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Faculdade de Farmácia


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Dissertation supervised by Professor Helena Soares and co-supervised by Professor João Gonçalves

Master Degree Biopharmaceutical Sciences

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*I have a deeply hidden and inarticulate desire for something beyond the daily life.*

Virginia Woolf

# ABSTRACT

The mammalian immune system has evolved to provide protection from environmental threats, eliminating pathogenic organisms and toxins whilst avoiding an excessive response in the presence of commensal organisms and bacteria. The disruption of this perfectly balanced mechanism of immune responsiveness often results in chronic inflammation and autoimmune disease. A better comprehension of the mechanisms that regulate immune tolerance is thus crucial to develop therapies that allow us to control exacerbated reactions in autoimmune illness.

Toll-like receptors (TLRs) detect conserved microbial patterns and largely contribute to the rapid initiation of the highly complex protective mechanism that is the immune response. Despite having been intensively studied for their expression and role in innate immune cells, recent evidence shows that T lymphocytes also express these receptors and that their expression may directly affect cell function. T lymphocytes are a key component of the adaptive arm of the immune system and respond to pathogen encounters by releasing cytokines that dictate the outcome of the inflammatory response. Particularly, previous data from our group has demonstrated that human $CD4^+$ T cells express TLR4, the molecular sensor of bacterial liposaccharide (LPS).

In the present work, we provide evidence that the activation of TLR4 has a direct impact in human $CD4^+$ T cells, without interference from innate immune cells. The chronic stimulation of these cells with non-polarizing TCR stimulation and LPS leads to enhanced activation, survival and proliferation. Furthermore, the same stimulation re-directed helper T cells into expressing lower levels of the pro-inflammatory cytokine interferon-gamma, but it increased the production of interleukin (IL) 17 and the immunoregulatory IL-10. Additionally, we isolated $CD4^+$ T cells from human liver samples that simultaneously expressed markers of tissue residency and TLR4, which may broaden these findings to tissue resident T cells.

Our results support the view that a consistent activation of TLR4 in $CD4^+$ T cells in response to commensal bacteria leads to re-direction of IL-17-producing cells to a more tolerogenic phenotype. These findings thus pave the way for new studies that focus on the role of direct TLR signaling in T cells and their contribution to immunotolerance.

**Keywords:** Toll-like receptor 4; Human helper T cells; T cell plasticity; Immune tolerance; CD4 tissue resident cells;

# RESUMO

O sistema imunitário dos mamíferos evoluiu de forma a proteger o hospedeiro e a eliminar organismos patogénicos, enquanto, simultaneamente evita uma resposta imune excessiva perante bactérias e organismos comensais. A disrupção do equilíbrio presente nos mecanismos desta resposta resulta frequentemente em inflamação crónica ou doenças autoimunes. Uma melhor compreensão dos mecanismos que regulam a tolerância do sistema imune é então crucial para o desenvolvimento de terapias que permitam o controlo de reações exacerbadas em situações de autoimunidade.

A resposta imunitária é geralmente dividida em duas partes, sendo estas denominadas resposta inata e resposta adaptativa. A reposta inata é o primeiro obstáculo encontrado por um microrganismo, e inclui tanto barreiras físicas composta por células epiteliais como células do sangue especializadas na destruição e fagocitose dos invasores. Enquanto que esta é uma resposta imediata mas pouco específica, a resposta inata, por outro lado, é altamente específica mas de ação mais lenta, demorando vários dias a ser desenvolvida.

Os linfócitos T são componentes chave do sistema imune adaptativo e respondem à presença de patógenos libertando citoquinas que recrutam e ativam diferentes tipo de células, ditando assim o desfecho da resposta inflamatória. Quando são ativados, as células T podem diferenciar-se em diferentes subtipos, que por sua vez vão mediar diferentes respostas. Enquanto que certos subtipos ditam respostas inflamatórias e levam a uma maior ativação da resposta imune, outros tipos de linfócitos T, intitulados células T reguladoras, atenuam esta resposta, evitando assim uma resposta exacerbada.

Um subtipo particular das células T, denominadas de células T auxiliares 17 (ou Th17), tem sido bastante estudado devido ao seu envolvimento em condições de inflamação crónica. Estas células caracterizam-se pela produção da interleucina 17 (IL-17), mas também pela sua capacidade de adotarem diferentes fenótipos em microambientes diferentes. Um acumular de estudos tem vindo a demonstrar que as células Th17 podem expressar citoquinas características de outros subtipos de células T, e têm até a capacidade de serem totalmente convertidas em células reguladoras. Os mecanismos que levam à conversão destas células em fenótipos inflamatórios ou reguladores podem ter consequências cruciais na falta de tolerância que está por detrás de doenças crónicas inflamatórias e devem por isso ser investigados.

As células do sistema inato utilizam recetores que reconhecem padrões moleculares característicos de diferentes patógenos. Entre estes recetores, os recetores do tipo Toll (TLRs) são os melhor descritos e contribuem para a rápida iniciação da resposta imunitária. No entanto, um acumular de estudos recentes demonstra que os linfócitos T também expressam estes TLRs e que a sua expressão pode afetar diretamente as funções da célula. Em particular, resultados anteriores do nosso grupo demonstraram que as células T $CD4^+$ humanas expressam TLR4, o recetor que reconhece lipopolissacarídeos (LPS) presentes na parede celular de bactérias.

Foi ainda demonstrado por outros estudos que a expressão do TLR4 em células T $CD4^+$ tem um papel importante em doenças inflamatórias crónicas tais como a colite, ou a esclerose múltipla. Contudo, os resultados variam de acordo com o modelo de doença utilizado, sugerindo que a presença de sinalização através do TLR4 tem consequências distintas de acordo com o microambiente onde a célula se insere. Neste projeto, foram isoladas células $CD4^+$ T e mantidas em cultura na presença de LPS, de forma a reproduzir o ambiente a que estas células estão sujeitas no intestino humano, onde existe uma enorme variedade de bactérias comensais. Sucintamente, amostras de sangue foram recolhidas de dadores saudáveis e procedeu-se ao isolamento das células mononucleares periféricas (PBMCs), sendo que a partir destas, os linfócitos T $CD4^+$ foram isolados por citometria de fluxo com uma pureza de aproximadamente 99.5%. Para além do LPS presente no meio de cultura celular, foram ainda utilizados anticorpos direcionados aos recetores CD3 e CD28, essenciais para a ativação dos linfócitos T.

Os nossos resultados indicam que esta estimulação crónica do TLR4, juntamente com a estimulação não-polarizante do recetor das células T (TCR), pode redirecionar as células $CD4^+$ T para um fenótipo mais tolerante. Mais especificamente, a cultura destas células durante 5 dias na presença de LPS levou a uma diminuição da produção da citoquina pró-inflamatória interferão-gama (IFN-γ) e a um aumento na produção de IL-10 e IL-17. Enquanto que a IL-10 é uma citoquina essencial para respostas anti-inflamatórias, a IL-17 é tipicamente produzida pelas Th17, um subtipo marcado pela sua plasticidade. De facto, é de esperar que um encontro crónico com bactérias exija um delicado equilíbrio entre a produção de citoquinas inflamatórias e reguladoras, de forma a manter uma resposta apropriada na presença de patógenos enquanto simultaneamente é evitada uma resposta exagerada perante bactérias comensais.

Adicionalmente, esta mesma estimulação levou ainda a um aumento da ativação, sobrevivência, e proliferação das células T. Foi ainda observado que esta influência na ativação e sobrevivência é mais significativa quando são utilizadas menores concentrações de anticorpo para estimular o recetor CD3. Assim, os resultados sugerem que o TLR4 pode funcionar como um sinal de co estimulação para o desenvolvimento e manutenção das células $CD4^+$ T na presença de sinais provenientes de patógenos. Desta forma, no presente trabalho demonstramos que a ativação do TLR4 tem um impacto direto em células $CD4^+$ T humanas, sem a interferência de células do sistema inato.

Numa segunda parte deste projeto, foram ainda isoladas células T $CD4^+$ de amostras de fígado humano, que expressavam simultaneamente marcadores de tecido e o recetor TLR4. Estas observações preliminares podem assim indicar que as nossas observações anteriores se alargam ainda a células que residem no tecido. De facto, as células T que residem em tecidos têm ganho bastante atenção ao longo dos últimos anos, devido ao seu papel na vigilância e manutenção da resposta imunitária. De certa forma semelhante ao intestino, apesar de a uma menor medida, também no fígado as respostas imunes requerem um equilíbrio entre respostas inflamatórias e tolerância imune. Isto deve-se ao facto da veia porta transportar muitas vezes moléculas derivadas de microrganismos provenientes do trato gastrointestinal. Adicionalmente, foi já demonstrado por outros estudos que o fígado apresenta vários mecanismos que contribuem para uma tolerância imunológica, tais como a ativação preferencial de células

com fenótipos reguladores e uma baixa expressão de TLR4 em células inatas. Desta forma, apesar de serem necessários mais estudos envolvendo células T $CD4^+$ residentes, é possível que a expressão do TLR4 nestas células também participe em mecanismos que contribuem para redirecionar as células imunes do fígado para um fenótipo mais tolerante.

Os resultados obtidos no decorrer deste projeto apoiam assim a ideia de que uma ativação consistente do TLR4 em células T $CD4^+$, em resposta a bactérias comensais, levam à redirecção das células produtoras de IL-17 para um fenótipo mais tolerante. Apesar de não nos ter sido possível desvendar quais os mecanismos moleculares que estão por detrás da influência do TLR4 nos linfócitos T, é essencial realizar mais estudos que o permitam fazer. Ao perceber quais as moléculas de sinalização celular que regem esta influência, tornar-se-á possível entender de que forma é regulado o equilíbrio entre respostas pró e anti-inflamatórias. É imperativo entender como é controlada a plasticidade das células do sistema imunitário de forma a desenvolver abordagens farmacêuticas que permitam tanto promover a tolerância na autoimunidade como quebrá-la em caso de infeção crónica.

Assim, estes resultados possibilitam a realização de novos estudos que se foquem no papel da ativação direta do TLR4 em linfócitos T e na sua contribuição para a tolerância no sistema imunitário.

**Palavras-chave:** Recetor do tipo Toll 4; células T auxiliares humanas; plasticidade de células T; tolerância imunitária; células CD4 residentes no tecido;

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# LIST OF ABBREVIATIONS

**AKT:** (PKT) Protein kinase B
**APC:** Antigen-presenting cell
**BCL:** B-cell lymphoma
**BCL-xL:** B-cell lymphoma extra-large
**BCR:** B cell receptor
**BFA:** Brefeldin A
**BSA:** Bovine serum albumin
**CCL:** Chemokine (C-C) motif ligand
**CD:** Cluster of differentiation
**CLR:** C-type lectin receptor
**CNS:** Central nervous system
**CXCR5: (**C-X-C) chemokine receptor type 5
**DAMP:** Danger associated molecular pattern
**DC:** Dendritic cells
**DMSO:** Dimethyl sulfoxide
**DNA:** Deoxyribonucleic acid
**EAE:** Experimental autoimmune encephalomyelitis
**ERK:** Extracellular signal-related kinase
**ETP:** Early thymic progenitors
**FACS:** Fluorescence-activated cell sorting
**FBS:** Fetal Bovine Serum
**Foxp3:** Forkhead box protein 3
**FSC:** Forward scatter
**HBSS:** Hank's Balanced Salt Solution
**ICOS:** Inducible T-cell co-stimulator
**IFN:** Interferon
**IL:** Interleukin
**IRF:** Interferon regulatory factor
**ITAM:** Immunoreceptor tyrosine-based activation motif
**IU:** International units
**KLF2:** Krüppel-like Factor 2
**LCK:** Lymphocyte-specific protein tyrosine kinase
**LPS:** Lipopolysaccharide
**MAPK:** Mitogen-activated protein kinase
**MHC:** Major histocompatibility complex

**MyD88:** Myeloid differentiation primary response protein 88
**NF-kB:** Nuclear factor kappa B
**NK:** Natural killer
**NLRP2:** NOD-like receptor family pyrin domain containing 2
**NLRs:** NOD-like receptor
**NOD:** Nucleotide-binding oligomerization domain
**PAMP:** Pathogen associated molecular pattern
**PBMC:** Peripheral blood mononuclear cell
**PBS:** Phosphate buffered saline
**PD-1:** Programmed cell death protein-1
**Pen/Strep:** Penicillin/Streptomycin
**PFA:** Paraformaldehyde
**PLL:** Poly-L-lysine
**PMA:** Phorbol 12-myristate 13-acetate
**RAR:** Retinoic acid receptor
**RIG-I:** Retinoic acid-inducible gene I
**RLR:** RIG-like receptor
**RNA:** Ribonucleic acid
**RORγT:** RAR-related orphan receptor gamma
**RPMI:** Roswell Park Memorial Institute medium
**S1PR1:** Sphingosine-1-phosphate receptor-1
**SSC:** Side scatter
**STAT:** Signal transducer and activator of transcription
**Tbet:** T-box transcription factor
**TCR:** T-cell receptor
**Tfh:** Follicular helper T
**TGF-β:** Transforming growth factor-beta
**Th:** T helper
**TIR:** Toll/interleukin-1 receptor
**TIRAP:** TIR domain-containing adaptor protein
**TLR:** Toll-like receptor
**TNF-α:** Tumor necrosis factor-alpha
**TRAF:** TNF receptor-associated factor
**TRAM:** TRIF related adaptor molecule
**Treg:** T regulatory
**TRIF:** TIR-domain containing adaptor-inducing interferon-β
**TRM:** Tissue resident memory
**ZAP70:** Zeta-chain-associated protein kinase

# 1| INTRODUCTION

## 1.1 The immune system

The mammalian immune system has evolved to provide protection from a wide range of environmental threats, discriminating between beneficial commensal organisms and pathogenic organisms and toxins. An interactive network of lymphoid organs, cells, humoral factors, and cytokines shape this highly complex protective mechanism that may be generally divided into two parts, determined by the speed and specificity of the reaction. These are titled the innate and adaptive responses and interact with one another in order to eliminate pathogens whilst avoiding an excessive response that would damage the host's own tissues[1].

### 1.1.1 Innate response

The innate immune response constitutes the first obstacle encountered by an invading pathogen, and it includes physical barriers such as epithelial cell layers that express tight cell to cell contacts, as well as proteins and bioactive molecules present in biological fluids. When the pathogen invades these barriers, an array of both hematopoietic and nonhematopoietic cells come into play and respond to damage or infection[2]. Most cells involved in the innate response are of hematopoietic nature - namely macrophages, dendritic cells, neutrophils, and natural killer cells. However, epithelial and parenchymal cells also play their part by producing enzymes and permeabilizing peptides that neutralize microorganisms, as well as chemokines that recruit granulocytes and T cells[3].

Inflammatory signals such as chemotactic factors and cytokines rapidly recruit neutrophils from the blood stream to the infection site. Neutrophils then recognize and phagocyte organisms that have been opsonized by antibodies or by the complement system. Lastly, the pathogens are destroyed through a combination of cytotoxic mechanisms.

Shortly after the recruitment of neutrophils, monocytes are recruited and migrate from peripheral blood to the inflammatory site where they convert into tissue macrophages and exert important functions to both innate and adaptive immunity[4]. Once activated, the main function of a macrophage is to recognize and phagocyte pathogens, using the production of nitric oxide as a major killing mechanism. However, these cells can adopt one of several phenotypes and release large amounts of cytokines according to

the signals they receive. While classically activated macrophages release pro-inflammatory cytokines such as interferon-gamma (IFN-γ) or tumor necrosis factor-alpha (TNF-α), alternatively activated macrophages acquire anti-inflammatory functions through the release of interleukin-10 (IL-10) and transforming growth factor-beta (TGF-β). Furthermore, after the pathogen is eliminated, monocytes and macrophages undergo phenotypic and functional changes to play crucial roles in tissue repair, for example, the production of extracellular matrix proteins[5]. These cells are also able to process microbial antigens and present them to T cells. However, the most relevant antigen-presenting cells are dendritic cells (DCs), thus playing an important role in the bridge between innate and adaptive immunity.

