Rute Regina Ferreira Gonçalves

Licenciada em Bioquímica

Toll-like receptor 4 and its direct impact on human CD4 T cells

Dissertação para obtenção do Grau de Mestre em
Genética Molecular e Biomedicina

Orientador: Helena Soares, PhD, CEDOC

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Projeto desenvolvido em CEDOC/FCM-UNL - Centro de Estudos de Doenças Crónicas da Faculdade de Ciências Médicas da Universidade Nova de Lisboa

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To my mom, family and beloved friends

for always supporting, helping and standing by me

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The results discussed in this thesis originated:

Posters:

- SINAL 2017 – 8th Meeting on Signal Transduction: "Bridging Innate and Adaptive Immunity – Toll-like Receptor 4 Signaling in human CD4 T cells". Rute Gonçalves and Helena Soares **(June 2017)**

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Abstract

Toll-like receptors are mainly expressed on innate immune cells and play an important role in immune responses against pathogens by transducing signals in response to microbial products. TLR4 is well known for recognizing Lipopolysaccharide (LPS), and it has been also detected on activated human CD4 T cells at the mRNA level. However, TLR4 signaling specificities and their contribution for T lymphocyte functions remain unknown. We hypothesized that in addition to cytokines, other tissue microenvironment factors, such as the presence of LPS-bearing commensal and pathogenic bacteria can have an impact on human T cell phenotype. We showed for the first time that TLR4 is expressed in human CD4 T cells, both at the plasma membrane and in endosomal compartments. We found that direct recognition of LPS by activated CD4 T cells promotes their survival. Our results show that TLR4 engagement ensues two distinct signaling pathways driving distinct functional outcomes. At the plasma membrane, TLR4 drives pAKT^{S473} and p38 MAPK^{Y182} activation, which might offer a mechanistic explanation for increased T cell survival. In addition, the plasma membrane is the site for TLR4 and TCR signaling cross-talk manifested by improved PKCθ^{S676} phosphorylation. Surprisingly, once located to the endocytic compartment, TLR4 intersects TCR signaling by increasing the phosphorylation of a residue that has been described to be under the exclusive control of TCR engagement, p38 MAPK$^{Y323.}$ By increasing the activation of p38 MAPK^{Y323}, TLR4 might impinge directly on CD4 T cell differentiation, due to the known role of this pathway in directing cytokine production. In this work we demonstrate how TLR4 signaling impinge on CD4 T cell functions namely manipulating cytokine production and the immune response. Our work might contribute to explain how immune plasticity is regulated by the tissue microenvironment, and how it might counteract chronic inflammations, an emerging field in immunology with elevated translational potential.

Keywords: Toll-like receptor 4; Human CD4 T cells; Helper T cell plasticity; Helper T cell counter-regulation; Tissue protection; novel therapeutic targets

Resumo

Os receptores do tipo Toll são maioritariamente expressos em células imunes inatas e desempenham um papel importante nas respostas imunitárias contra agentes patogénicos através da transdução de sinais em resposta a produtos microbianos. O TLR4 reconhece o Lipopolissacarídeo (LPS), e foi também detectado ao nível do mRNA em células T humanas ativadas. No entanto, as especificidades da sinalização do TLR4 e a sua contribuição para as funções dos linfócitos T permanecem desconhecidas. Levantou-se a hipótese que, além de citoquinas, outros fatores como a presença de bactérias comensais e patogénicas portadoras de LPS, pudessem afetar o fenótipo das células T. Mostrámos pela primeira vez que o TLR4 é expresso em células T CD4 humanas, tanto na membrana plasmática como em compartimentos intracelulares. Descobrimos que o reconhecimento direto do LPS por células T CD4 ativadas promove a sua sobrevivência. Os resultados mostram que a ativação do TLR4 segue duas vias de sinalização que geram comportamentos funcionais distintos. Na membrana plasmática, o TLR4 ativa o pAKT^{S473} e o p38 MAPK^{Y182}, o que pode oferecer uma explicação mecanicista para o aumento da sobrevivência das células T. Além disso, na membrana plasmática ocorre o cruzamento das sinalizações do TLR4 e do TCR manifestado pelo aumento da fosforilação do PKCθ^{S676}. Surpreendentemente, quando localizado no compartimento intracelular, o TLR4 intersecta a sinalização do TCR aumentando a fosforilação de um resíduo que foi descrito como estando sob controlo exclusivo do TCR, p38 MAPK^{Y323}. Ao aumentar a ativação do p38 MAPK^{Y323}, o TLR4 pode afetar diretamente a diferenciação de células T CD4, devido ao envolvimento desta via na produção de citoquinas. O nosso trabalho poderá contribuir para explicar o modo como a plasticidade das células T é regulada pelo microambiente, e o modo como esta pode contrariar inflamações crónicas, um novo domínio emergente na área da Imunologia com elevado potencial translacional.

