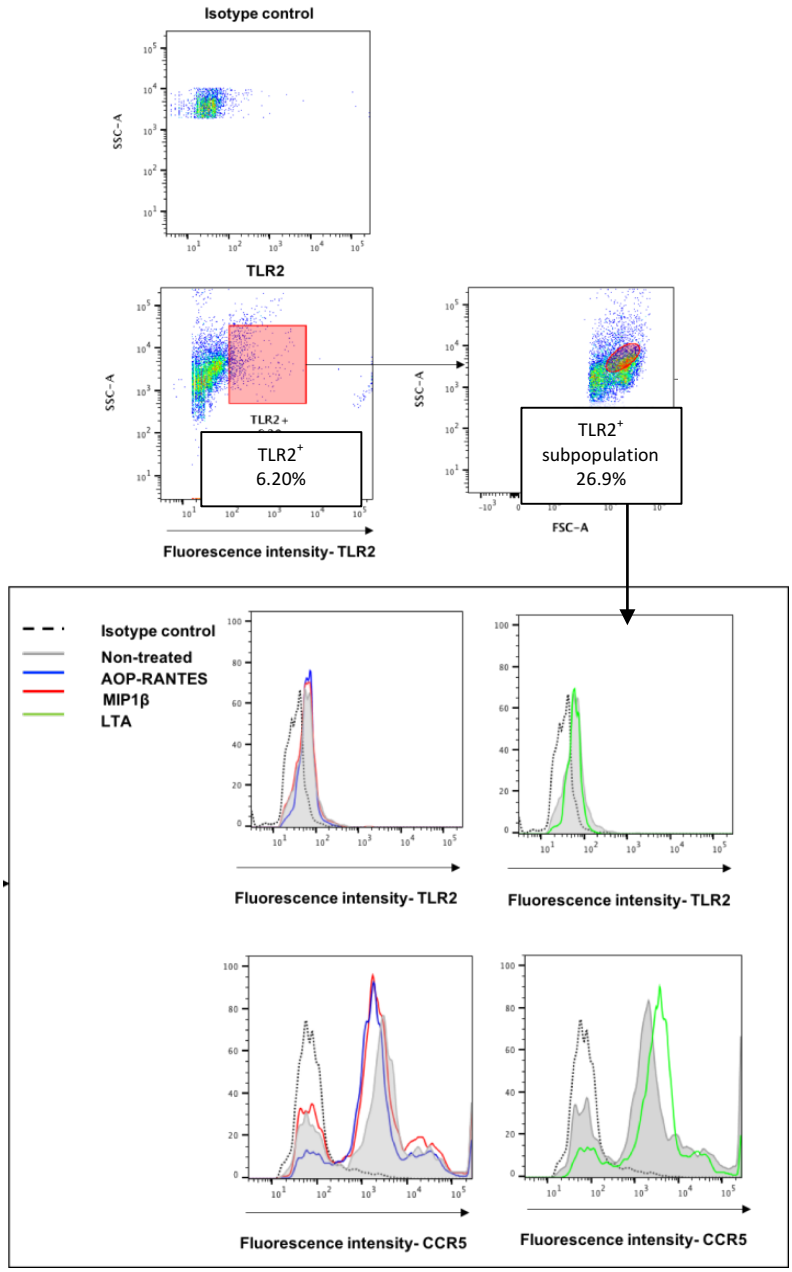

Figure 21: Downmodulation experiment. Cells were removed from culture on Day 3, suspended in binding medium and treated with the TLR2 ligand LTA, the CCR5 ligands RANTES or MIP-1β, or binding medium alone and incubated for 2 hours to allow ligand binding. Cells were then single-stained for TLR2 and CCR5. FSC-A vs FSC-H was used to isolate singlet populations (not shown). TLR2 fluorescence intensity vs SSC was used to identify the TLR2-expressing T cell subpopulation by comparison to an isotype control, and a gate was set on this population based on FSC vs SSC. Histograms of each stimulation condition were overlaid on an appropriate isotype control (dotted black histograms) and non-treated cells (grey shaded histograms).

4. Discussion

This project aimed to examine potential TLR2 and CCR5 expressing T cell populations. We wished to examine the phenotypes of the cells, and to ascertain the conditions that favoured their growth and expansion, before investigating a potential cross-talk pathway between the two receptors.