DCs are widely distributed as immature cells within all tissues and lymphoid organs, and express a variety of chemokine receptors that allow them to respond to stimuli and be attracted to areas of inflammation. Once activated, they upregulate co-stimulatory molecules and migrate to lymphoid organs, the spleen and the lymph nodes, where they present antigens to T-helper cells via major histocompatibility complex (MHC) class II molecules[6].

In turn, Natural Killer (NK) cells recognize threats using negative selection – while T and B cells are activated after recognition of an antigen present in MHC class II molecules, NK cells are activated when MHC class I molecules are not recognized on the host cell, thus detecting infected cells in which the expression of surface markers is altered. Once activated, NK cells exert cytotoxic activity mainly by the release of perforin, serine proteases and granzymes, which induce apoptosis in the target cells or by caspase-dependent apoptosis[7].

The immune response is activated due to pattern recognition receptors (PRRs), which help the cells identify the "non-self" by recognizing pathogen-associated molecular patterns (PAMPs). The innate system is also able to recognize damage-associated molecular patterns (DAMPs), which are molecules that are released during infection and inflammation and when recognized promote pro-inflammatory and profibrotic pathways [8]. PRR engagement in innate immune cells triggers intracellular signaling cascades, which include adaptor molecules, kinases, and transcription factors, that ultimately result in the synthesis of cytokines, chemokines, cell adhesion molecules and immunoreceptors.

PRRs are divided into four families that differ in ligand recognition, signal transduction and sub-cellular localization. Toll like-receptors (TLRs) are transmembrane proteins with more than 10 distinct sub-families identified in humans, with each recognizing distinct PAMPs derived from viruses, bacteria, fungi and protozoa. C-type lectin receptors (CLRs) are found at the plasma membrane and recognize glycans from fungi and some bacteria, and its engagement mediates endocytosis and influences intracellular signaling pathways through classical immunoreceptor tyrosine-based activation motifs (ITAMs). Cytosolic PRRs that recognize microorganisms independently of TLRs are broadly divided into retinoid acid-inducible gene I (RIG-I)-like receptors (RLRs) and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs). RLRs are RNA helicases that recognize viral RNA released into the cytoplasm and trigger antiviral responses via type I IFN induction. NLRs comprise more than 20 sub-families that recognize a broader spectrum of PAMPs, including viral and

bacterial DNA, where different receptors have different functions – for example, NOD2 induces autophagy while NOD1 activates the nuclear factor kappa B (NF-κB) and mitogen-activated protein kinases MAPK signaling pathways, but others, including NLRP2, act as negative modulators of the NF-kB pathway[9].

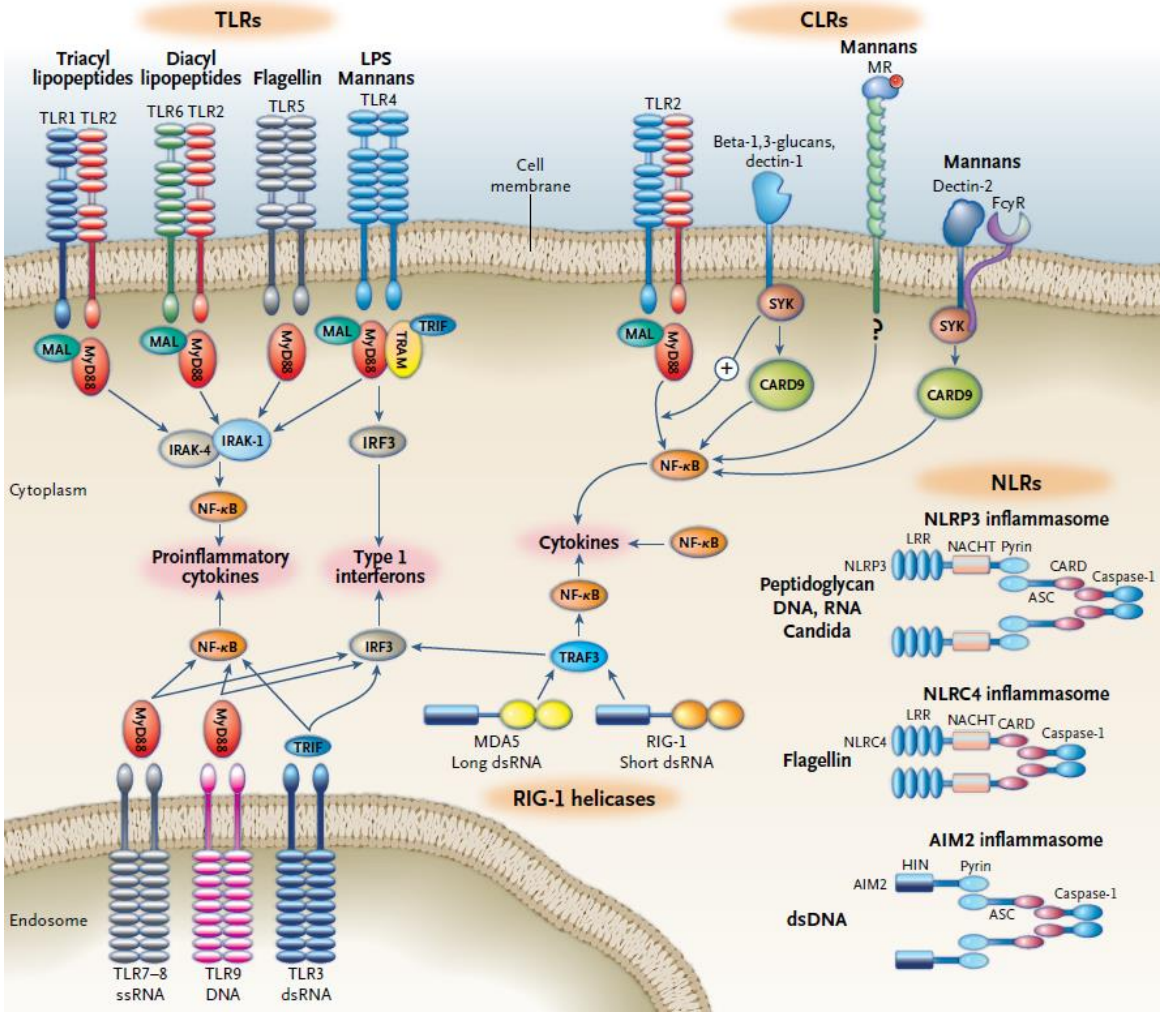


**Figure 1**-Erro! Marcador não definido. – **Major classes of pattern recognition receptors and their ligands.** TLRs are a large family of transmembrane proteins that recognize flagellin and lipoproteins from pathogens extracellularly, and pathogenic RNA and DNA intracellularly. CLRs recognize glycans and, like TLRs, lead to the production of cytokines. RIG-1 receptors are intracellular receptors of RNA that lead to the production of cytokines. NLRs are involved in the formation of inflammasomes.
**(**Netea, M. G. & Van Der Meer, J. W. M, 2011)

### 1.1.2 Toll-Like Receptors

Toll-like receptors (TLRs) are the major and most extensively studied type of PRRs. Their function is to recognize conserved molecular patterns characteristic of pathogens and initiate signaling pathways that will both activate an innate immune response and prepare an adaptive response[10]. Although the expression of these receptors is most predominant in cells of the innate response, such as macrophages and DCs, recent work has indicated a role for direct TLR-signaling in cells of the adaptive immune response, namely T and B lymphocytes[11,12].

There are 10 described families of TLRs in humans (TLR1-TLR10), and these may be located either at the cell surface or intracellularly, in endosomes and lysosomes[13]. Whereas cell surface TLRs primarily recognize microbial membrane components, including lipids, lipoproteins and proteins, intracellular TLRs are activated by nucleic acids derived from bacteria and viruses. These transmembrane receptors possess an extracellular leucine-rich repeat domain responsible for ligand recognition and a highly conserved intracellular toll/interleukin-1 receptor-like (TIR) domain crucial for signal transduction. Different TLRs lead to specific responses, which is explained by the differently recruited TIR-domain containing adaptor molecules. TLRs signaling pathways can be largely classified as MyD88-dependent pathways, or as TRIF-dependent pathways[14]. The MyD88 pathway is universally used by all TLRs, expect TLR3, and leads to the activation of NF-kB and MAPK and consequent induction of inflammatory cytokines. On the other hand, TRIF is used by TLR3 and TLR4 and uses the activation of interferon regulatory factor 3 (IRF3) and NF-kB to induce both inflammatory cytokines and type I interferon. TRAM and TRIP are two other adaptor molecules that recruit TRIF to TLR4 and MyD88 to TLR2 and TLR4, respectively.

It is important to note that while these pathways are relatively well described in innate immune cells, a lot less is known of T cells, where TLRs have also been found to be expressed and influence cell function[15].

Importantly, TLR4 is the only described TLR capable of using all four adaptors and activate both pathways[16]. Initially, cell membrane-bound TLR4 recruits TIRAP to facilitate the recruitment of MyD88 and consequently trigger the activation of MAPK and NF-kB and thus induce the production of proinflammatory cytokines. However, TRL4 also suffers dynamin-dependent endocytosis and TRAM and TRIF adaptor molecules form a complex at the endosome where the TRIF-dependent pathway is initiated, leading to the expression of the anti-inflammatory cytokine IL-10 and the regulatory IFN-β[14]. Additionally, several studies have come to demonstrate that IL-10 production in macrophages and DCs relies on the activation of extracellular signal–regulated kinase (ERK) and enhanced p38 signalling[17].

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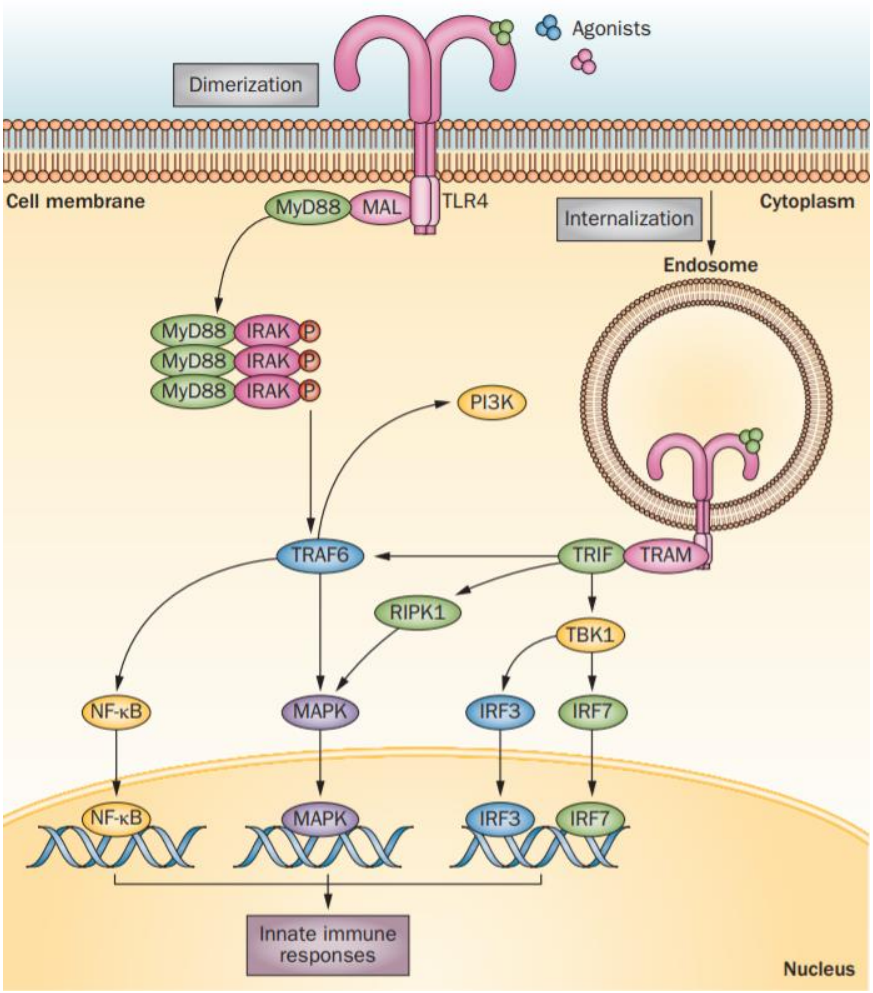


**Figure 1-1 TLR4 signaling in innate immune cells.** Engagement at the cell membrane activates the MyD88-dependent pathway that leads to the activation of transcription factors such as NF-kB and consequently express pro-inflammatory cytokines. TLR4 can also be internalized and signal through the TRIF-dependent pathway, leading to the activation of IRFs and consequent release of regulatory molecules such as IFN-γ. (Gómez et al., 2014)

### 1.1.3 Adaptative response

The adaptive arm of the immune response comes into play several days after pathogen encounter, when the innate response is ineffective in eliminating the infection. The characteristic cells of this response are T and B lymphocytes, responsible for the cell-mediated response and the humoral response, respectively. In contrast to the innate cells that recognize generic patterns present in a wide range of microorganisms, the adaptive system allows a stronger response, tailored for a specific pathogen[18]. In this manner, lymphocytes are capable of recognizing pathogens that have evolved to evade innate responses, including bacteria that have a protective capsule that conceals PAMPs

expressed at the surface or viruses that carry no invariant molecules and are rarely directly recognized by macrophages.

T cells derive from hematopoietic stem cells in bone marrow that later migrate to mature in the thymus, hence their denomination. They express an infinitude of unique antigen-binding receptors on their surface, named T-cell receptors (TCRs) that can bind specific foreign peptides presented by Antigen-presenting cells (APCs) through the major histocompatibility complex (MHC). When a T cell encounters the APC presenting the corresponding antigen, its activation begins, leading to a process of clonal expansion that creates an array of cells that will recognize the same antigen[19]. The antigen presentation process leads to the proliferation and differentiation of T cells into either cytotoxic ($CD8^+$) T cells, that are primarily involved in the destruction of infected cells, or helper ($CD4^+$) T cells, that maximize the immune response by activating other cells. Importantly, after the infection is cleared, some T cells are retained as memory cells that can rapidly differentiate into effector cells when they encounter the same antigen. The functions and characteristics of these cells will be discussed in further detail in the next section.

B lymphocytes start their maturation in the bone marrow and then migrate through the blood to secondary lymphoid organs. Similarly to T cells, each B cell expresses only one unique type of B cell receptor (BCR) to ensure the specificity of the response[20]. The large repertoire of receptors is accomplished through the rearrangement of the Ig gene segments and nucleotide insertion mechanism during the maturation of the cell. Unlike T cells, B cells are able to directly recognize foreign antigens, which leads to their activation and consequent proliferation and differentiation into antibody-producing plasma cells and memory B cells. These plasma cells secrete only one type of antibody, specific for the antigens that lead to its activation, once more highlighting the high specificity of the adaptive response. The secreted antibodies bind to the surface of pathogens, marking them for destruction by opsonization and promoting phagocytosis by immune effector cells. Memory B cells will survive long past the infection and quickly respond to re-infection by antibody production, whilst plasma cells are short-lived and undergo apoptosis when the pathogen is eliminated[20]. Both T and B lymphocytes that would react to “self” antigens either suffer apoptosis or become anergic during their development.

Adaptive immunity is thus mainly characterized by its high specificity towards the pathogen and its ability to keep memory cells that protect the host against re-infection, sometimes during an entire life span.