Palavras-chave: Receptor do tipo Toll 4; Células T CD4 humanas; Plasticidade de células T auxiliares; Contra-regulação de células T auxiliares; Proteção de tecidos; Alvos terapêuticos

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Abbreviation List

AKT	(PKB) Protein kinase B
AP1	Activator protein 1
APC	Antigen presenting cell
BAD	BCL-2 associated death promoter
BCL-X_L	B-cell lymphoma extra-large
BCR	B cell receptor
BFA	Brefeldin A
BSA	Bovine Serum Albumin
CD*x*	Cluster of Differentiation *x*
cGAS	Cyclic GMP-AMP synthase
CLR	C-type lectin receptor
CPZ	Chlorpromazine
CREB	Cyclic AMP-responsive element-binding protein
cSMAC	Central supramolecular activation cluster
DAMP	Dangerous Associated Molecular Pattern
DC	Dendritic cell
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic Acid
EAE	Experimental autoimmune encephalomyelitis
ER	Endoplasmic reticulum
ERK	Extracellular signal–regulated kinase
FACS	Fluorescence-activated cell sorting
FBS	Fetal Bovine Serum
FOXO1	Forkhead box protein O1
Foxp3	Forkhead box P3
GM-CSF	Granulocyte-macrophage colony-stimulating factor
IFI16	Interferon gamma inducible protein 16
IFN-γ	Interferon gamma
IKKε	IκB kinase ε
IL-*x*	Interleukin-*x*
IRAK	IL-1R-associated kinase
IRF*x*	Interferon regulatory factor *x*
ITAM	Immune-receptor tyrosine-based activation motif
iT_{reg}	Induced regulatory T cell
IU	International units
JNK	JUN N-terminal kinase
LPS	Lipopolysaccharide

MAL	MyD88-adapter-like
MAPK	Mitogen-activated protein kinase
MHC	Major histocompatibility complex
mTOR	Mammalian target of rapamycin
MyD88	Myeloid differentiation primary response protein 88
NF-kB	Nuclear factor kappa B
NOD-like receptor	Nucleotide-binding oligomerization domain receptor
nT_{reg}	Natural regulatory T cell
PAMP	Pathogen Associated Molecular Pattern
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
Pen/Strep	Penicillin/Streptomycin
PFA	Paraformaldehyde
PI3K	Phosphoinositide 3-kinase
PKCθ	Protein kinase C-theta
PLL	Poly-L-Lysine hydrobromide
PMA	Phorbol 12-myristate 13-acetate
PtdIns(3,4,5)P_3	Phosphatidylinositol 3,4,5-triphosphate
PtdIns(4,5)P_2	Phosphatidylinositol 4,5-biphosphate
qPCR	Real-time polymerase chain reaction
RIG-I-like receptor	Retinoic-acid-inducible gene 1-like receptor
RORγT	RAR-related orphan receptor gamma
RPMI 1640	Roswell Park Memorial Institute medium 1640
RT	Room temperature
Syk	Spleen tyrosine kinase
Tbet	T-box transcription factor
TBK1	TANK-binding kinase 1
TCR	T-cell receptor
TGF-β	Transforming growth factor beta
Th*x*	Helper T cell type *x*
TIR	Toll/interleukin-1 receptor
TIRAP	TIR domain-containing adaptor protein
TLR	Toll-Like Receptor
TNFα	Tumour Necrosis Factor α
T_R1	T regulatory type 1 cell
TRAF	TNF receptor-associated factor
TRAM	TRIF related adaptor molecule
TRIF	TIR-domain-containing adapter-inducing interferon-β
VEGF	Vascular endothelial growth factor
ZAP70	Zeta chain of T cell receptor associated protein kinase 70kDa

1 INTRODUCTION

1.1. Immune system

The immune system constitutes the defense of the organism. Its main functions are to eliminate pathogenic agents and to maintain the body homeostasis. It is one of the most complex systems comprising both unspecific and specific components that, although distinct, cooperate and synergize to create a controlled protection to disease (Kennedy 2010). The immune system is an interactive network of lymphoid organs, cells, humoral factors and cytokines and, differently from other systems, it is highly dispersed throughout the organism. In fact, its elements vary from physical barriers, such as the epidermis, to the most complex and specific components, such as the lymphocytes (Parkin and Cohen 2001).