In order to fulfil these objectives, we used PBMC-derived cells in order to isolate small numbers of $TLR2^+$, $CCR5^+$ and $TLR2^+CCR5$ cells within a T cell subpopulation, designated Subpopulation A. Receptor kinetics were examined over a period of 9 days in culture to investigate changes in receptor expression. Stimulation experiments were also performed to see if Subpopulation A could be expanded through the addition of mitogens, cytokines and receptor-ligands taken from a review of literature. Finally, a downmodulation experiment was carried to explore a potential cross-talk pathway.

The existence of a $TLR2^+$ subpopulation was confirmed through single staining for flow cytometry. Co-staining was carried out to define the subpopulation, which was found to contain CCR5, CD4, CD8, CD25, CD45RO and FoxP3 positive cells (see Figure 22). Triple-staining was also used to try and identify a $FoxP3^+$ $TLR2^+$ $CCR5^+$ regulatory T cell population. It was found that 0.96-22.6% of Subpopulation A comprises $FoxP3^+$ $TLR2^+$ cells. However, as this percentage represents relatively few cells, and numbers of $TLR2^+$ $CCR5^+$ cells are also very small, it was not possible to differentiate cells that co-express FoxP3, TLR2 and CCR5 from background fluorescence.

It was found that $TLR2^+$ cells were activated, as they were only detectable after PHA stimulation, a lectin mitogen that cross-links the TCR, resulting in expression of the IL2 receptor, indicating an activated state of these T cells. Staining for CD25, a component of the IL2 receptor, found that 100% of Subpopulation A was $CD25^+$, suggesting that the whole population was activated. CD45RO and TLR2 co-staining was carried out, and, 47-89.4% of $TLR2^+$ cells within Subpopulation A were found to express CD45RO. Despite donor variability in expression, it appears that the majority of $TLR2^+$ cells belong to a memory T cell population.

As TLR2 expression could be associated with expression of CD25, FoxP3 and CD25, it is therefore reasonable to conclude that at least some of these cells express all of these receptors, although only a minority seem to express FoxP3. Figure 22 summarises potential combinations of CCR5, CD45RO, CD25 and FoxP3 within these subpopulations. However, more complex staining will need to be carried out in the future out to assess whether all four receptors are actually co-expressed on these cells.