**Table 1-1** Major differences between the innate and adaptative responses of the immune system

| | Innate | Adaptative |
|---|---|---|
| **Major cell types** | Monocytes, Macrophages, NK, DCs | B and T lymphocytes |
| **Action time** | Immediate effector response | Delayed response |
| **Specificity** | **Low specificity:** triggered by generic pathogen patterns | **Very specific:** targets a specific pathogen |
| **Memory** | No memory – although some cells present trained immunity | Retains memory and improves response after re-exposure |
| **Receptors** | PRRs | TCR and BCR |

## 1.2 T cell mediated immunity

T cells are produced and complete their development in the thymus and posteriorly circulate between the bloodstream and peripheral lymphoid tissue as naïve T cells[21]. In the thymic cortex, cells named early thymic progenitors (ETP) differentiate into double negative cells that will later follow a selection process and differentiate into either $CD4^{-}CD8^{+}$ or $CD4^{+}CD8^{-}$ cells. The expression of either CD4 or CD8 on the surface of T-lymphocytes distinguishes them and is related to different functions: whilst $CD8^{+}$ cells, known as cytotoxic T-cells, directly kill infected or malignant cells, $CD4^{+}$ cells generate cytokines that activate neighboring cells and chemokines that recruit new immune cell subsets[22].

$CD4^{+}$ T cells, also termed helper T cells, are extremely important in mediating the immune response, as dramatically demonstrated in AIDS patients, where the depletion of these cells leads to infections by pathogens that are harmless under normal circumstances[23]. These cells help activate macrophages at sites of infection by secreting IFN-γ, which binds to receptors on the macrophage surface. The presence of T helper cells at the infection site also helps recruit monocytes and neutrophils by releasing cytokines that increase their production, by secreting chemokines that direct their migration and also by activating endothelial cells to express cell adhesion molecules that cause the phagocytic cells to adhere at the site. The secretion of IFN-γ by T helper cells also increases the efficiency with which a cytotoxic T cells kills virus-infected target cells. Additionally, $CD4^{+}$ T cells can help stimulate B cells to differentiate into either effector cells that secrete antibodies or memory cells[24]. The stimulation of B cells leads to the secretion of antibodies that coat extracellular pathogens and activate the complement system, as well as bind to basophils and eosinophils that release local mediators.

Cytotoxic T cells, however, are particularly important in the defense against intracellular pathogens and in tumor surveillance. These cells directly eliminate infected or malignant cells by the release of perforins, that form a pore in the membrane of the target cells, and granzymes that enter the cells and cleave its proteins. Furthermore, they destroy infected cells by activating a caspase cascade by Fas/FasL interactions that result in apoptosis. $CD8^+$ T cells also secrete TNF-α, a proinflammatory cytokine that can initiate apoptotic signaling, and IFN-γ that, as already mentioned, activates effector cells[25].

During their maturation, T cells acquire receptors, named T-cell receptors (TCR), that recognize a specific antigen when presented by MHC molecules. These receptors contain two variable antigen-binding chains which are highly polymorphic and thus allow the recognition of a great variety of peptides. The rearrangement of the TCR gene, with the binding and transcription of several gene segments, is a critical step in the development of mature T-lymphocytes. The somatic recombination of genes encoding the variable segments of the antigen-binding chains is responsible for the vast variety of TCRs present in the human body[21].

For an immune response to happen, naïve T cells must be activated in response to an antigen, through multiple coordinated interactions between TCRs and the MHC molecules present in APCs[26]. The activation requires antigen recognition, mediated through adhesion molecules expressed on the naïve T cells, which leads to several pathways that culminate in the secretion of interleukin-2 (IL-2), as well as in the expression of integrins that promote cellular adhesion and in the expression of anti-apoptotic proteins. For T-cells to be activated, a co-stimulatory signal that induces the activation of the nuclear transcription factor NF-κB and consequently potentiates the production of IL-2 is also required. In the case of helper T cells, CD28 is expressed on their surface and interacts with either CD80 or CD86 on the APC. A third signal has been identified as a requirement for a productive T cell response, which will determine the type of responder cell that arises from the naïve T cell. This last signal comes in the form of inflammatory cytokines, such as IL-1, IL-12 and IFN-α/β. Hence, a process of clonal expansion begins and leads to proliferation of T cells with identical receptors that are thus able to uniquely recognize the antigen responsible for its activation[27].

### 1.2.1 Subsets of CD4 T cells

In response to a pathogen encounter, the immune cells of the host release several inflammatory cytokines that will influence the differentiation of antigen-stimulated T-cells into different T helper subsets. These subsets are defined by the cytokines they produce and the expression of characteristic lineage-defining transcription factors, and each one mediates different responses.

T helper (Th) 1 subset will develop in the presence of large amounts of IL-12 produced by APCs and IFN-γ secreted by NK cells. The t-box transcription factor (T-bet) is the key regulator to induce full differentiation of the Th1 subset, by enhancing the production of IFN-γ. Th1 cells are predominant in responses towards intracellular pathogens and secrete IL-2, TNFα and IFN-γ, thus helping in macrophage activation, nitric oxide production and $CD8^+$ T cell proliferation[28].

On the other hand, the presence of both IL-4 and IL-2 are critical for the differentiation of the Th2 subset, which depends on signal transducer and activator of transcription 6 (STAT6). This subset has been described to be predominant in the response against extracellular pathogens and release IL-5, IL-13 and IL-4, which stimulate B lymphocytes to further mature, to produce antibodies and even to acquire a memory phenotype[29].

The more recently discovered Th17 subtype is characterized by the expression of the cytokine family IL-17, and it has been shown to be involved in the pathogenesis of several autoimmune disorders, such as rheumatoid arthritis, experimental autoimmune encephalomyelitis (EAE), colitis, and lupus. Given the great importance of this subset in pathologies of the immune system, its functions are described in further detailed in the next section.

Yet another distinct subset is termed follicular T helper (Tfh), mainly localized in secondary lymphoid organs, where they interact with B cells and help in the formation of germinal centers. These cells are identified by the high expression of PD-1 in combination with CXCR5. They also express high levels of inducible T-cell co-stimulator (ICOS) and its master transcription factor B-cell lymphoma-6 (BCL-6), and have shown to be essential for the formation of most high affinity antibodies and memory B cells[30].

Regulatory T cells (Tregs) are majorly regulated by the transcription factor forkhead box protein 3 (Foxp3) and serve to regulate the effector functions of T cells and avoid damage to the host. They act by regulating APC stimulatory molecules, by inducing T cells apoptosis through granzyme and perforin release, and by expressing inhibitory cytokines such as IL-10 and TGF-β. Importantly, these cytokines re-establish immune quiescence after the invading pathogen is eliminated and help maintain self-tolerance[31].

The complexity of the immune response is thus partly demonstrated by the plethora of different functions that $CD4^+$ T cells exert, where the heterogeneity of the populations is assured by the stem-cell like properties of naïve T cells that can differentiate into any type of effector, regulatory or memory cells.

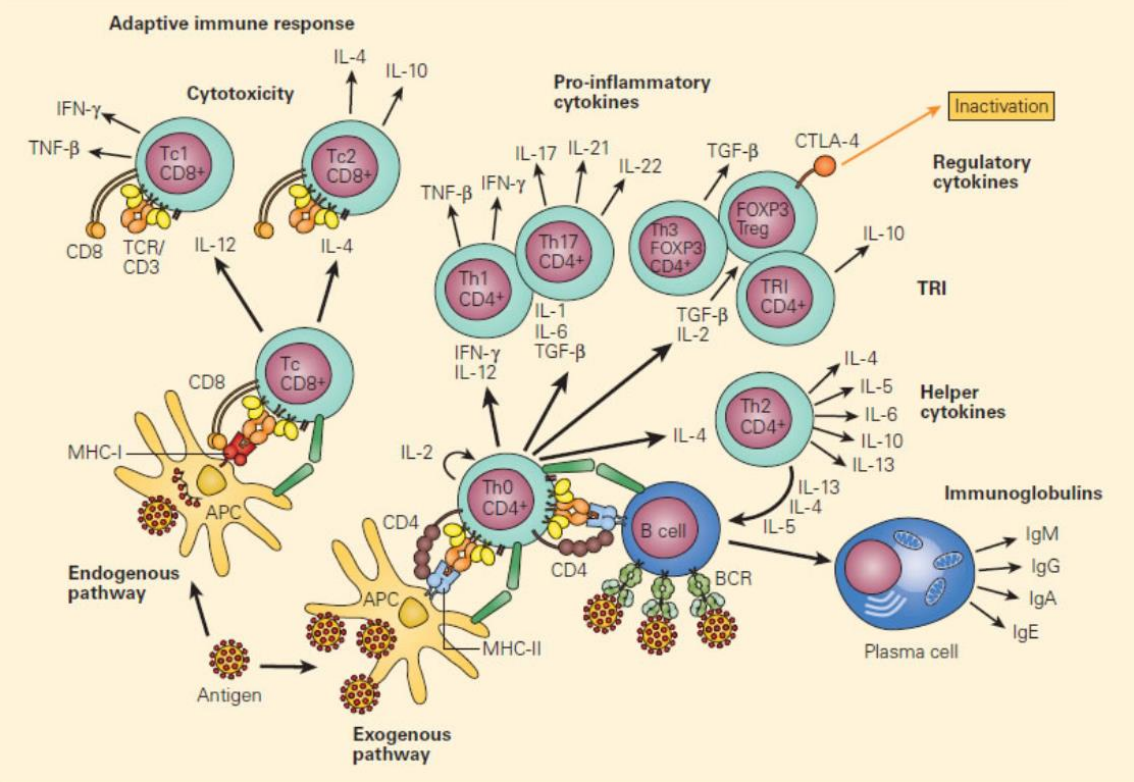


**Figure 1-2 T cell activation and differentiation** (Adapted from Bellanti, JA, 2012). APCs present antigens to $CD8^+$ T cells through MHC class I (endogenous pathway) and to $CD4^+$ T cells via the MHC class II (exogenous) pathway. In both cases, T cell activation requires MHC-mediated antigen presentation, co-stimulatory signals like CD28, and the response to environmental cytokines. After activation, T cells proliferate and differentiate into several subsets, under the inductive influence of cytokines that ultimately determine their effector functions.

### 1.2.1.1 Th17 cells

IL-17 producing Th17 cells are classified as an inflammatory subset and have increasingly been studied in the context of several chronic inflammatory conditions, including psoriasis and inflammatory bowel disease[32,33,34]. The presence of TGF-β and IL-6 is essential for the initial differentiation of naïve T cells into Th17, whilst IL-23 is needed for the stabilization of the linage[35]. This differentiation is accompanied by the upregulation of the lineage-defining factor RORγT, which is dependent on STAT3[36]. The production of IL-17 is key in the recruitment and activation of neutrophils, and also leads to the induction of inflammatory mediators and antimicrobial peptides. Additionally, Th17 cells also produce IL-22, a cytokine with important functions in the defense at mucosal surfaces as well as tissue remodeling and repair. Interestingly, the combined production these two cytokines appears to further enhance their inherent pro-inflammatory properties, as shown in a model of airway inflammation[37].

In fact, Th17 cells have been extensively associated with many inflammatory disorders, such as rheumatoid arthritis, asthma, multiple sclerosis and lupus[38]. In the case of EAE, the mouse model of multiple sclerosis, the development of the disease was suppressed in IL17 knockout mice, suggesting that this cytokine plays a critical role in this disease[39]. However, the blockage of IL17 to treat inflammatory bowel disease actually resulted in disease aggravation[40].

Indeed, Th17 and their effector cytokines are intensively studied for the plasticity of this subset. Under inflammatory conditions, human Th17 cells may shift to a Th1-like phenotype and start releasing IFN-γ[41], an important activator of macrophages. These cells may also acquire a Treg phenotype in response to *in vitro* polarization induced by the presence of IL-27[34]. Furthermore, mouse intestinal Th17 cells have been demonstrated to express the anti-inflammatory cytokine IL-10 and fully convert into Treg functional cells[42]. The opposite has also been observed: in specific conditions, Tregs can be converted into $Foxp3^+$ IL-17-releasing $CD4^+$ cells that gradually lose the expression of Foxp3 and fully differentiate into Th17 cells[43,44,45]. Likewise, Th1 cells have been observed as being able to convert into Th17 cells in the presence of TGF-β[46]. Particularly, populations of IL10-expressing Th17 cells, despite being a small percentage of Th17, are able to negatively regulate immune responses, inhibiting autoimmune inflammation[47]. Taken together, these studies suggest that Th17 cells may contribute either to pathogenesis or immune tolerance, according to the complexity of the surrounding microenvironment.

Hence, although there is great evidence that this subset plays an important part in autoimmune disorders, it is still unclear what drives these cells to become pathogenic against the host. The mechanisms that influence the conversion of the Th17 cell phenotype require further studies in order to understand how they could be used to develop therapies that restore immune tolerance in chronic inflammation.

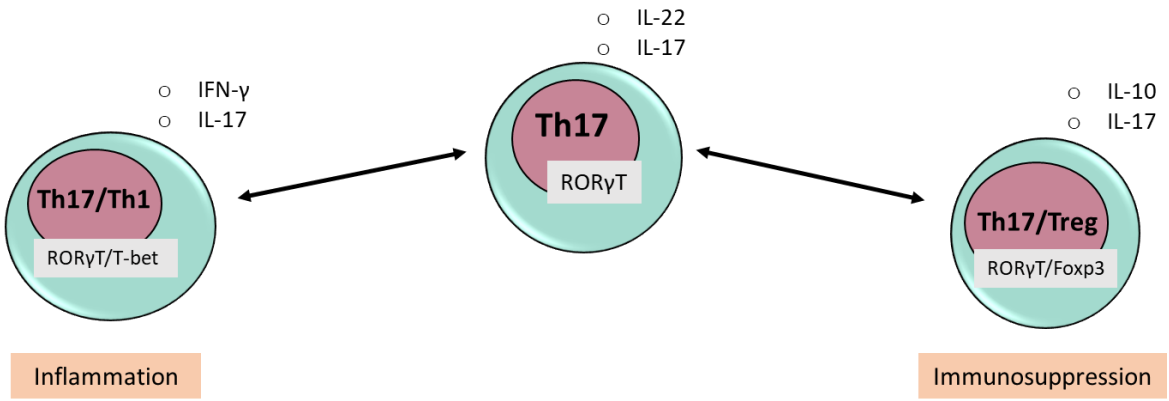


**Figure 1-3 Conversion of Th17 cells.** Cells of the Th17 subset are extremely heterogenous, produce various cytokines that are characteristic of different subsets and may convert into other subsets under different microenvironments.

### 1.2.1.2 Gamma-delta T cells

Gamma–Delta (γδ) T Cells are a minor but unique population of T lymphocytes that express the γδ TCR surface receptor and are located preferentially in mucosal and epithelial tissues, where they persist as resident cells, but also exist in secondary lymphoid organs and other peripheral tissues. While they account for only about 1 to 10% of T lymphocytes in the blood, they are the major subpopulation of T lymphocytes in other locations, such as the small intestine, where they account for 35 to 60% of all T lymphocytes[48].

The majority of circulating γδ T cells lack the expression of CD4 and CD8, which correlates with the fact that antigen recognition in these cells happens in the absence of MHC[49]. They may be divided in two groups according to functionality: a lymphoid-homing population that may be primed in circulation and suffer clonal expansion in a conventionally adaptative manner, or a population that responds rapidly to pathogens in an innate-like manner in different tissues. Accordingly, γδ T Cells are capable of attacking target cells either directly, through the release of perforin and granzymes, or indirectly, through antibody-dependent cellular cytotoxicity[50].

Tissue-resident γδ T cells can boost the innate response by producing IL-17 that leads to the recruitment and activation of neutrophils, or by producing CC-chemokine ligand 2 (CCL2) that in turn recruits monocytes and macrophages. They also promote B cell responses and antibody class switching and even contribute to downregulate inflammation by releasing immunosuppressive cytokines[51]. This functional plasticity is maintained in the periphery, thus allowing γδ T cells to employ different effector functions according to the inflammatory context.

Interestingly, a role for TLR4 signaling in γδ T cells has emerged in some reports. Its expression has been shown in human γδ T cells[52], and the stimulation of these cells with lipopolysaccharide (LPS) led to increased proliferation, IFN-γ release and cytotoxic potential[53]. Another report has proved that a heterogeneous population of γδ T cells responds to LPS in a TLR4-dependent manner whilst showing the crucial role of TLR4 in the initiation of autoimmune inflammation[54].