The immune system can be divided in three lines of defense:

- The first line of defense are physical, chemical and biologic barriers that prevent the entry of pathogens into the body;
- The second line of defense is constituted by phagocytes and other defense mechanisms (e.g. complement) that comprise the rapid response of the innate immunity;
- The third line of defense consists of lymphocytes, which are highly specific cells capable to create memory and to ensue more efficient immune responses upon re-exposure.

Upon encounter with the invading pathogen, the first line of defense sets up physical, chemical and enzymatic barriers to avoid its entrance into the body. If those barriers fail or are insufficient, the innate immunity immediately comes into play, eliciting a very quick and broad immune response that often culminates in the elimination of the pathological agent. The players of the innate immunity are the monocytes, macrophages, dendritic cells (DCs), neutrophils, eosinophils, basophils and mast cells. Innate immune cells are endowed of multiple defense mechanisms capable of recognizing and neutralizing pathogens, such as phagocytosis, defensins and the complement system. The innate immune system is capable of recognizing self from nonself through the expression of receptors that are able to recognize and bind common and preserved components present in pathogens but not in the host cells. In some cases, these receptors do not recognize microbial products directly but detect their presence indirectly through the recognition of ‘‘stress signals’’ molecules produced by cells that have become infected (Melvold and Sticca 2007). Albeit, efficient at rapidly eliminating pathogens, the innate immune response is not perfect: on one hand the strong inflammatory response ensued by the innate immune cells lead to damage of healthy

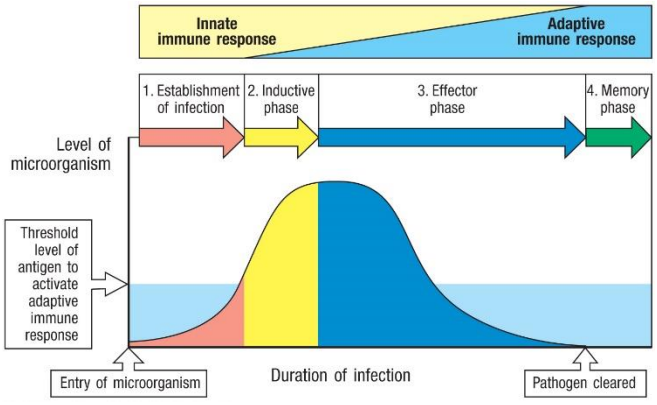


Figure 1.1 - Dynamics of innate and adaptive immunity. From (Janeway et al. 2001). After the entrance of the microorganism, 1st and 2nd lines of defense are set up, leading to the influx of antimicrobial proteins, phagocytes, and complement to the local of infection, where they work alongside to destroy the pathogen. If they fail to clear the infection, and the threshold level of antigen is reached, the inductive phase begins and professional antigen presenting cells (APCs) migrate to the lymph nodes where they trigger the adaptive immunity. That way, innate and adaptive immunity build an efficient response against the invader. After pathogen clearance, immunological memory is mounted by the adaptive system, ensuring a faster and stronger immune response after a re-exposure to the same microorganism.

tissues, and on the other hand it cannot eliminate pathogens that quickly evolve to avoid their detection and clearance by the immune system.

Whenever the 2nd line of defense fails to neutralize the infection, the adaptive immune system takes action and mount a latter but highly specific response against the invading pathogen *(Figure 1.1)*. This system possesses more sophisticated defense mechanisms, it has the capability to recognize ever changing pathogens and to mount a stronger immune response upon re-exposure. Thus, the defining feature of the adaptive immune system is the memory, i.e. the capacity to mount a faster and stronger immune response, capable of avoiding reinfection with the same microorganism (Purves et al. 2000).