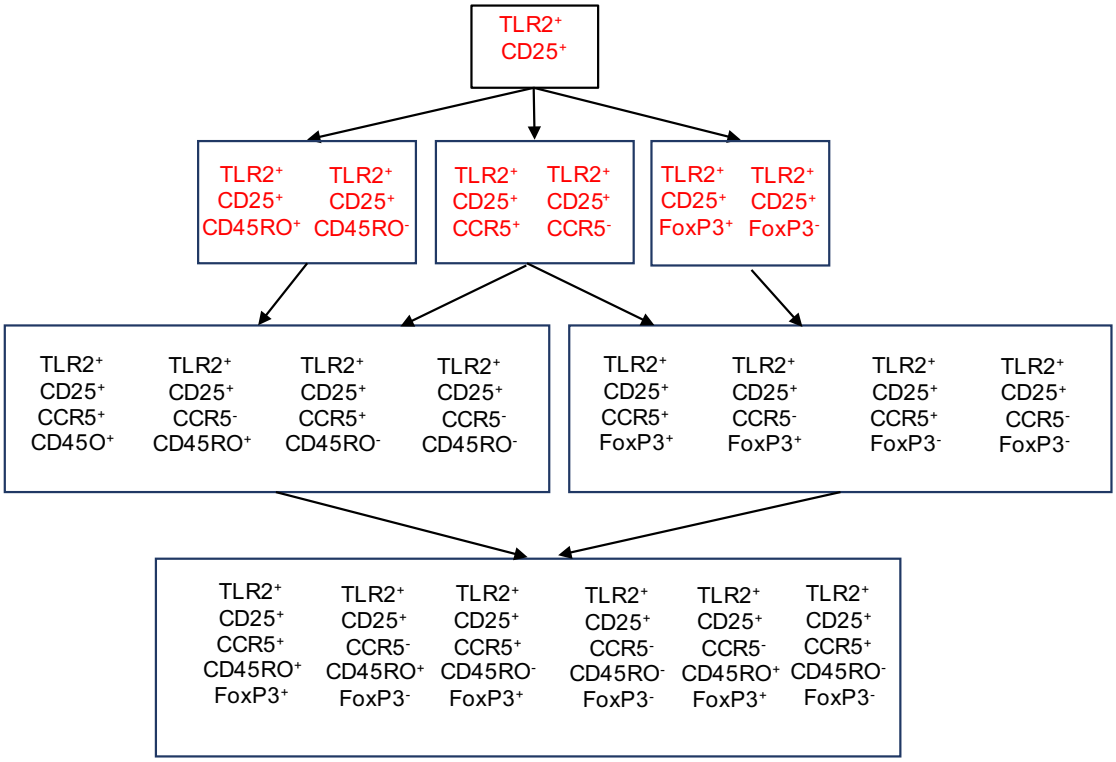

Figure 22: Combinations of possible phenotypes within Subpopulation A. Red text denotes marker combinations that have been observed in Subpopulation A; black text denotes potential phenotypes that may exist in the subpopulation.

There are two main subsets of cells that appear to be present in the $TLR2^+$ subpopulation; memory and regulatory T cells, identified by CD45RO and FoxP3 expression respectively. Both of these markers have been observed to be expressed by both $CD4^+$ and $CD8^+$ T cells; however, memory and regulatory phenotypes are more commonly associated with $CD4^+$ helper T cells. Expression of TLR2 has been previously reported in these subsets (see Table 2). [65,85,86,87,88] Additionally, subsets of regulatory T cells have also been reported to express CD45RO; Table 4 lists phenotypic markers, chemokine receptors and transcription factors specific to subsets of regulatory T cells, adapted from a review by Rosenblum et al, including CD45RO.[96] As the cells in Subpopulation A have been

observed to express CD45RO, FoxP3 and CD25, resting, activated effector or memory regulatory T cells could be responsible for the phenotypic markers present in this subpopulation. FoxP3 and CD45RO have been reported to be expressed by all three subsets, and this appears to be the case with our cells; CD25 expression is found in all of the cells; however, it is unclear where the CD25 expression we have seen in Subpopulation A falls in terms of being high expressing or variable as reported in the review from Rosenblum et al.

	Regulatory T cell subset		
	Resting	**Activated effector**	**Memory**
Phenotypic markers	$CD25^{hi}$	CD25 expression variable	$CD25^{hi}$
	$CD44^{hi}$	$CD44^{hi}$	$CD44^{hi}$
	$CD45RA^{hi}$	$CD45RA^{low}$	$CD45RA^{low}$
	$CD45RO^{low}$	$CD45RO^{hi}$	$CD45RO^{hi}$
	$CD69^{low}$	$CD69^{hi}$	CD69 expression unknown
	L-$selectin^{hi}$	L-$selectin^{low}$	L-$selectin^{low}$
	$CD127^{low}$	$CD127^{low}$	
			$CD27^{hi}$
	$BCL\text{-}2^{hi}$	$BCL\text{-}2^{low}$	$BCL\text{-}2^{hi}$
	$CTLA4^{low}$	$CTLA4^{hi}$	$CTLA\text{-}4^{hi}$
	$HLA\text{-}DR^{low}$	$HLA\text{-}DR^{hi‡}$	HLA-DR expression not defined
	$ICOS^{low}$	$ICOS^{hi}$	$ICOS^{hi}$
	$Ki67^{low}$	$Ki67^{hi}$	$Ki67^{low}$
		$KLRG1^{hi}$	KLRG1 expression not defined
Chemokine receptors	$CCR7^{hi}$	$CCR7^{low}$	$CCR7^{low}$
Transcription factors	$FOXP3^{hi}$	$FOXP3^{hi}$	$FOXP3^{hi}$

Table 4: List of markers of regulatory T cell subsets, adapted from Rosenblum et al. Green cells denote receptor expression and levels observed on cells in Subpopulation A; blue cells denote receptor expression observed on cells in Subpopulation A where relative levels of receptor expression are unknown.

Whilst Subpopulation A appears to express higher levels of CD25 than Subpopulation B, it is not clear whether a separate high-expressing subpopulation exists that we have not seen in our samples. This means that the specific phenotype of these cells remains uncertain, and further investigation should be carried out to more definitively characterise this subpopulation.