These unique population of T cells has also been recently studied in the context of several immune diseases and in cancer. Particularly, tumor-infiltrating γδ T cells are the immune population which more highly correlates with a favorable prognostic in several cancer types[55]. As an example, tumor-infiltrating effector-memory γδ T cells can control tumor growth through distinct cytotoxic mechanisms in melanomas[56]. Furthermore, the stimulation of effector-memory γδ T cells isolated from rheumatoid arthritis patients induced IFN-γ and IL-17 secretion, sustained $CD4^+$ T cell activation and aggravated the disease[57]. On the other hand, psoriasis patients appears to have a redistribution of a subpopulation of γδ T cells from blood to the skin, which normalized after successful treatment with psoriasis-targeted therapy[58].

In this manner, γδ T cells show a remarkable plasticity, sharing attributes of the adaptive or innate response, or both, depending on the particular context[59], but our current understanding of their many roles in the pathogenesis of chronic inflammatory diseases is still limited.

## 1.3 Tissue resident T cells

Despite having been first defined in blood and lymphoid tissues, we now know that a considerable part of the immune system stably occupies tissues and cannot be analyzed in blood, thus being hidden from many analyses. Many immune cells have the capacity to reside in tissue, such as macrophages, natural killer cells, memory B cells and plasma cells, all of these being essential in immune surveillance and organ homeostasis.

As previously mentioned, naïve T cells that are activated by APCs undergo clonal expansion and differentiate into effector cells that clear the infection. The cells that remain long after pathogen clearance are denominated memory T cells – these may be central memory T cells, that either circulate or enter secondary lymphoid tissues, or effector memory T cells, that in turn may enter peripheral tissues. However, recently, a new population termed tissue-resident memory T cells ($T_{RM}$) has been described to persist in non-lymphoid organs, for instance the skin, the brain, the lungs, the kidney and the liver[60].

These cells use combinations of selectins, chemokine receptors, and integrins to target their entry into specific tissues – as an example, CD62L expression is essential for T cells to enter the lymph nodes[61], while CCR9 and the integrin α4β7 are essential for cells to reside in the small intestine[62].

Recent reports show a population of $CD8^+$ T cells isolated from mouse intestinal epithelium that had constitutive cytolytic activity, minimal proliferation and an unique array of differentiation markers that did not exist in any cell of the blood or spleen[63–65]. A different report using a mouse model of psoriasis demonstrated that T cells in the tissue are able to execute effector function without any intervention of circulating cells.[66] Furthermore, it has been reported that $CD4^+$ T cells residing in normal skin were mostly polarized as Th1, where a population of T regulatory cells were also present[67].

The identification of rigorous and reliable markers for tissue residency is still an open debate in the field. While most $T_{RM}$ cells are positive for CD69, many $CD8^+$ tissue resident cells also express CD103[68]. While CD69 inhibits S1PR1 mediated egress from the tissues[69], CD103 docks cells to epithelial E-cadherin[70]. However, tissue resident cells lacking both CD69 and CD103 expression have also been identified[71]. Furthermore, $CD4^+$ tissue resident T cells have shown to rarely express CD103[60]. Despite this, it is in fact shown that a number of $T_{RM}$ populations utilize CD69 and CD103, as well as the cytokine IL-15 for maintenance and survival[68,72]., and thus most studies still use the simultaneous expression of the two surface markers to identify these cells.

$T_{RM}$ cells help in tissue surveillance and monitoring, and when activated, these cells produce cytokines such as IFN-γ, TNF-α and IL-2, and activate DCs, NK cells and activate bystander functions of CD8 T cells[73–75]. Furthermore, they appear to have an important role in tumor surveillance – numerous studies have correlated the number of $CD103^+$ tumor infiltrating T cells with a better cancer prognosis[76–78]. Importantly, $CD4^+$ $T_{RM}$ cells were shown to have a role in the mouse model for asthma[79]. Other mice experiments revealed that $CD4^+$ $T_{RM}$ cells can play important roles in the protection

against infections by diverse pathogens in the reproductive mucosa, the lungs, and skin[80–82].

One question that immediately arises, is if this local memory should be taken into advantage to generate more effective vaccines. it is also important to note that, while in normal conditions these cells promote a stronger immunity, they may also recognize self-antigens and cause tissue damage. Indeed, many recent reports point to a role for $T_{RM}$ cells in many auto-immune diseases, including inflammatory bowel disease and multiple sclerosis. Hence, the study of these resident populations is critical in order to paint a clear picture of the immune response as a whole, as well as to better understand the underling mechanisms of various diseases.

## 1.4 The liver as an immunological organ

The liver is uniquely supplied by both arterial and venous blood, with the blood that enters through the portal vein carrying from the gastrointestinal tract microbe-derived molecules, harmless food antigens and even potential gut-derived pathogens[83,84]. The healthy functioning of this organ requires an equilibrium between tolerance to harmless antigens and immunity to pathogens.

In the healthy liver, hepatocytes and Kupffer cells (a population of liver-resident macrophages) express PRRs and consequently recognize, phagocyte and destroy DAMPs, avoiding an inflammatory response that would usually happen in the presence of such antigens[85]. The liver-resident immune cells include APCs, myeloid cells, and lymphoid cell populations, even though some of these liver-resident populations are still not well described. The lymphoid cells present in the liver include NK cells, mucosal associated invariant T cells and γδ T cells. Mostly importantly, $CD4^+$ and $CD8^+$ T cells, as well as B cells, are found in the liver. Among these, the most commonly found are $CD8^+$ T cells and memory T cells[86].

Liver-resident APCs preferentially produce tolerogenic cytokines, particularly IL-10, upon antigen encounter[87]. The interactions between intrahepatic $CD4^+$ T cells and these APCs have been related to this immunotolerant phenotype in several studies, which despite being necessary to avoid dysregulated inflammation, is sometimes associated with permissiveness to chronic infections and weak antitumor responses[88,89].

Importantly, liver-resident DCs have reduced TLR4 expression[90] in order to maintain a tolerogenic microenvironment in the presence of the microbial products that enter through the portal vein. As a consequence, there is a lower capacity to activate naïve T cell proliferation, and T cell priming by these DCs preferentially results in an IL-10 and IL-4 mediated response[91].

During fibrosis, the liver microenvironment is altered and T cell priming by liver-resident APCs is enhanced, thus promoting an inflammatory shift[92]. The effects of liver inflammation on $CD4^+$ T cell priming are yet to be fully comprehended, but may help us understand how to manage chronic liver diseases.

γδ T cells comprise another important subset of liver-resident cells – these are present at a higher frequency in the liver than in any other organ or tissue. A recent study showed that intrahepatic γδ T cells are enriched for clonally expanded effector T cells, while naïve γδ T cells were generally absent in the liver. Furthermore, hepatic γδ T cells predominantly produce IL-17, and the homeostasis of this IL17-producing population appears to be sustained by the antigens that originate from the microbiota in the gut, in a process that relies on the lipid presentation molecule CD1d[93].

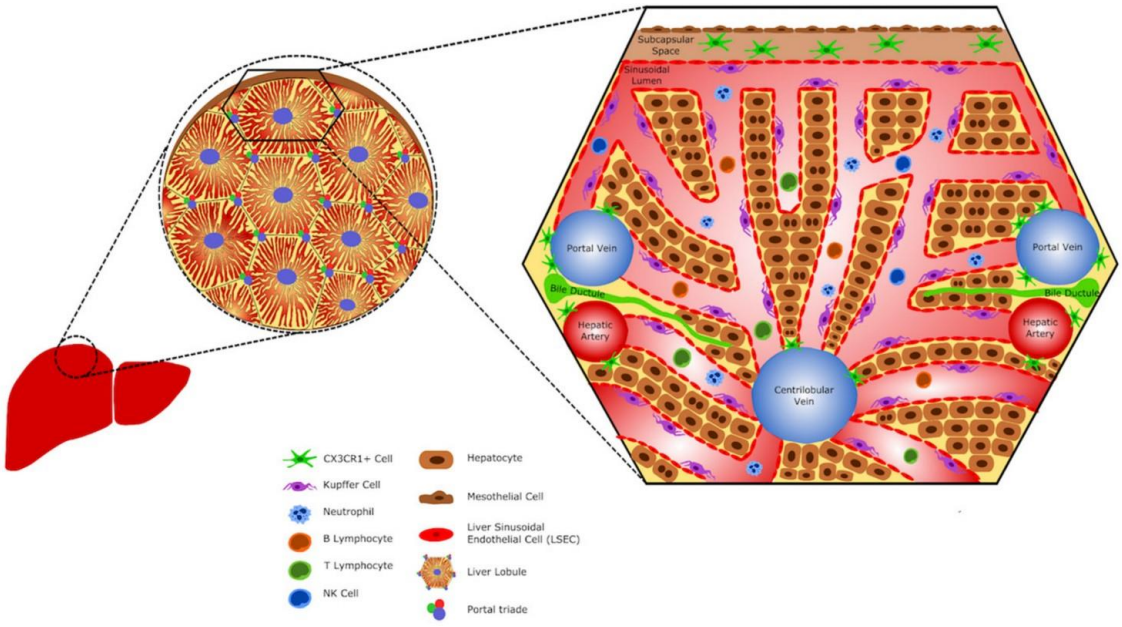


**Figure 1-4 The hepatic immune cells.** The liver harbors a large population of immune cells: DCs surround large blood vessels, Kupffer cells are the most common immune cells in this organ and neutrophils, B lymphocytes and T lymphocytes are present in fewer numbers but circulate in the sinusoids. (Freitas-Lopes, MA et al 2017)

## 1.5 TLR4 in T cells

Although its expression and function is best characterized in innate immune cells, mainly DCs and macrophages, increasing data indicates that TLRs are also expressed and activated in T cells, as reviewed in [15]. Moreover, it was reported that TLRs ligands directly promote activated $CD4^+$ T cell survival in murine and in human cells[94,95,96].

Concerning TLR4, it has been demonstrated that TLR4 triggering through LPS treatment inhibited ERK 1/2 activation and consequently down-regulated IFN-γ production in a model for experimental colitis in mice through the induction of MAPK phosphatases and consequent decrease of TCR signaling. In this model, TLR4 promoted naïve T cell proliferation and survival, but there was not a clear role for TLR4 activation

in T cell differentiation[97]. Additionally, IL-17 was up-regulated by LPS stimulation. However, it is relevant to mention that in the same study, and in several others[98,99], the profile of expressed cytokines due to LPS induction in vivo did not correspond to the same profile in vitro.

Furthermore, while one study showed that $Il10^{-/-}Tlr4^{-/-}$ $CD4^+$ T cells in a colitis mice model resulted in disease intensification[97], the use of an EAE model resulted in an opposite effect, in which the presence of TLR4 promoted inflammation of the central nervous system (CNS)[54]. More studies have demonstrated a protective role for TLR4 in colitis[99,100] and further investigation has shown that the knockdown of the adaptor protein MyD88 reduced disease severity, suggesting that the MyD88-dependent pro-inflammatory response may promote chronic inflammation in colitis. One possible explanation for this apparent inconsistency in results may be the influence of the micro-environment that surrounds cells: the pathogen-rich environment of the gut may cause a different response from that of a much more controlled, sterile CNS environment.

Taking all this into account, there is accumulating evidence that $CD4^+$ T cell functions are influenced by TLR signaling pathways, and that this influence may play an important role in cases of chronic inflammation. Also, the environment surrounding the cells may be relevant to determine the outcome of TLR signaling, not only the cytokines present during the immune response, but also factors such as the LPS present in bacterial cell walls. To understand how T cell differentiation may play a role in chronic inflammatory or autoimmune diseases, and to discover new therapeutic targets, it is crucial to study the exact molecular mechanisms underlying this interaction.

# 2 | AIMS OF THE STUDY

$CD4^+$ T cell subsets are characterized by their particular cytokine profiles and the expression of transcription factors. The Th17 subset is one of the most studied due to its involvement in the pathogenesis of several chronic inflammatory disorders. Recent studies show that Th17 lymphocytes are able to acquire a Th1 phenotype, characterized by the release of interferon gamma (IFN-γ) and tumor necrosis factor beta (TNF-β), which leads to a phagocyte-dependent inflammation, but may also convert into a regulatory phenotype and hence help suppress inflammation. However, the mechanisms underlying this change remain very poorly understood. Furthermore, accumulating evidence suggests that the expression of toll-like receptors (TLRs) in T cells are able to influence the cell metabolism, as well as its proliferation and survival. TLR4 is the only of these receptors that is known to be expressed both at the cell membrane and at the membrane of endosomal compartments, and thus mediate two distinct immune responses. Signaling pathways initiated by T-cell receptors (TCRs) are thought to influence TLRs expression through a crosstalk that is yet unknown.

Previous data from our group detailed that human $CD4^+$ T cells isolated from blood express TLR4 and that this expression is improved in response to TCR activation [unpublished data]. We propose here that the activation of TLR4 signaling in the presence of extracellular signals, namely the lipopolysaccharides (LPS) present in bacteria, influences the cell phenotype and leads to the release of anti-inflammatory cytokines such as IL-10. This influence is particularly relevant in locations where there is an abundance of inflammatory signals that arise from commensal bacteria, as is the case of the gut.

Consequently, the principal aim of this study is to determine how TLR4 activation by LPS affects $CD4^+$ T cell function, and for this purpose, three major tasks were established:

- Determine how TLR4 engagement influences T cell activation, survival and proliferation
- Assess the role of TRL4 activation in T cell differentiation
- Investigate the interaction between TCR and TLR4 signaling pathways

# 3 | MATERIAL AND METHODS

## 3.1 Reagents

**Table 3-1** List of reagents used in the assays

| Reagent | Supplier | Catalogue number |
|---|---|---|
| Biocoll Separating solution | Merck | L 6715 |
| Bovine Serum Albumin (BSA) | Thermo Scientific | SH 30574.02 |
| Brefeldin A (5mg/mL) | Sigma-Aldrich | B7651 |
| CellTrace™ Violet | Introvigen | C32571 |
| Dimethyl sulfoxide (DMSO) | Sigma-Aldrich | W387520 |
| Ebioscience™ Foxp3 Kit | Thermo Fisher | 00-5523-00 |
| Fetal Bovine Serum (FBS) | Merck | S 0415 |
| Fluoromount-G | ShouthernBiotech | 0100-01 |
| DAPI Fluoromount-G | ShouthernBiotech | 0100-20 |
| Interleukin-2 (IL-2) | NIH AIDS Reagent Program | 136 |
| Ionomycin (2.5mg/mL) | Merck | 407952 |
| Lipopolysaccharide (LPS) (1.7mg/mL diluted in sterile PBS) | Sigma-Aldrich | L2137 |
| LIVE/DEAD™ Fixable Aqua Dead Cell Stain | Thermo Fisher | L34966 |
| Paraformaldehyde (PFA) | Sigma-Aldrich | P6148 |
| Penicillin-Streptomycin | Thermo Fisher | 15140122 |
| Phorbol 12-myristate 13-acetate (PMA) | Sigma-Aldrich | P8139 |
| Phosphate Buffered Saline (PBS) | VWR | 19B1156576 |
| Poly-L-Lysine hydrobromide | Sigma-Aldrich | P6282 |
| RPMI Medium 1640 | Gibco, Thermo Fisher | 11875093 |
| Saponin (5%, diluted in distilled water) | Carl Roth | 6857.1 |

## 3.2 Antibodies

**Table 3-2** List of antibodies used in flow cytometry

| Antibody | Origin species | Final concentration | Supplier | Catalog number |
|---|---|---|---|---|
| Biotin anti-human **CD284 (TLR4)** | Mouse IgG2a | 5 µg/ml | BioLegend | 312804 |
| APC/Cy7 anti-human **IL17-A** | Mouse IgG1 | 3 µg/ml | BioLegend | 512319 |
| Pacific Blue anti-human **IFN-γ** | Mouse IgG1 | 5 µg/ml | BioLegend | 502521 |
| PerCP/Cy5.5 anti-human **CD4** | Mouse IgG1 | 2 µg/ml | BioLegend | 300530 |
| FITC anti-human **CD4** | Mouse IgG1 | 4 µg/ml | BioLegend | 300506 |
| APC/Cy7 anti-human **CD103** | Mouse IgG1 | 1 µg/ml | BioLegend | 350228 |
| Pacific Blue anti-human **CD3** | Mouse IgG1 | 4 µg/ml | BioLegend | 344824 |
| APCCy7 Anti-human **CD3** | Mouse IgG2a | 0.5 µg/ml | BioLegend | 300318 |
| FITC anti-human **γδ TCR** | Mouse IgG1 | 2 µg/ml | Biolegend | 331207 |
| Anti-human **CD28** | Mouse IgG1 | 2 µg/ml | Biolegend | 302914 |
| Anti-αβ**CD3** | Mouse IgG1 | As described in 3.5 | Biolegend | 300402 |
| PE Anti-human **CD62L** | Mouse IgG1 | 4 µg/ml | Biolegend | 304840 |
| APCCy7 anti-human **CD103** | Mouse IgG1 | 1 µg/ml | Biolegend | 350227 |
| A647 Anti-human **pERK** Phospho (Thr202/Tyr204) | Mouse IgG2b | 0.5 µg/ml | Biolegend | 675503 |
| Anti-human **pAkt (Ser473)** | Rabbit IgG | 0.5 µg/ml | Biolegend | 649001 |
| Anti-rabbit IgG **A647** | Goat | 1.3 µg/ml | Life technologies | 1696456 |
| Anti-mouse IgG2b **Pe-Cy7** | Goat | 1 µg/ml | Abcam | ab130790 |

## 3.3 Isolation of peripheral blood mononuclear cells from healthy donors

Healthy donors authorized the collection of whole blood by certified staff at CEDOC – NOVA Medical School of Lisbon. This protocol was approved by the ethics committee of NOVA Medical School. Written informed consent was obtained from all donors.