Along with those refined mechanisms that allow the immune system to recognize and act against non-self and/or "stress" proteins, the immune tolerance is highly required. When this discrimination fails, the immune system activates destructive immune reactions against body's own components, and autoimmune diseases arise. This can be prevented by tolerance mechanisms to self-proteins, including: (i) Central tolerance by clonal deletion of autoreactive lymphocytes in the thymus or bone marrow. During that process, T cells undergo a positive selection that ensures T cells only recognize antigen in association with major histocompatibility complex (MHC) molecules. Finally, those previously selected T cells meet with self-peptides, and self-reactive ones are elected to die via programmed cell death and excluded from the T cell repertoire (negative selection) (Parkin and Cohen 2001); (ii) Peripheral tolerance acts after T cells are exported into the periphery. Some self-reactive T cells might escape through central tolerance mechanisms if they don't have sufficient affinity for the self-antigen, if they only recognize tissue-specific antigens, or even if they only react at latter times of development. Peripheral tolerance resort to deletion and inactivation (anergy) of self-reacting peripheral lymphocytes; (iii) Regulatory T cells are capable to suppress or downregulate induction and proliferation of effector T cells. Those regulatory cells are known to modulate the immune system, maintain tolerance to self-antigens, thus preventing autoimmune diseases (Abramson and Husebye 2016).

Immune system hyper-responsiveness can also lead to exacerbated immune responses against foreign compounds like what happens in allergic rhinitis and asthma.

On the opposite end of the spectrum there is the immunodeficiency where the immune system is deficient in mounting an effective immune response, resulting in recurring and life-threatening infections.

Altogether, the immune system, as a complex network, needs to work under a strict balance. Its overall functions are to recognize, defend and memorize, protecting the host from hazardous agents and disease, thereby preserving the integrity of the body.

1.2. Immune recognition

1.2.1. Innate Immunity

The immune response is trigged by exposure to a foreign compound. In normal circumstances, it is a controlled response that works to eliminate pathological microbes and toxic or allergic proteins, but also, at the same time, must avoid self-destruction and the clearance of commensal and beneficial microbes. That is the reason why during the immune response there is a shift towards specialized immune receptors capable to distinguish between self- and non-self-proteins, whose specificity increases in the course of the response (Melvold and Sticca 2007). Whereas the adaptive immune

system uses a large repertoire of receptors encoded by rearranging genes to recognize an infinite variety of antigens, innate immunity depends upon germ-line encoded receptors to recognize pathogenic features that are common and evolutionary conserved.

After exposure, cytokines produced by phagocytes [Interleukin-1 (IL-1), IL-6 and Tumour Necrosis Factor α (TNFα)] activate the acute phase response and the body generates active and unspecific proteins, such as the C-reactive protein produced by liver cells, in order to immediately act against microorganisms or to improve trauma resolution (Di Napoli et al. 2011). This acute response leads the activation of the coagulation system, and the production of the complement, lactoferrin and transferrin, lysozyme and cytokines. Those components act by neutralizing bacteria, increasing vasodilatation and vascular permeability, creating a boost and powerful influx of even more phagocytic cells. Inflammation, fever, loss of appetite and somnolence are some of the long-range effects of this stage of immune response to infection and trauma (Janeway et al. 2001).

As stated above, monocytes, macrophages and DCs are some of the key players of the innate immune system. Each one of them has its specific function in the resolution of the infection. Even though they lack the high specificity of adaptive immune cells, they still have the capability to distinguish self from nonself. They express immune receptors that allows them to recognize conserved patterns and structural motifs only presented in microorganisms - Pathogen Associated Molecular Patterns (PAMPs) – and “danger signals” that result from tissue damage - Dangerous Associated Molecular Patterns (DAMPs) (Jin et al. 2012).

The recognition of microorganisms is mediated by several families of innate immune receptors that survey the extracellular space, endolysosomal compartments and the cytoplasm for signs of infection or tissue damage and gives rise to very rapid responses. These are nucleotide-binding oligomerization domain receptors (NOD-like receptors), C-type lectin receptors (CLRs), retinoic-acid-inducible gene 1(RIG-I)-like receptors, Scavenger receptors, Toll-Like Receptors (TLRs) and the most recently described deoxyribonucleic acid (DNA) sensors Interferon gamma inducible protein 16 (IFI16) and cyclic GMP-AMP synthase (cGAS) (Joosten et al. 2016). They have a number of different functions: some stimulate ingestion of the pathogen, some are chemotactic receptors and others induce effector molecules that contribute to the priming of innate immune responses and that influence the initiation and nature of any subsequent adaptive immune response.