CCR5 is known to be expressed by a variety of regulatory T cells, and plays an important role in cell migration.[97] Notably, whilst TLR2 expression has been observed in regulatory T cells, it has not yet been attributed to resting, activated effector or "memory regulatory" T cell subsets. [55] We were unable to establish with confidence a $FoxP3^+$ $TLR2^+$ Treg subpopulation that also expressed CCR5. However, nearly all $TLR2^+$ cells in Subpopulation A for the fresh PBMCs were also $CCR5^+$ at day 3, whereas the percentage of $FoxP3^+$ cells within the $TLR2^+$ cells was less than 1%. CCR5 may well be expressed by these $TLR2^+$ regulatory T cells, however, a larger number of cells would need to be characterised in the future to assess whether this is the case.

A large proportion of the $TLR2^+$ cells were $CD45RO^+$, but not $FoxP3^+$, meaning the majority of the $TLR2^+$ population fall into the memory phenotype. This has been reported by Komai Koma et al and Thibault et al, who both suggest that TLR2 is constitutively expressed by $CD45RO^+$ T cells.[65,88] Thibault et al also observed that these $CD45RO^+$ $TLR2^+$ cells are anergic, which is in line with the results from our experiments, and explains why we are unable to expand them. Co-staining with CD45RO and CCR5 was not possible with the antibodies available in the lab; however, in the fresh PBMCs at day 3, 89% of TLR2-expressing cells in Subpopulation A were $CD45RO^+$. It can therefore be inferred that Subpopulation A is TLR2/CD25/CCR5/CD45RO cells. Notably, the CD45RO/CD25 phenotype was also reported to express TLR2 and CCR5 by Thibault et al.[88] However, as this has only been observed in fresh PBMCs from one donor, and to a much lesser extent with defrosted cells, this should be investigated further with more fresh samples.

It is clear that the small $TLR2^+$ subpopulation in our experiments is a mixture of cells, likely to contain $TLR2^+$ $CD25^+$ $CCR5^+$ $CD45RO^+$ cells, and also a very small number of $TLR2^+$ $CCR5^+$ $CD25^+$ $CD45RO^+$ $FoxP3^+$ cells. These cells include both $CD4^+$ and $CD8^+$ cells, although we did not have the time to investigate the relationship of CD4 and CD8 to the other markers; however, a higher proportion of cells seem to be CD4-positive.

PHA/IL2 stimulation proved to be the only successful method of generating a significant number of TLR2-expressing cells; other methods used, such as stimulation with anti-CD3, did not. Addition of IL2 does not seem to enhance this expression, with the numbers of $TLR2^+$ cells declining after day 3, and the $TLR2^+$ $CCR5^+$ subpopulation disappearing from culture in fresh samples. It is possible that this relates to cross-linking of the TCR by PHA; Komai Koma et al. report that TLR2 expression is TCR-activation dependent.[65] However, anti-CD3 stimulation also cross-links the TCR, and we did not see the same numbers of TLR2-expressing cells when this method was used (see Figure 20). It seems that PHA stimulation is able to induce TLR2-expression via a different route. PHA is a plant-derived lectin rather than a T cell-specific antigen, and there is evidence that it also activates cells via other pathways as well as cross-linking the TCR. PHA has been reported to stimulate CD2, with cell expansion occurring in a CD3-independent fashion.[98] There is evidence that regulatory T cells are dependent on CD2-stimulation in order to differentiate into antigen-specific Tregs.[99] This causes cell anergy, which could explain the failure of our cells to expand. In addition, naïve T cells have been observed to express CD45RO after CD2-stimulation.[100] This could explain the differences we have observed in PHA and anti-CD3-stimulated cells. Future experiments should be undertaken to explore whether TLR2 is expressed when T cells are stimulated with anti-CD2, and if this leads to generation of the phenotypes that we observe in our cells.

The function of these $TLR2^+$ cells is also unclear. Jin et al observed that PAMP binding to TLR2 has the ability to activate T cells.[101] It is possible that the TLR2 stimulation-pathway is an alternative, antigen-independent means of activation for these cells. This could be advantageous in the case of regulatory T cells for general antigen-independent recruitment to sites of bacterial entry, where they can exert an anti-inflammatory effect on other leukocytes, preventing unnecessary tissue damage from occurring. Evidence for this

is conflicting; TLR2 has been seen to enhance suppressive function of Tregs, whereas Nyirenda et al suggest it reduces suppressive function.[102,85]

The case for this pathway in memory T cells is less compelling; by their very nature, memory T cells are required to be antigen-specific in order to allow a response to previously-encountered antigen. It is possible that their role relates to cell recruitment and migration rather than activation, but it is unclear why this would be the case.