Following blood collection, peripheral blood mononuclear cells (PBMCs) were isolated from the blood using a Ficoll density gradient as described in **[**[101]**]**. Isolated cells were either kept in over-night culture in complete RPMI 1640 medium (RPMI 1640, 10% FBS, 2mM L-glutamine and 1% Pen/Strep) with 5 IU/mL IL-2 at 37%, or frozen in FBS with 10% DMSO at -80°C for 48 hours and consequently kept at -150°C until use.

Before every assay, PBMCs were thawed and cultured overnight in complete RPMI medium with IL-2 at 5 IU/mL.

## 3.4 Isolation and purification of CD4 T lymphocytes

After over-night culture, PBMCs were labelled for flow cytometry: cells were washed with FACS buffer (PBS with 2% FBS), labelled with a FITC-conjugated CD4 antibody at 4 µg/mL, then washed twice in FACS buffer and sorted in BD FACS Aria II. Lymphocytes were gated based on FSC-A and SSC-A region[101] and then the CD4 positive population was selected and collected in complete RPMI medium. Cells were isolated with a purity of approximately 99,5% as a critical step to exclude any contamination with other cells, particularly professional APCs.

## 3.5 Culture and stimulation of CD4$^{m}$T lymphocytes

Purified $CD4^+$ T cells were kept in culture at a concentration of $2x10^6$ cells/mL in complete RPMI 1640 medium with IL-2 at 20 IU/mL in 96 round U-bottomed plates coated with PLL at 2 µg/mL or with both PLL and TCR [UCHT1] (Biolegend, used at 0.1, 5 or 10 µg/mL) and CD28 (Biolegend, used at 2 µL/mL) stimulation antibodies. Cells were kept in culture for either 5 or 6 days, depending on the particular assay. In some conditions, LPS, the TLR4 ligand, was added at 1.8 µg/mL.

## 3.6 T cell survival and proliferation assay

After 6 days in culture, $CD4^+$ T cell viability was assessed in either the presence or absence of LPS. Cells were washed twice with PBS and incubated with Fixable Viability Dye eFluor™ 506 (Invitrogen) or Fixable Viability Dye eFluor™ 780 (Invitrogen) for 20 minutes at 4°C and then analyzed by flow cytometry in BD DACS Canto II.

For the proliferation assay, cells were labelled on day 1 with CellTrace™ violet (Invitrogen) and analyzed by flow cytometry on day 6.

## 3.7 Cytokine production

In order to assess cytokine production after TLR4 stimulation, $CD4^+$ T cells were kept in culture for 5 days and then treated with PMA (50 ng/mL), Ionomycin (500 ng/mL) and BFA (2 µg/mL) for 4 hours. Next, cells were washed twice with PBS and incubated with LIVE/DEAD™ Fixable Aqua Dead Cell Stain for 20 minutes at 4°C. Cells were fixed using PFA (10 mg/mL) for 20 minutes at room temperature, washed with PBS and then permeabilized using saponin (1 mg/mL) for 20 minutes at room temperature. After permeabilization, cells were stained with antibodies against cytokines: IL-17A at 3.3 µg/mL, IFN-γ at 10 µg/mL and IL-10 at 0.4 µg/mL, for 30 minutes at room temperature, then washed with FACS buffer and analyzed by flow cytometry (BD FACS Canto II).

## 3.8 Phosphoprotein analysis

After 6 days in culture, $CD4^+$ T cells were stimulated with either LPS (1.8 µg/mL), or with stimulating antibodies for TCR (10 µg/mL) and CD28 (2 µg/mL) and a cross-linking antibody (anti-mouse IgG1 at 10 µg/mL). The stimulation times varied according to the molecule in study. After the corresponding time-points, the stimulation was stopped by placing the eppendorfs on ice and adding 500 µL of cold PBS. Next, cells were stained with the corresponding antibodies using the same conditions as described in the previous step.

## 3.9 Isolation of intrahepatic T lymphocytes

Liver samples were collected from patients undergoing liver resection at Curry Cabral Hospital in Lisbon. A small sample from both healthy and embolized tissue was obtained, kept on ice and used in the following assays. All patients authorized the

collection of the samples and their use for scientific research at CEDOC – Nova Medical School.

Samples were dissected into fragments of approximately 3mm$^2$ each and transferred into a solution containing HBSS buffer (containing calcium and magnesium) and 0.05% of collagenase II and incubated for 20 minutes at 37°C. Next, in order to obtain a cell suspension, the fragments were filtered through a 70µM cell strainer and the obtained suspension was diluted in PBS. Then, a Ficoll gradient was applied, as an attempt to separate the immune cells from the hepatocytes. The Ficoll layer that usually contains the PBMCs was removed for isolation and washed twice in PBS.

Cells were stained with LIVE/DEAD$^{TM}$ Fixable Aqua Dead Cell Stain for 20 minutes at 4°C and then with the primary surface antibodies, also for 20 minutes. When necessary, secondary antibodies were incubated for 20 minutes at room temperature. Samples were immediately analyzed by flow cytometry in BD FACS CANTO II.

# 4 | RESULTS

## 4.1 CD4 T cell isolation and purification

Innate cells recognize molecular patterns characteristic of pathogens through receptors such as TLRs. The engagement of these receptors marks the beginning of a complex network of cellular activation which, among many other outcomes, results in $CD4^+$ T cell activation. This activation happens due to the interactions between APCs and T cells, and is translated in $CD4^+$ T cell proliferation, increased survival, and under polarizing environments, differentiation. However, accumulating evidence indicates that certain TLRs are also expressed in B and T lymphocytes, and that their activation may have a direct impact on the proliferation and function of these cells. Importantly, previous studies from our group have already shown that TLR4 is expressed on human $CD4^+$ T cells, and that stimulating these cells with its ligand (LPS) for 5 days increases TLR4 intracellular expression [unpublished data].

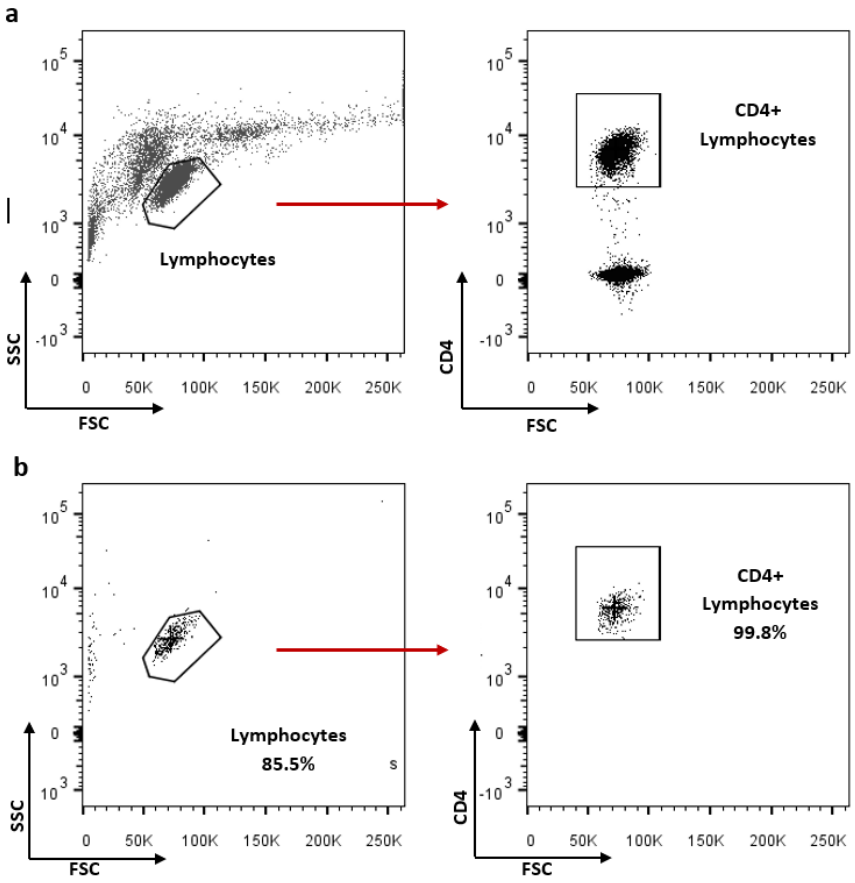


**Figure 4-1 Sorting strategy**. (a) Lymphocytes were gated based on FCS-A and SSC-A values and then sorted based on CD4 expression. (b) A small sample of the sorted cells was always analyzed a second time by flow cytometry in order to unsure the purity of the sample.

In the present study, the effect of TLR4 in these adaptive cells was further analyzed. Firstly, the purification of CD4 T lymphocytes was ensured – PBMCs previously isolated from healthy donors were analyzed by flow cytometry, lymphocytes were selected using a strict gating strategy based on FSC and SSC parameters and those expressing the CD4 receptor were sorted with high purity (figure 4.1). In this manner, it was guaranteed that the results were not affected by the presence of professional APCs that express high levels of TLR4[102]. As shown in figure 4-1b, when a small sample of the sorted cells was re-analyzed, approximately 99% of cells were CD4 positive lymphocytes.

## 4.2 TLR4 activation increases CD4 T cell survival and proliferation

T cell activation requires not only the engagement of TCR by MHC molecules but also additional co-stimulation via CD28 receptor, and their survival further requires the presence of cytokines from the IL-2 family. Without these signals, naïve T cells will become anergic or suffer apoptosis. Secondary co-stimulatory receptors, such as CD40L or OX-40, can also help sustain T cell responses, increasing survival and proliferation. Accordingly, we wanted to assess if the activation of TLR4 in $CD4^+$ T cell could also influence the cells in a similar manner. Survival and proliferation assays were performed in sorted $CD4^+$ T cells that were kept in culture with CD3 and CD28-stimulating antibodies and the TLR4 ligand, LPS. Surely, cumulative flow cytometry results show that, after 5 days, cells that were cultured in the presence of LPS showed increased survival (figure 4-2c).

Furthermore, the presence of LPS appears to be enough to stimulate $CD4^+$ T cells and contribute to their activation, given the shift in the FSC-A parameter (figure 4-2a), which can be used as a rough measure of cell size. Different concentrations of antibody for TCR activation were used, and LPS was able to increase cell survival with both lower and higher stimulations. Interestingly, the effect on cell survival appears to be more striking with a lower TCR stimulation.

During an immune reaction, lymphocytes require rapid expansion in order to form a large pool of effector cells and establish an appropriate response. Accordingly, T cell activation is usually accompanied by higher proliferation rates. Taking this into account, the next evident step was to evaluate the effect of TLR4 engagement on cell proliferation. A simple CellTrace proliferation assay shows that adding LPS to the culture media for 6 days drops median fluorescence values by more than half, hence correlating with a higher percentage of proliferative cells (figure 4-3).

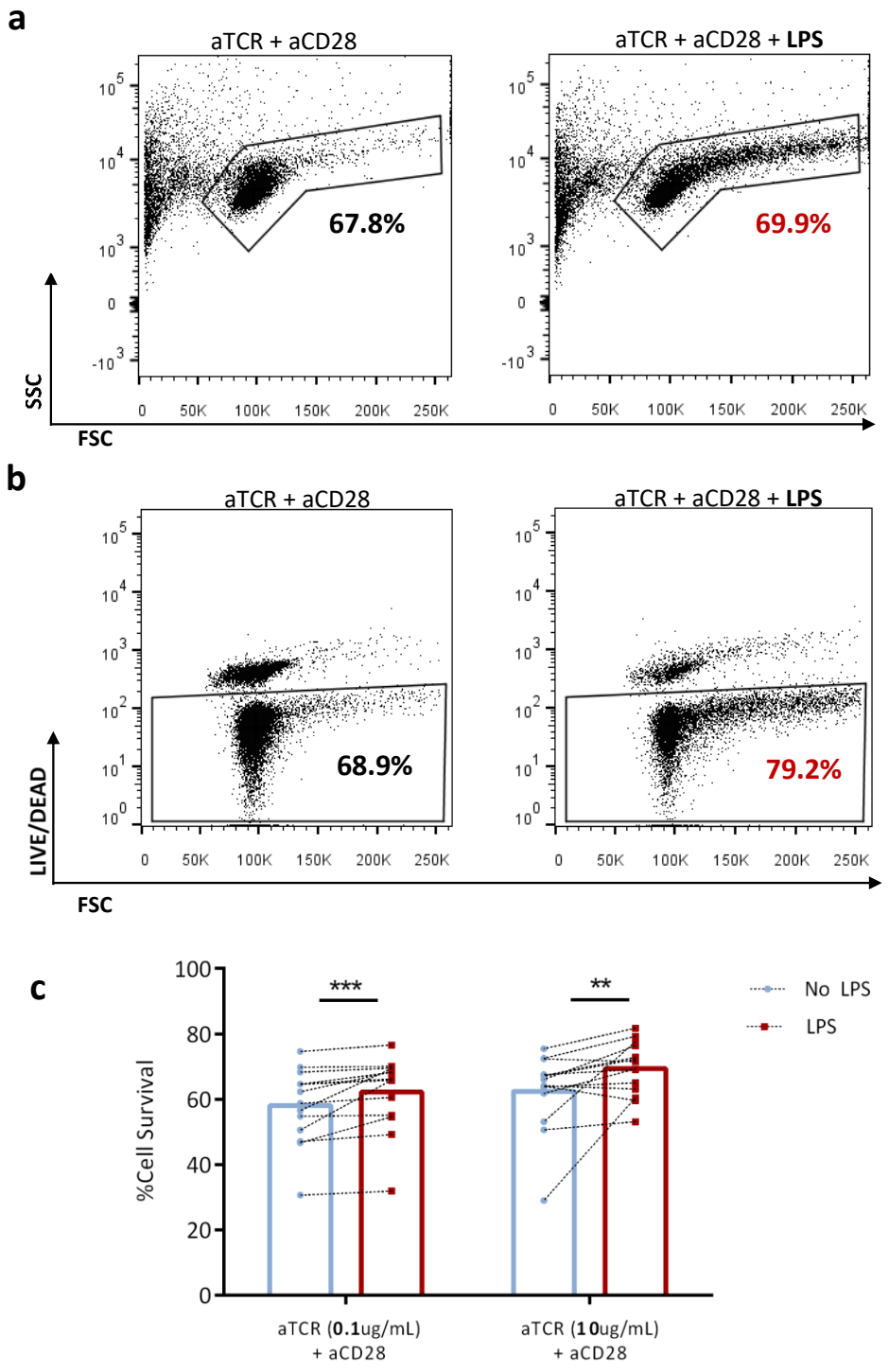


**Figure 4-2 LPS increases CD4 T cell survival**. Cells were cultured for 5 days with coated antibodies for CD28 at 2 µg/mL and TCR at either 0.1 or 10 µg/mL, stained with LIVE/DEAD™ Fixable Aqua Dead Cell Stain and analyzed in BD FACS CANTO II. Representative flow cytometry analysis illustrating the effect of LPS in T cell activation (a) and T cell survival (b). (c) Pooled data from 14 independent experiments. Wilcoxon matched-pairs test was used for comparison between two pairs. **p<0.005 ***p<0.0005

So far, our results show that the direct engagement of TLR4 on purified CD4$^+$ T cells imparts on T cell activation, leading to an increase in cell survival and proliferation. Indeed, these results are in line with several recent reports showing that several TLRs, such as TLR2, 5 7 and 8, are expressed in T lymphocytes and can directly influence the activation state of the cell[103,95,54]. Concerning TLR4, Reynolds *et al.* have reported that treating mice naïve T cells with LPS lead to enhanced proliferation at suboptimal doses of anti-CD3[54]. Importantly, we have confirmed these findings in primary human T cells.