TLRs are the best characterized family in terms of known ligands, established localization, downstream signalling pathways and functional relevance (T Kawai and Akira 2006). TLRs are expressed in a variety of mammalian cell types, however their functional relevance is best studied in antigen presenting cells (APCs) as macrophages and DCs. They can be classified and divided depending on their ligands and subcellular sites. Some localizations are stable, whereas others can be more flexible. TLR 1, 2, 6, 5 and 4 are localized in the cell surface, thus recognizing accessible molecules in the membrane of most pathogens, like lipopolysaccharide (LPS), lipoproteins, fibronectin and flagelins. On the other hand, TLR 3, 7, 8 and 9 are localized within various endosomal compartments, where they recognize microbial nucleic acids (Kabelitz and Medzhitov 2007) *(Figure 1.2)*.

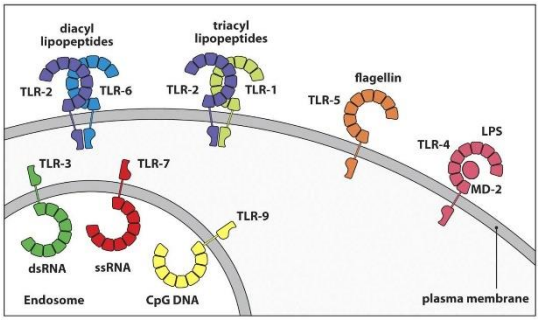


Figure 1.2 - Toll-like receptors: Subcellular localization and ligands. From (Janeway et al. 2001) Toll-like receptors are the best characterized family of innate immune receptors. Some TLRs are expressed at the plasma membrane, while others are located within endosomal compartments. They are responsible for the recognition of various microorganisms, signs of infection and/or tissue damage.

Binding of TLRs on phagocytic cells triggers their activation and they become enlarged, increase their production of antimicrobial products, and start to ingest and degrade microbes. Meanwhile, some activated phagocytic cells activate the adaptive system and the adaptive immune response is finally generated. Eventually, if the innate immune response fails to neutralize the infection, the adaptive immune system takes action ensuing a highly specific and long-lasting immune response against the pathogen.

1.2.2. Adaptive Immunity

The adaptive immunity is mediated by lymphocytes, specialized cells with highly specific receptors for the antigen. Lymphocytes derive from lymphoid committed progenitors, of which 25% remain in the bone marrow and differentiate into naïve B lymphocytes. The remaining ones travel to the thymus and become naïve T lymphocytes. The thymus is a specialized primary organ that provides an inductive environment for T cell development from hematopoietic progenitor cells. In addition, one of the most important roles of the thymus is the induction of central tolerance selecting a functional and self-tolerant T cell repertoire (Jin et al. 2012). Naïve B and T cells migrate into the lymph nodes where eventually they will be activated by antigen-primed DCs.

In fact, DCs are the main innate immune cells that ensue the crosstalk between the innate and the adaptive immune response. Naïve DCs, as specialized phagocytic cells, are capable to recognize exogenous pathogens and/or foreign proteins through specialized receptors such as TLRs. That induces the activation of intracellular signalling pathways and the production of inductive cytokines (Ley 2014) *(Figure 1.3 A)*.