The fact that we were unable to observe downmodulation with the $TLR2^{+}CCR5^{+}$ T cell subpopulation suggests that the role of TLR2 on T cells may differ from TLR2 on monocytes, and a communication pathway between TLR2 and CCR5 does not exist in T cells. However, it is also possible that we did not have enough cells present in our sample to see this effect. Additionally, the downmodulation experiments previously conducted by the Signoret lab on monocytes were undertaken between days 7-21; we used PBMCs at day 3. It is possible that in the future, with greater number of cells, they could be isolated and cultured alone over a longer period of time to see if any downmodulation can be seen.

Another question raised is why this cell population would be anergic. It has been reported that this is the case with regulatory and memory T cells. However, the reason for this is yet to be elucidated. There is the possibility that the cells are not anergic at all, and the reduction in expressing cells is due to T cell plasticity, a phenomenon whereby T cells are able to change their phenotype in response to stimulation.[103] If PHA stimulation of CD2 is what is causing the expression of TLR2 and the expression of memory and regulatory phenotypes in these cells, then a lack of stimulation in the following days may cause a reversal of this state. In this case, sustained expression of TLR2 would expected with re-stimulation with PHA or anti-CD2. Further experiments in order to explore this possibility should be undertaken in the future.

At this point, we have established a crude method for the consistent identification of a $TLR2^{+}$ T cell subpopulation. However, there is a clear need to expand these cells in order to achieve full phenotyping and functional characterisation. There are several methods that could be attempted in the future to achieve this. As we have established that addition of PHA is the best method for generating this subpopulation (potentially via a CD2-

mediated pathway), it is possible that re-stimulation with PHA or anti-CD2 every 3 days could maintain or expand the cells. Other stimulation conditions could also be trialled, as we now have a better idea of the phenotype of these cells. IL-7/IL-15 stimulation is of particular interest, as it has been shown to generate a memory regulatory T cell, a potential phenotype for our $TLR2^{+}CCR5^{+}$ subpopulation.[104]

Isolating these cells for culture alone could also be of benefit; the majority of studies that have reported TLR2-expressing T cells have isolated certain subsets, such as $CD4^{+}$ cells, in their experiments (see Tables 2A and 2B). This could be achieved through the use of magnetic bead-based cell selection, or use of fluorescence-activated cell sorting, and could help to expand the population of interest.

4.1 Conclusions

The experiments we have carried out have confirmed $TLR2^+$ and $TLR2^+$ $CCR5^+$ T cells exist in our samples, and that these cells are a mixed population. They consist of Tregs, memory cells and a mix of $CD4^+$ and $CD8^+$ cells. The majority of $TLR2^+$ cells appear to be $TLR2^/CCR5$ double-expressing, and the subpopulation seems to be $CD25^+$, based on staining for CD25 as well as the fact that the $TLR2^+$ is only identifiable after PHA stimulation.

We were unable to expand our cells, and this impacted our ability to fully characterise the subpopulation. It does appear, however, that this is a transient phenotype that disappears from culture. This could be directly caused by the withdrawal of PHA stimulus. Alternatively, the temporary nature of the phenotype could be functional; it is possible that these cells require further cytokine or antigen stimulation after activation in order to proliferate. Observation of the behaviour of this subpopulation *in vivo* could elucidate the cause of this transient phenotype.

Other groups have reported the existence of this subpopulation, and have reported that it other methods of stimulation can be used to induce TLR2 expression, including anti-CD3, TLR2 ligands (such as LTA) or IFN-α (see Tables 2A and 2B). However, we were unable to see any increase in TLR2 expression with these methods. One explanation for this could be our use of live/dead stain in order to discriminate between viable positive cells and non-specific antibody binding to dead cells or cell fragments. Fixable live/dead stain was not readily available at the time when the papers in Table 2 were published, and there is no mention in any of the studies of using staining to assess cell viability. It is also possible that TLR2 expression was easier to identify, as the authors of the papers used isolated cell subpopulations, such as pure $CD45RO^+$ or $CD4^+$ cells. As the sample size is so small, differences in sample quality, and variation in numbers of cell subsets between donors has a large impact on our results. In addition, the presence of monocytes made the characterisation of the TLR2+ subpopulation more problematic, especially in the fresh total PBMCs. The use of isolated cells could resolve these issues, and should be used in

the future to see if differences in expression can be seen between PHA/IL2 stimulation and the alternative stimulation methods set out in Tables 2A and 2B.