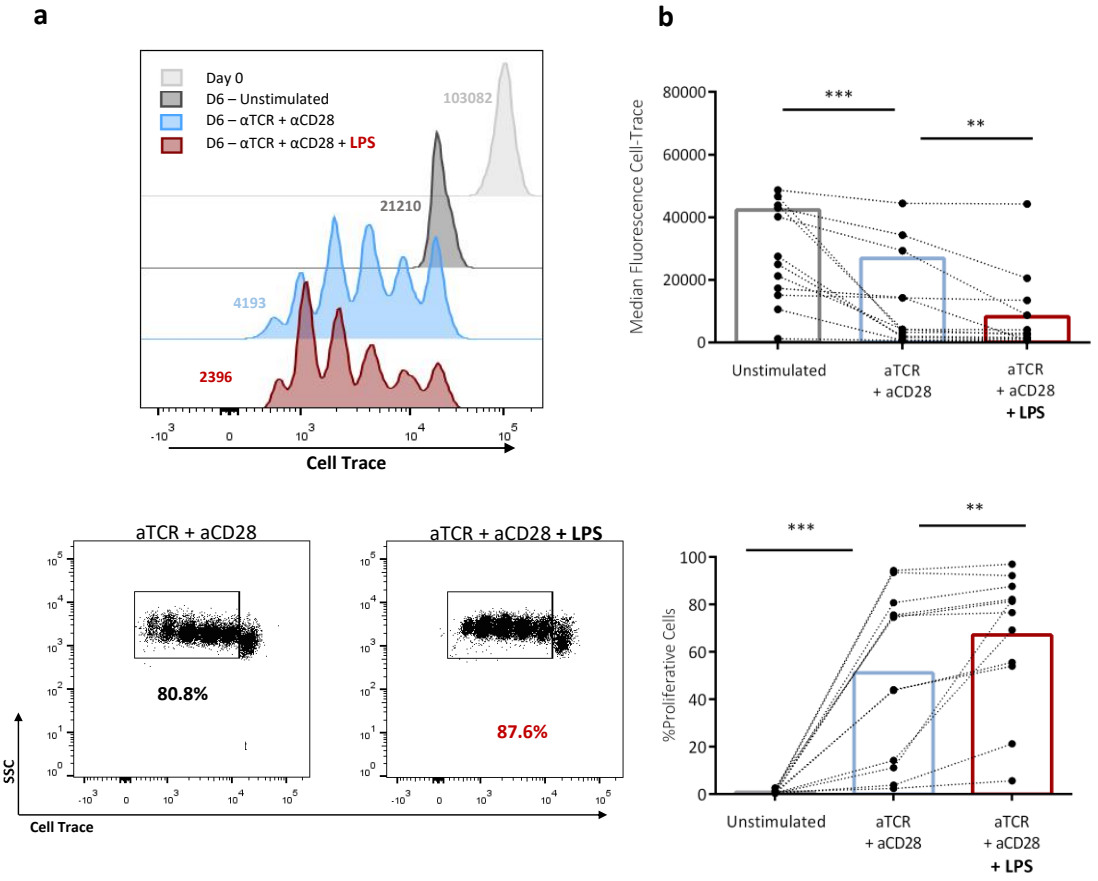


**Figure 4-3 TLR4 engagement on CD4 T cells promotes proliferation**. CD4$^+$ T cells were stained with CellTrace$^{TM}$ violet, kept in culture for 6 days with coated antibodies for CD28 at 2 µg/mL and for TCR at 5 µg/mL and analyzed in BD FACS CANTO II. (a) representative histogram with median fluorescence values and dot plot with percentage of proliferative cells. (b) cumulative data from 10 independent experiments. Wilcoxon matched-pairs test was used for comparison between two pairs. **p<0.005 ***p<0.0005

Considering that the effect of LPS appears to be more striking at lower concentrations of anti-CD3, it is possible that TLR4 may function as a co-stimulatory signal for T cell development and maintenance in the presence of danger signals. Furthermore, previous data from our lab, as well as results from others[104] show that LPS only affects T cell activation in the presence of CD3 stimulation. Results from our group have additionally shown that TCR stimulation increases TLR4 expression, which helps explain the need for TCR stimulation in order to see an effect in the presence of LPS. It

is also highly possible that this requirement for a simultaneous TCR stimulation may happen as a mechanism to avoid unspecific or excessive activation.

## 4.3 Chronic LPS stimulation influences the cytokine production profile of CD4 T cells

### 4.3.1 IFN-γ production decreases

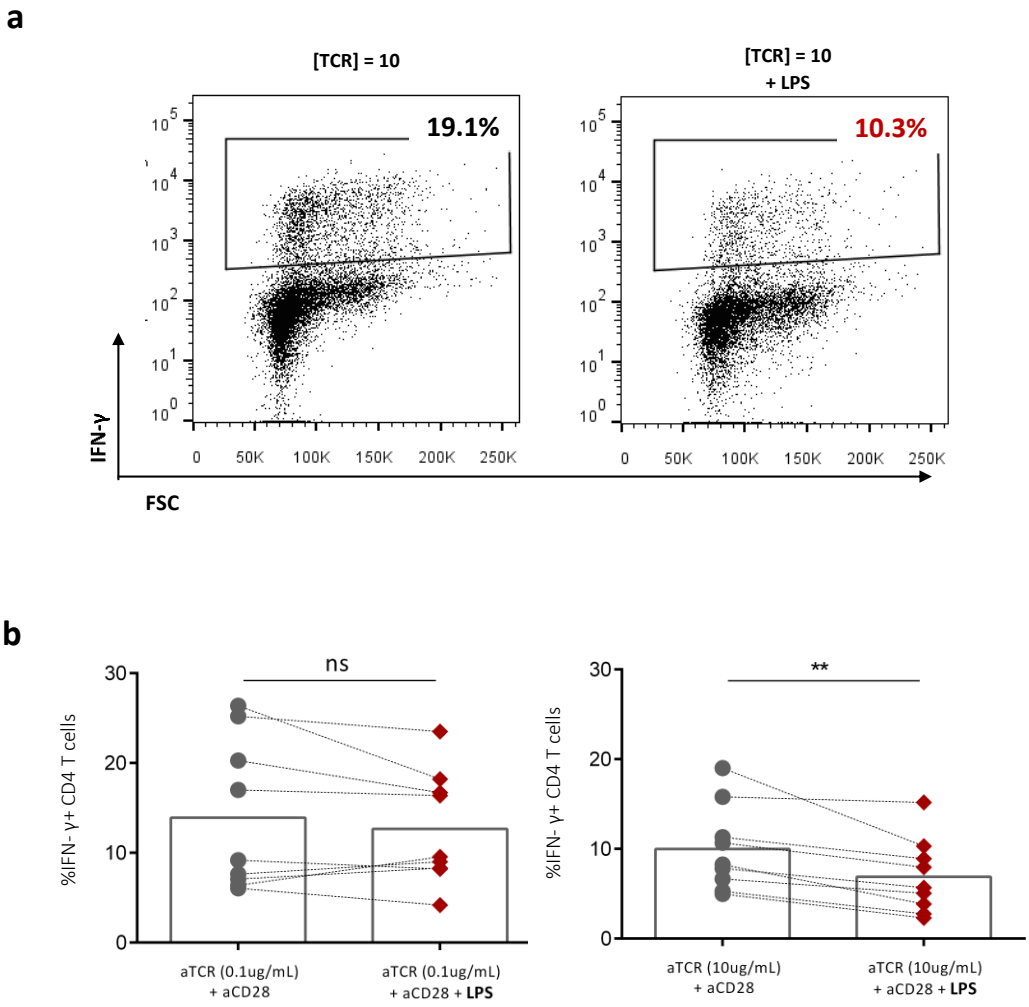


**Figure 4-4 LPS stimulation decreases IFN-γ production in human CD4 T cells**. Cells were kept in culture for 5 days with coated antibodies for CD28 at 2 µg/mL and for TCR at different concentrations. (a) Representative dot plots illustrating the percentage of cells producing IFN-γ (b) Cumulative data from 9 independent experiments. Wilcoxon matched-pairs test was used for comparison between two pairs. **p<0.005

Having established a clear impact of TLR4 engagement in $CD4^+$ T cell survival and proliferation, the next question that arises is if cell functions are also affected. T cells that differentiate into different subsets and their consequent effector functions are characterized by signature cytokines and expression of specific transcription factors. Accordingly, the expression profile of certain cytokines was assessed in the presence and absence of LPS stimulation.

The flow cytometry analysis showed that stimulating purified $CD4^+$ T cells for 5 days with LPS lead to a decrease in the percentage of cells that are producing IFN-γ (figure 4-4). Curiously, this influence also appears to be dependent on the strength of TCR stimulation. This dependence may be explained by the fact that TCR strength controls the polarization of $CD4^+$ T cells: stronger TCR signals direct $CD4^+$ T cell polarization towards a Th1 profile, marked by a higher production of IFN-γ[105]. Interestingly, recent studies have reported that absence of TLR4 signaling leads to an increase in intestinal inflammation mediated by an enhanced production of IFN-γ by $Foxp3^+$ cells[104,106]. Therefore, it is highly possible that LPS is able to downregulate the production of this proinflammatory cytokine when its production is emphasized.

### 4.3.2 Chronic TLR4 engagement increases IL-17 and IL-10 production

As discussed earlier, IL-17-producing cells are associated with the pathogenesis of many autoimmune diseases, but the study of their role has had conflicting reports. Furthermore, given the effect of TLR4 engagement on IFN-γ production, one could postulate that the presence of LPS could be re-directing $CD4^+$ T cells to a less inflammatory profile. To pursue this hypothesis, we further analyzed the production of the cytokines IL-17 and IL-10.

A flow cytometry analysis was able to show that a chronic LPS stimulation leads to a small but consistent increase in the frequency of cells that produce both IL-17 and IL-10. (figure 4-5). Although in most cases the production of these cytokines was not simultaneous, in some (as represented in figure 4-5), there is a shift into a double producing population. Once again, this effect is dependent on the strength of TCR stimulation and in this case appears to be more relevant when a lower concentration of plate bound TCR antibody is used. (figure 4-6). However, these results are not surprising if we consider that low-strength TCR signals promote Th17 responses[107].

Whilst Il-10 is a cytokine with a broad immunoregulatory function and an established tissue-protecting role, IL-17 is a key marker of the Th17 subset. The production of IL-17 has been shown to have a pathogenic role in many organ-specific autoimmune diseases, as reviewed in [[108]]. However, Th17 is also marked by its plasticity and importance in the gut microenvironment[109], where IL-10 also plays an important role in Treg mediated suppression of intestinal inflammation[110]. These cells are capable of expressing high levels of signature cytokines from other subsets and shift their phenotype. In fact, IL-17 producing cells have been shown to start producing IL-10 and fully convert into functional Treg cells[42].

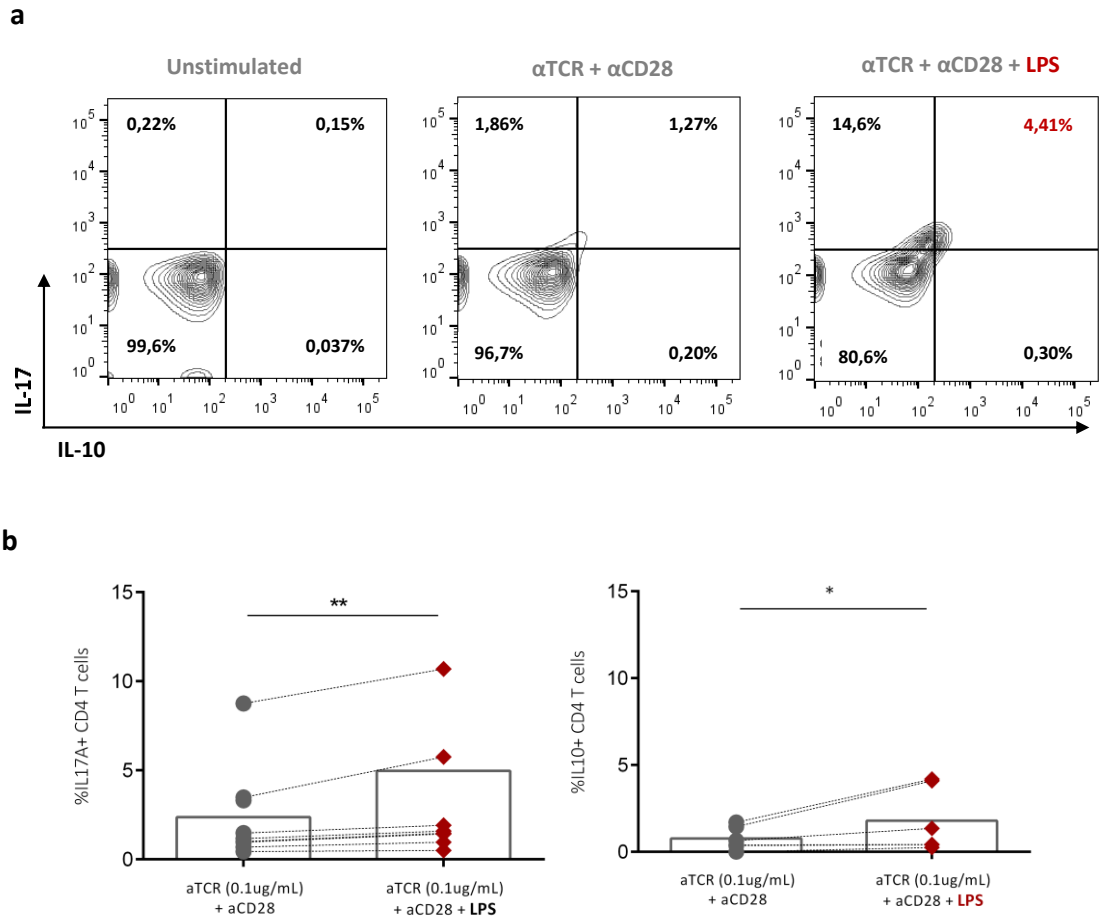


**Figure 4-5 - Chronic LPS stimulation leads to an increase in CD4 T cells producing IL-17 and IL-10.** Cells were kept in culture for 5 days with coated antibodies for CD28 at 2µg/mL and for TCR at 0.1µg/mL. (a) Representative contour plots showing percentage of cells producing both IL-10 and IL-17. (b) Cumulative data from 9 (IL-17) and 5 (IL-10) independent experiments. Wilcoxon matched-pairs test was used for comparison between two pairs. *p<0.05 **p<0.005

It has been established that IL-17-producing cells play a role in the regulation of chronic inflammation in both the central nervous system and in the gut. Furthermore, it has been described that Th17 cells express high levels of TLR4 mRNA[111]. However, the role of TLR4 in these cells is conflicting: the loss of TLR4 signaling in a colitis model lead to disease intensification, while in a EAE model it lead to the abrogation of disease symptoms. It is important to note that these diseases take place in two highly different environments, where the pathogen-rich environment of the gut contrasts with the sterile microenvironment of the central nervous system. In our work model, the culture of primary human cells for several days in the presence of LPS intends to mimic the chronic encounter with bacteria that happens in the gut. According to the results presented above, it appears that this chronic stimulation may lead $CD4^+$ T cell to downregulate

inflammatory responses, as shown by the decrease in IFN-γ production. Furthermore, the increase in IL-10 production supports this idea by showing that TLR4 activation can simultaneously lead to an upregulation of an immunosuppressive response.