The significance of this cell population is unclear; however, we do have an idea of some of the phenotypes that can be found within the $TLR2^+$ subpopulation. TLR2 is thought to play a role in regulating the suppressive function of Tregs, and there is evidence that CCR5 has a similar function. This could be an important pathway when considering the role of regulatory T cells in the pathogenesis of cancer and autoimmune disease. A better understanding of the pathways that control the ability of Tregs to suppress other immune cells could allow the development of drugs that mediate their anti-inflammatory action. Possible uses for this could include disrupting the recruitment of Tregs by tumours, or the prevention of the excessive inflammation characteristic of autoimmune conditions.[44] However, careful investigation of these pathways would be needed in order to assess the clinical implications and potential for adverse effects caused by the disruption of Treg function, given the links between loss of Tregs and autoimmune diseases such as lupus erythematosus and rheumatoid arthritis.[105] This would require large numbers of cells belonging to the identified subpopulation, and therefore a method for their expansion would need to be established. Additionally, this subpopulation does not encompass all of the regulatory T cell population, and so may not be a suitable target for systemic therapies targeting Treg dysfunction.

The role of TLR2 on memory T cells is less obvious, and possibly relates to the maintenance of their memory function. However, dysfunction of these cells is known to cause autoimmune reactions, as the cells continue to cause inflammation long after antigen stimulation has ceased.[106] Again, an understanding of a communication pathway involving TLR2 would be of benefit, and could possibly provide a therapeutic target for the regulation and control of dysfunctional cells.

The downmodulation experiment that we carried out did not produce evidence of a communication pathway between TLR2 or CCR5. However, the fact that we found all of the $TLR2^+$ cells in our fresh sample to also express CCR5 suggests that there possibly is a link between the two receptors. It is possible that there is a pathway that links TLR2 and CCR5 expression, but that it is different than the one seen in monocytes. Conversely,

when the Signoret lab examined the monocyte downmodulation pathway, the small subpopulation of $TLR2^+$ T cells was not detectable when compared to the high levels of TLR2 expression on monocytes.[62] It is therefore possible that our threshold for detection of downmodulation on these T cells is simply not low enough to pick up small changes in TLR2 or CCR5 expression, and that a cross-talk pathway does indeed exist for these cells.

It is clear there are many questions raised by our experiments; whilst our methods are relatively crude, we have established that this previously-reported subpopulation can indeed be found in fresh and frozen PBMCs. Furthermore, we have found CD25 to be a possible marker of this subpopulation, and have confirmed that there is expression of CCR5, CD45RO and FoxP3 within these cells. We have been able to build a solid basis for future research, and it is likely that further investigation of these cells will yield interesting results.

5. Abbreviation list

APC: Antigen-presenting cell

BCR: B cell receptor

BM: Binding medium

CCR5: CC receptor 5

CD: Cluster of differentiation (e.g. CD4, CD8)

DAMP: Damage-associated molecular pattern

FBS: Fetal bovine serum

FoxP3: Forkhead box P3

FSC: Forward scatter

FSC-H: Forward scatter- height

FSC-H: Forward scatter- area

HEPES: (N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid)

IFNα: Interferon-alpha

IL: Interleukin (e.g. IL-2)

LPS: Lipopolysaccharide

LTA: Lipotechoic acid

MAC: Membrane attack complex

MHC: Major histocompatibility complex

MRSA: Methicillin-resistant *Staphylococcus aureus*

PAMP: Pathogen-associated molecular pattern

PBMCs: Peripheral blood mononuclear cells

PBS: Phosphate-buffered saline

PHA: Phytohaemagglutinin

PRR: Pattern-recognition receptor

SSC: Side scatter

SSC-H: Side scatter- height

SSC-H: Side scatter- area

Th17: T helper 17

TCR: T cell receptor

TLR2: Toll-like receptor 2

Treg: Regulatory T cell

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