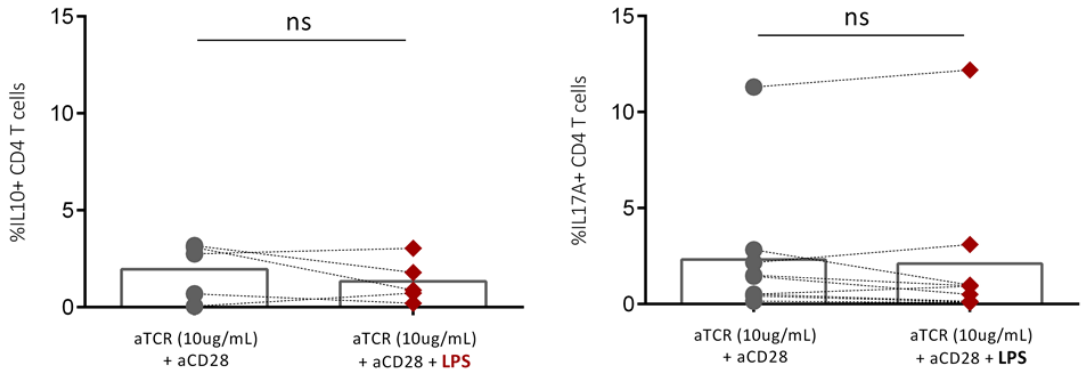


**Figure 4-6 LPS influence on cytokine production depends on the strength of αTCR stimulation.** Graphs show cumulative data of 5 and 9 independent experiments: cells were kept in culture for 5 days with coated stimulating-antibodies for CD28 at 2 µg/mL and for TCR at 10 µg/mL, and analyzed by flow cytometry.

Our results also show that the chronic presence of LPS lead to an increase in IL-17-producing cells. Although IL-17 producing cells are usually implicated in a pro-inflammatory response, increasing evidence indicates that human Foxp3$^+$ Tregs can secrete IL-17 while still exerting suppressive activity[112,113]. Moreover, while Th17 and IL-10 secreting CD4$^+$ T cells are usually seen as two different populations, the simultaneous expression of both cytokines by the same Th17 cells has been described[114]. This highlights the already known plasticity of the Th17 subset. Indeed, it makes sense that a chronic encounter with bacteria requires a fine equilibrium between regulatory and inflammatory cytokines, as a way to maintain both responsiveness and tolerance. Hence, our data not only support previous studies showing the importance of direct TLR4 signaling in the function of CD4 T lymphocytes, but also provide a possible explanation for apparent conflicting results.

## 4.4 Interaction between TCR and TLR4 signaling pathways

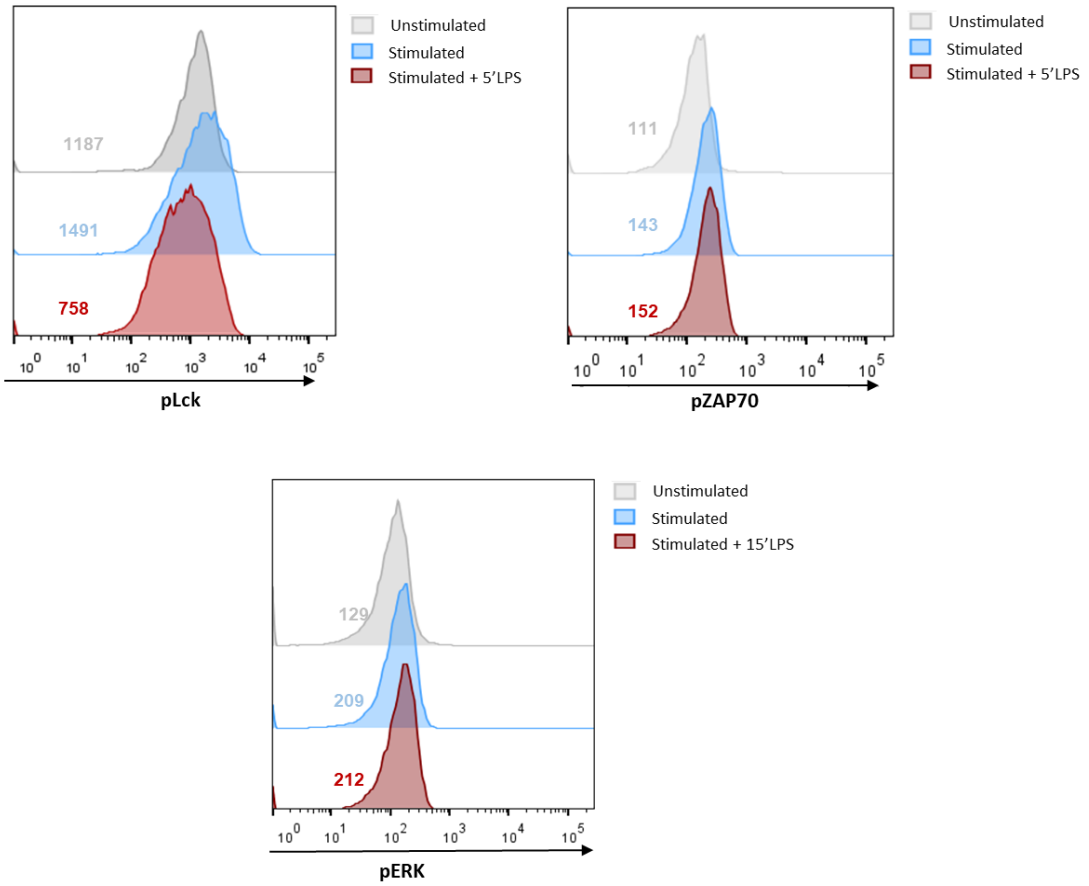


**Figure 4-7 Flow cytometry analysis of phospho-proteins from the TCR signaling pathway.** Purified $CD4^+$ T cells were cultured for 5 days, either unstimulated or with TCR stimulation at 5 µg/mL and CD28 at 2 µg/mL, and then re-stimulated with LPS at 1.8 µg/mL. After fixation and permeabilization, cells were stained with antibodies for the corresponding molecules and analyzed in BD FACS CANTO II.

Having already established that the engagement of TLR4 is capable of affecting $CD4^+$ T cells in their survival and proliferation, as well as affect the production of certain cytokines, the next step would be to try to understand which signaling pathways are being affected in this process.

A "phospho flow" experiment was constructed as an attempt to identify which phospho-proteins were activated in the presence of the TLR4 ligand. Firstly, and simply to unveil which TCR signaling pathways could be affected by TLR4 activation, cells that were kept in culture for 5 days were re-stimulated with LPS during the necessary time for their activation. Secondly, to understand if TLR4 internalization affects any of these molecules, cells were re-stimulated for 30 minutes with LPS, which is the necessary time for TLR4 internalization[115] and then re-stimulated with anti-CD3 and anti-CD28. After the incubation time, cells were immediately placed on ice and then fixed in order to

prevent the levels of phosphorylation to change. The samples were analyzed by flow cytometry as shown in figure 4-7 and 4-8.

The candidate molecules were ones that are present in TCR signaling from the membrane, and could therefore be co-stimulated by TLR4 activation, such as lymphocyte-specific protein tyrosine kinase (Lck) and zeta-chain-associated protein kinase (ZAP70). These kinases are activated following TCR activation and lead to consequent signaling pathways that result in T cell activation, proliferation and differentiation[116]. We also analyzed ERK activation due to its essential role in transcriptionally regulating IL-10 expression[117,17]. Furthermore, TLR4 signaling in $CD4^+$ T cells has been shown to downregulate IFN-γ production through ERK 1/2 inhibition[104], making this molecule a relevant candidate for the cross-talk between TCR and TLR4 signaling.

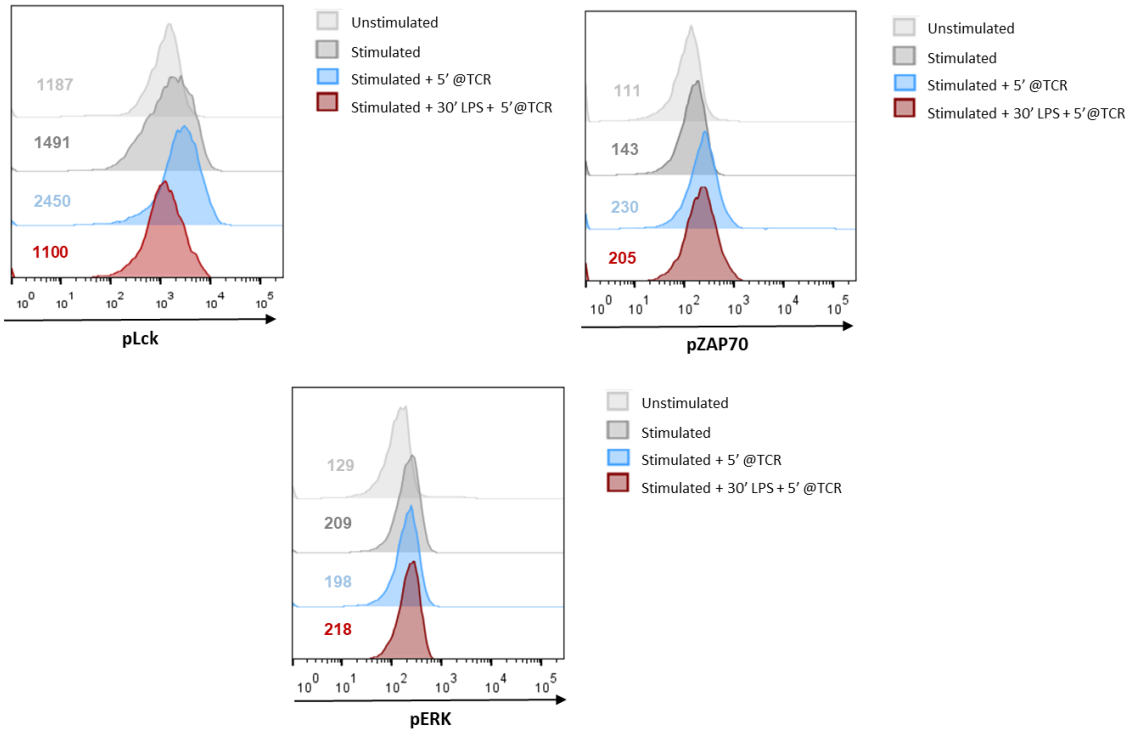


**Figure 4-8 - Flow cytometry analysis of phospho-proteins from the TCR signaling pathway.** Purified $CD4^+$ T cells were cultured for 5 days and were either kept unstimulated or with TCR stimulation at 5µg/mL and CD28 at 2µg/mL. On day 5, cells were re-stimulated with either the same antibodies, or incubated with LPS for 30 minutes and then re-stimulated with TCR and CD28 soluble antibodies. After fixation and permeabilization, cells were stained with antibodies for the corresponding molecules and analyzed in BD FACS CANTO II.

However, even though there is a shift in the activation of the phosphoproteins from the unstimulated to the chronically TCR-stimulated conditions, especially with pLck, the re-stimulation doesn't appear to be enough to further improve the activation. Furthermore, repeating these experiments did not produce consistent results. In this

manner, we could not conclude if these molecules are being activated by TLR4 engagement. Nonetheless, these molecules are strong candidates to regulate TLR4 activation in $CD4^+$ T cells and their activation should be the subject of further investigation.

Moreover, there are other molecular pathways through which TLR4 may be signaling to affect T cell survival and function. TLR9, for instance, is also expressed in CD4 lymphocytes and has been shown to affect cell survival inducing NF-kB-dependent upregulation of the anti-apoptotic Bcl-xL[94]. The conserved kinase AKT is able to restore the function of BCL-xL[118] and could also be a candidate for regulating the crosstalk between TLR4 and TCR.

Our group has previously shown that TLR4 in T cells is expressed both at the membrane and intracellularly [unpublished data], in a similar manner to what happens in macrophages. As mentioned before, it is well described that in macrophages TLR4 internalization guides the production of type I interferons and IL-10 through IFR3 activation. Hence, it would be interesting to examine if the production of the regulatory cytokine observed here (figure 4-4 and 4-5) is also being driven by TLR4 internalization. As an example, this could be achieved by using an inhibitor of endocytosis with subsequent analysis of cytokine production. Inspecting the activation of the transcription factor IRF3 and its translocation to the nucleus could also be relevant to understand if similar mechanisms are being used in $CD4^+$ T cells.

Altogether, our results so far have shown that the directed activation of TLR4 in primary human $CD4^+$ T cells leads to enhanced activation, survival and proliferation. Furthermore, a chronic stimulation with the TLR4 ligand LPS lead to the upregulation of a tolerogenic phenotype, as shown by the decrease of IFN-γ and increase of IL-10. Nonetheless, the signaling pathways that control this outcome require further elucidation.

## 4.5 Identification of a population of liver-resident T cells

### 4.5.1 γδ T cells are present at a high frequency in the liver

A second part of this project focuses on tissue-resident cells that are rapidly gaining attention due to their importance in local immune surveillance. Specifically, cells isolated from the liver were studied, an organ where a controlled immune response is critical to maintain homeostasis. Samples were collected from both the regenerative and the fibrotic tissue of patients undergoing liver resection. After lymphocyte isolation, a flow cytometry analysis showed that γδ T cells are present in both samples at a high frequency (figure 4-9). In both samples, gamma-delta lymphocytes represent about 30% of all lymphocytes, whilst $CD4^+$ T cells represent only about 5%. Interestingly, the fibrotic tissue shows a population which expresses higher levels of the gamma-delta receptor.

Several recent studies have already demonstrated that these distinct T cells are present in higher proportions in the liver, as already mentioned. Moreover, reports have shown that γδ T cells accumulate in fibrotic liver and contribute to IL-17 production in

different experimental models of chronic liver injury[119,120], which may explain the population we found expressing higher levels of this particular receptor (figure 4-9).

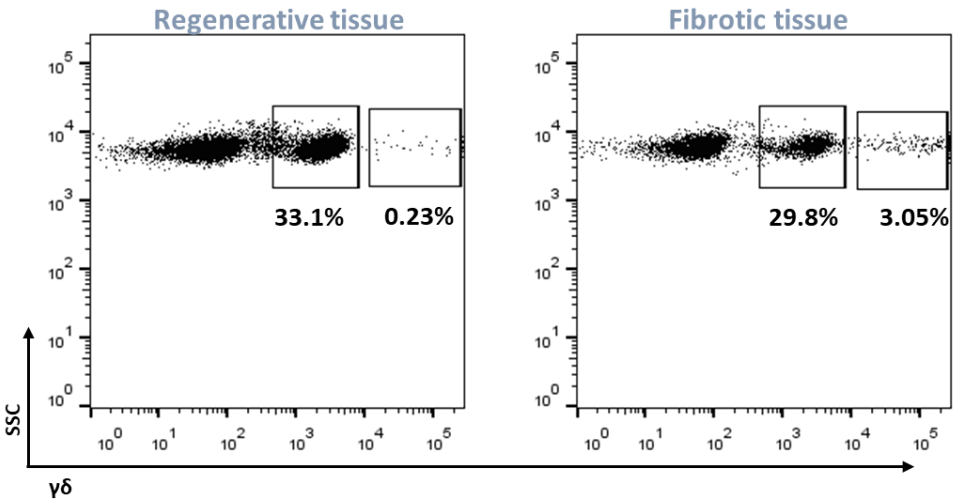


**Figure 4-9 Gamma delta T cells in the liver.** Representative FACS assay of lymphocytes isolated from both regenerative and fibrotic tissue of patients undergoing liver resection. The shown populations are gated on $CD3^+$ cells. Plots representative of 2 independent experiments.

The functional effect of γδ T cells on liver disease progression appears to depend on the subset involved and these cells may consequently have a protective or a pathogenic role in liver diseases. Importantly, these unconventional cells are known to contribute to liver inflammation, as reviewed in[121]. γδ T cells also accumulate in tumor-bearing liver and recent work suggests that they may contribute to anti-tumoral immune responses[122,123]. Our preliminary results therefore contribute to recent research showing that γδ T cells are enriched in tissue that is undergoing fibrosis. This project can be extended by functional studies to further characterize this population by their transcriptional and cytokine profile. In this manner, further studies to define γδ T cells can help understand their role in chronic inflammation.

## 4.5.2 Liver-resident CD4 T cells expressing TLR4

Moreover, the expression of TLR4 in liver cells was assessed in this project. The used strategy consisted of isolating lymphocytes, using the simultaneous expression of CD103 and CD69 as markers for tissue residency, and finally gating on $CD4^+$ $CD3^+$ cells. Inside this population, the expression of TLR4 was detected. Even though the isolation method should be improved in order to obtain a larger number of cells for analysis, these preliminary results tell us that is it highly probably that liver resident $CD4^+$ T cells are expressing TLR4.

The tissue-resident population of $CD4^+$ T cells we identified here is in fact a very small percentage of the initial population. However, this is easily explained by several reports stating that few $CD4^+$ $T_{RM}$ cells actually expressed CD103[60]. Further complicating

manners is the fact that CD4$^+$ T cells per se represent a small fraction of the immune cells present in the liver. Nonetheless, their presence in the liver has been shown to play a critical role for the tolerance of inflammatory signals that enter the liver via the portal vein[124]. As such, these cells should be the subject of further investigation. To do so, different markers of tissue residency should be used. While the identification of reliable markers is still an open debate in the field, there are some other molecules that are differently expressed in cells that reside in the tissue. Of note, these cells lack molecules that enable the egress from the tissue and migration to the lymph nodes, such as S1Pr1, CCR7 and CD62L, while most express CD69 and in some cases, CD49a and CD103[125]. Considering this, different combinations of these markers should be tested in order to identify CD4$^+$ resident T cells.

A recent report shows that infection of mice with *Bordetella pertussis* induced the development of CD4$^+$ TRM cells in the lung[126], supporting the idea that these cells accumulate in tissues to contribute to long-term local protection. In fact, other reports show that bacterial infections at various mucosal sites (lungs, gut and genital tract) induce the differentiation of CD4$^+$ TRM cells[80,127,128].

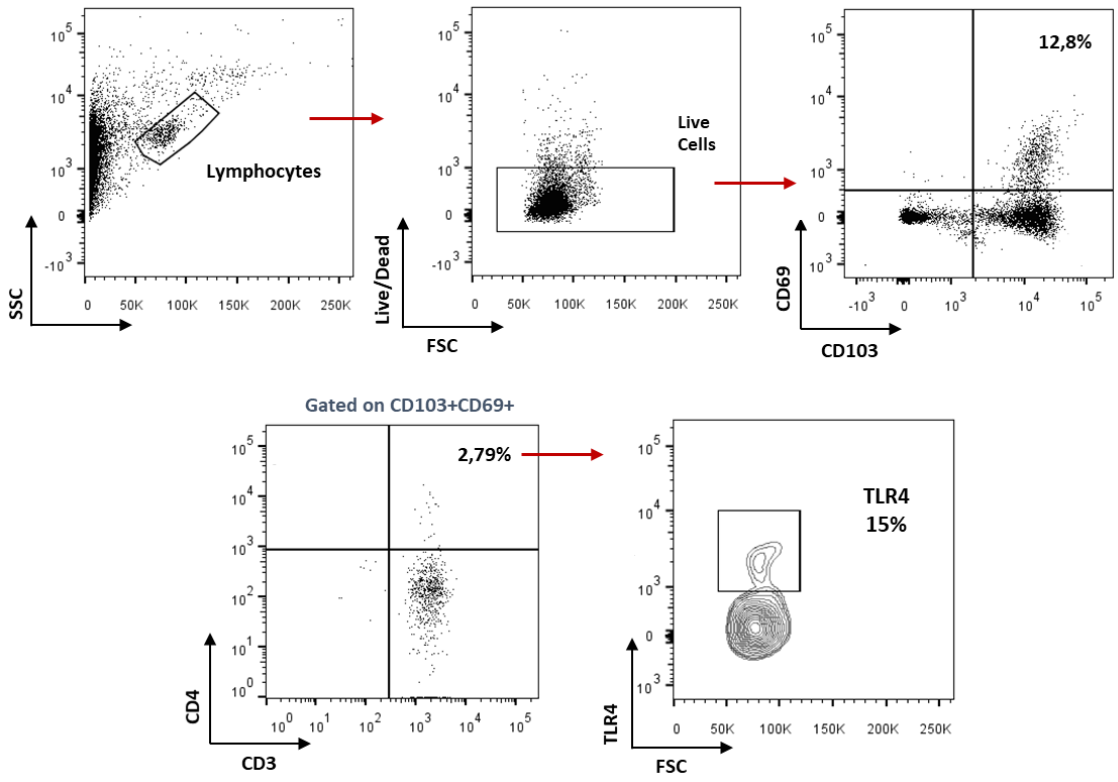


**Figure 4-10 CD4 T cells reside in the liver.** Lymphocytes were isolated from liver samples of patients undergoing liver resection, stained with the respective antibodies and analyzed by flow cytometry. A gating strategy was applied in order to identify resident cells expressing both CD4 and CD3 surface markers.

As mentioned above, the liver is often in contact with antigens derived from gut commensal microbiota carried in the portal vein. This means that immunotolerance is required to avoid damage to host in response to such antigens. Indeed, the healthy liver seems to have mechanisms that prevent extensive T cell stimulation, which includes the differentiation into non-Th1 and non-Th17[129], as well as the generation and activation of regulatory subsets. Considering our previous results regarding the role of TLR4 in $CD4^+$ T cells, along with our preliminary results showing TLR4 expression in immune liver cells, it is highly possible that a similar mechanism happens in the liver. As happened with $CD4^+$ T cell isolated from the blood, TLR4 may also participate in mechanisms that redirect these immune liver cells into a more tolerogenic phenotype.

# 5 | DISCUSSION

The human body is in constant contact with a large number of microorganisms, commonly known as the microbiota, that colonize the skin and mucosal surfaces. A complex interplay between the microbiota and the host immune system is crucial to maintain homeostasis. In this study, we focused on the consequences of the persistent activation of TLR4, the cellular sensor of bacterial LPS, in $CD4^+$ T cells.

Commensal bacteria can indirectly control pathogen colonization by a variety of mechanisms, among which is the stimulation of the adaptive response. Particularly, commensals are able to stimulate Th17 cells[130,131] and induce the differentiation and activation of Tregs[132,133]. Th17 cells tend to preferentially accumulate in the intestine, indicating that their development might be regulated by gut-intrinsic mechanisms[134]. In fact, several studies demonstrate that intestinal Th17 cells are greatly reduced in germ-free mice[130]. Furthermore, commensal bacteria tend to have anti-inflammatory activity. The accumulation of Treg cells that produce IL-10 was shown to be promoted by the microbiota and attenuate the pathogenesis of colitis[135]. More specifically, the polysaccharide A of *Bacteroides fragilis* was found to protect mice from colitis by promoting the accumulation of cells that produce IL-10[136]. Indeed, Treg cells are key players of immune tolerance, suppressing inflammatory responses in mucosal interfaces that are constantly exposed to commensal gut microbiota or to allergens.

While there is growing evidence that accumulation of both Th17 and Tregs in the gut is promoted by gut commensal bacteria, the mechanisms by which it happens remain poorly understood. In the present study, we demonstrate that the direct stimulation of primary human $CD4^+$ T cells with the TLR4 ligand LPS leads to an increase in the production of IL-17 and IL-10, and a decrease in IFN-γ. Hence, our results provide evidence that direct TLR4 activation may be one of the factors responsible for redirecting the development of IL-17 and IL-10-producing cells. To further support this notion, previous results for our group [unpublished] have demonstrated that the same LPS stimulation upregulates the expression of Foxp3, the linage-defining transcription factor for the Treg subset. Considering this, it is possible to conclude that the chronic presence of LPS, a major component of bacterial cells walls, helps promote the differentiation of the Treg subset, therefore helping explain how commensal bacteria tend to support anti-inflammatory responses. This influence is particularly relevant to maintain tolerance and limit inflammatory responses to resident bacteria that would otherwise lead to inflammatory bowel disease, or even responses to innocuous food antigens that happen in patients with celiac disease.

Immune tolerance is not only relevant in the gut, but also in other mucosal interfaces of the body that are constantly exposed to antigens, such as the respiratory tract. While the pathophysiology of allergic diseases is complex and may be influenced by many factors, it is shown that allergen-specific immunotherapy induces a state of tolerance

based on the increase of Treg functions[137]. Indeed, Treg-specific deletion of IL-10 promotes allergic inflammation[138]. Furthermore, it has been shown that there are higher levels of IL-10 being expressed in healthy subjects when compared with patients with rhinitis and asthma[139]. Looking at the case of allergic responses and their treatments, it is easy to observe that persistent antigen exposure leads to the induction of immune tolerance. In fact, exposure to high levels of allergens induce IL-10 production by Treg cells, accompanied by the amelioration of clinical symptoms[140]. One study, that focused on the exposure to bee venom, showed an in vivo switch in allergen specific IL-4 and IFN-γ-producing T cells towards IL-10 producing cells[141]. Indeed, our own results showed that a constant antigen exposure lead to a decrease in IFN-γ production whilst increasing IL-10 production. In this manner, we can postulate that TLR4 activation on T cells plays a role in several contexts of Treg-based immune tolerance. Hence, our results show one of the several mechanisms that could be behind the redirection of immune responses to a tolerogenic profile in the presence of persistent antigen exposure.

The surface of our skin is also inhabited by billions of bacteria, fungi and viruses that are in a constant dialogue with the cells of the immune system. Most of these microorganisms behave as commensal or mutualistic under steady-state conditions. Recent evidence even suggests that skin-resident bacteria engage host immunity and activate specific immune cell populations, such as IL-17$^+$ CD8$^+$ T cells that protect against cutaneous infections[142,143]. Furthermore, the capacity of T cells to produce IL-17 is severely compromised in the absence of skin commensal bacteria[142]. One example of an immune dysregulation with consequences on the skin is psoriasis, an inflammatory skin disease. Th17 cells have been widely associated with the pathogenesis of this disease, and IL-17 particularly contributes to skin barrier disruption and to amplify the inflammatory response[144–146]. In psoriasis, the regulatory capacity of Tregs and IL-10 is either diminished or absent[147,148]. It has also been reported that psoriasis-associated tissue has an evident increase in IFN-γ-producing T cells, as well as in tissue-resident T cells[149]. Hence, even though the influence of the skin commensal bacteria on the immune response is not as striking, the redirection of the immune response in this organ is equally relevant to avoid inflammatory diseases.

In our study, we attempted to further broaden our findings into tissue-resident memory cells present in the liver. We found that a population of CD4$^+$ T cells expressing tissue-residency markers simultaneously expressed TRL4. Even though these are preliminary results of an on-going project, the importance of these cells in the liver cannot be ruled out.

As discussed earlier, the liver is exposed to food-derived antigens and even to potential antigens that arise from gut-derived bacteria. As such, the balance of immune tolerance is particularly important in this organ, where high levels of anti-inflammatory cytokines drive a tolerogenic environment[90,150]. Additionally, is has been described that TLR4 engagement in NK cells and subsequent activation of the MyD88 pathway results in enhanced secretion of IL-10 by Kupffer cells, the liver-resident macrophages[151]. Although CD4$^+$ T cells are a smaller population of immune cells in the liver, one cannot rule out the hypothesis that TLR4 present in tissue-resident T cells may participate in mechanisms that promote immune tolerance.

The present work provides evidence that direct and chronic TLR4 signaling in $CD4^+$ T cells is able to not only contribute to their activation but also to re-direct these cells to a tolerogenic phenotype. These findings may be relevant in several environments where there is a permanent antigen exposure, such as the gut, the respiratory tract or even the liver, where an in-balance of such mechanisms lead to disease. In conclusion, our results allow us to step further in the understanding of the complex mechanisms that are required for a balanced immune response.

In the future, it would be relevant to understand the exact molecular mechanisms that drive the TLR4 signaling in $CD4^+$ T cells. Although our assays could not produce a relevant difference in the activation of phospho molecules, the activation of candidate molecules should be further investigated. As mentioned in chapter 4 of this thesis, there are key molecules that deserve further attention in the future, such as IRF3, which is known to be activated in innate cells upon TLR4 activation and internalization.

Another important step would be to understand the role of the tissue resident $CD4^+$ T cells we have identified. To do so, it might be necessary to explore the use of different tissue markers, such as a higher expression of CXCR6 and a lower expression of CCR7 and CD62L[152]. The discovery that these cells also expressed TRL4 may lead us to hypothesize that TLR4 may play a similar immunotolerant role in tissue surveillance. As such, similar analysis should be performed in tissue resident $CD4^+$ T cells.

# 6 | CONCLUSION

Innate cells activated by toll-like receptor signaling lead to the induction of an inflammatory environment that consequently activates $CD4^+$ T cells, which is reflected in increased survival and proliferation. Accumulating evidence indicates that toll-like receptor 4, the LPS sensing receptor, is also expressed in T cells and its activation may directly affect cell function, even though its role is not yet fully understood. Coincidently, T helper cells producing the typically inflammatory cytokine IL-17 are able to re-direct their phenotype to a regulatory on, through mechanisms that are still unknown.

With this project we demonstrate that the activation of TLR4 in human $CD4^+$ T cells directly influences cell function. Stimulating these lymphocytes for 5 days with both LPS and non-polarizing TCR stimulation resulted in enhanced activation, survival and proliferation. Furthermore, this same stimulation lead to a decrease in the percentage of cells expressing the pro-inflammatory cytokine IFN-γ, whilst increasing IL-10 and IL-17 production. These results support the idea that TLR4 may act to co-stimulate $CD4^+$ T cells while simultaneously redirecting them to a more tolerogenic phenotype. Moreover, we found that a small population simultaneously expressed both IL-17 and IL-10, thus further emphasizing the current view that Th17 cells show a remarkable plasticity. Despite not having disclosed the molecular pathways responsible for this impact on cell function, it is essential to conduct further studies that allow us to do so.

Additionally, we isolated $CD4^+$ T cells from human liver samples that simultaneously expressed markers of tissue residency and TLR4. Even though these are preliminary results that require additional experiments, it is likely that TLR4 present in liver-resident $CD4^+$ T cells helps maintain immunotolerance, similarly to what we have unraveled in lymphocytes isolated from the blood. Regulation of immunotolerance is particularly crucial in tissues such as the pathogen-rich environment of the gut, or in the liver, where antigens arrive from the portal vein.

Lastly, this work paves the way for a better understanding of how helper T cells contribute to the balance between tolerance and responsiveness that is required to provide protection whilst avoiding damage to the host's own tissue. A disruption in this balance and consequent inappropriate activation of immune cells is the cause behind autoimmune diseases. Comprehending how these cells are regulated is essential to develop pharmaceutical approaches that allow control over immune tolerance, both to promote tolerance in autoimmunity or to break tolerance in chronic infection.

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