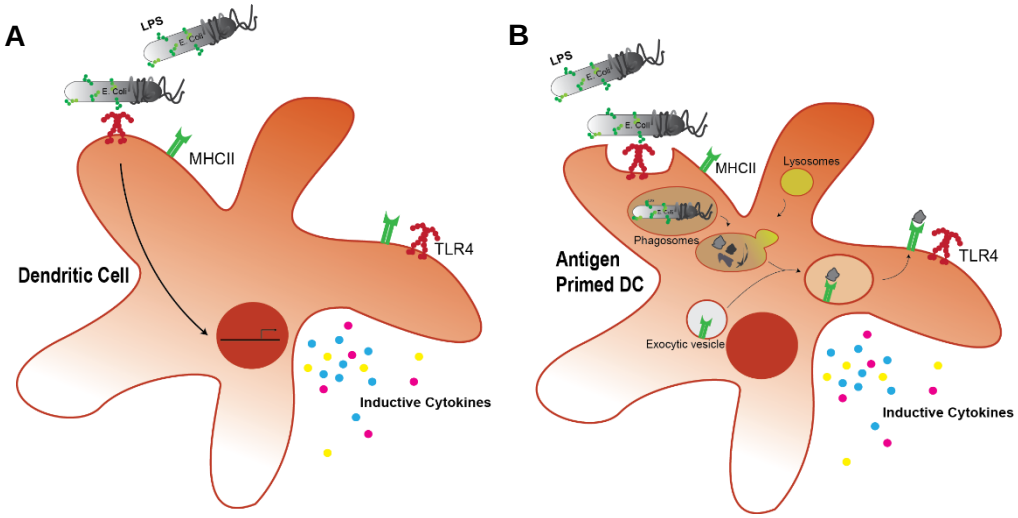


Figure 1.3 *(in previous page)* **– Activation and priming of naïve DCs after pathogen encounter. Based on (Janeway et al. 2001).** DCs recognize exogenous pathogens through specialized receptors such as TLRs. **A** - TLR4 recognizes LPS in Gram-negative bacteria inducing the activation of intracellular pathways and the production of inductive cytokines. **B** –After TLR engagement, the pathogen is internalized and phagocytosed. Degraded pathogen-derived proteins are then loaded on MHC class II molecules and delivered to the plasma membrane. By this time, DCs are loaded with specific antigens.

The internalized pathogens/proteins are processed inside the cell by acid-dependent proteases in endosomes. Meanwhile, immature MHC class II molecules are in the endoplasmic reticulum (ER) with their peptide-binding site blocked by Ii, an invariant chain that prevents them from binding self-peptides and/or peptides from intracellular pathogens. Those intracellular peptides follow a different endogenous pathway in which they are processed by the proteasome, loaded onto MHC class I molecules in the ER, and released through the Golgi apparatus to the plasma membrane where they will activate effector $CD8^+$ T cells. Otherwise, immature MHC class II molecules are exported from the ER in vesicles that fuse with late endosome/lysosome compartments containing the degraded pathogenic proteins, which are then loaded onto MHC class II antigen grove. This exogenous pathway ends up by delivering the MHC class II loaded peptides to the plasma membrane and to helper $CD4^+$ T cells activation (Kennedy 2010), (Aichinger and Lechler 1995) *(Figure 1.3 B)*. Activated DCs migrate into the lymph nodes and they start a process called as “Antigen presentation”, activating naïve B and T lymphocytes in both B and T zones, respectively *(Figure 1.4)*. From that moment, the adaptive immunity is initiated.

Naïve T lymphocytes are activated after antigen recognition and co-stimulation leading to cell proliferation that ensures a sufficient number of lymphocytes with the same specificity, an alteration of gene expression and differentiation in cells specialized to fight the infection at task. Antigen recognition is achieved through the engagement of the T-cell receptor (TCR) by the antigen loaded MHC molecules in the APC. TCR is highly specific for the antigen-MHC complex and this specificity derives from gene recombination. The TCR cytoplasmic tail contains a sequence known as ITAM (immune-receptor tyrosine-based activation motif), essential for the initiation of signal transduction (Guy et al. 2013). For T cell full activation, besides TCR engagement, it is also required interaction between co-stimulatory molecules cluster of differentiation 28 (CD28) on the T cell, and B7 family molecules on the APC. If there are no co-stimulatory signals, T lymphocytes become anergic, a crucial mechanism in the maintenance of peripheral tolerance (Kennedy 2010).

The process of clonal expansion takes 3-7 days and ends up with the production of both effector and memory T lymphocytes, all with the same antigenic specificity.

T cells take part of the cell-mediated immunity, acting against intracellular and phagocytosed microbes. They can be divided into Helper ($CD4^+$) and Cytotoxic ($CD8^+$) lymphocytes. $CD8^+$ lymphocytes are capable of killing infected cells and eliminate reservoirs of infection. $CD4^+$ lymphocytes are not usually capable of killing cells, instead they are considered the orchestrators of the specific response (Kennedy 2010). After thymus differentiation, $CD4^+$ T cells can be divided into $Foxp3^-$ effector T cells and $Foxp3^+$ natural regulatory T cells (nT_{regs}) (Fontenot, Gavin, and Rudensky 2003). Through the release of cytokines and other soluble messengers, effector $CD4^+$ T cells can activate macrophages allowing them to degrade the internalized pathogen, they can act by inducing B cell proliferation and determining antibody secretion, complement activation and neutrophil chemotaxis. nT_{regs} are a stable

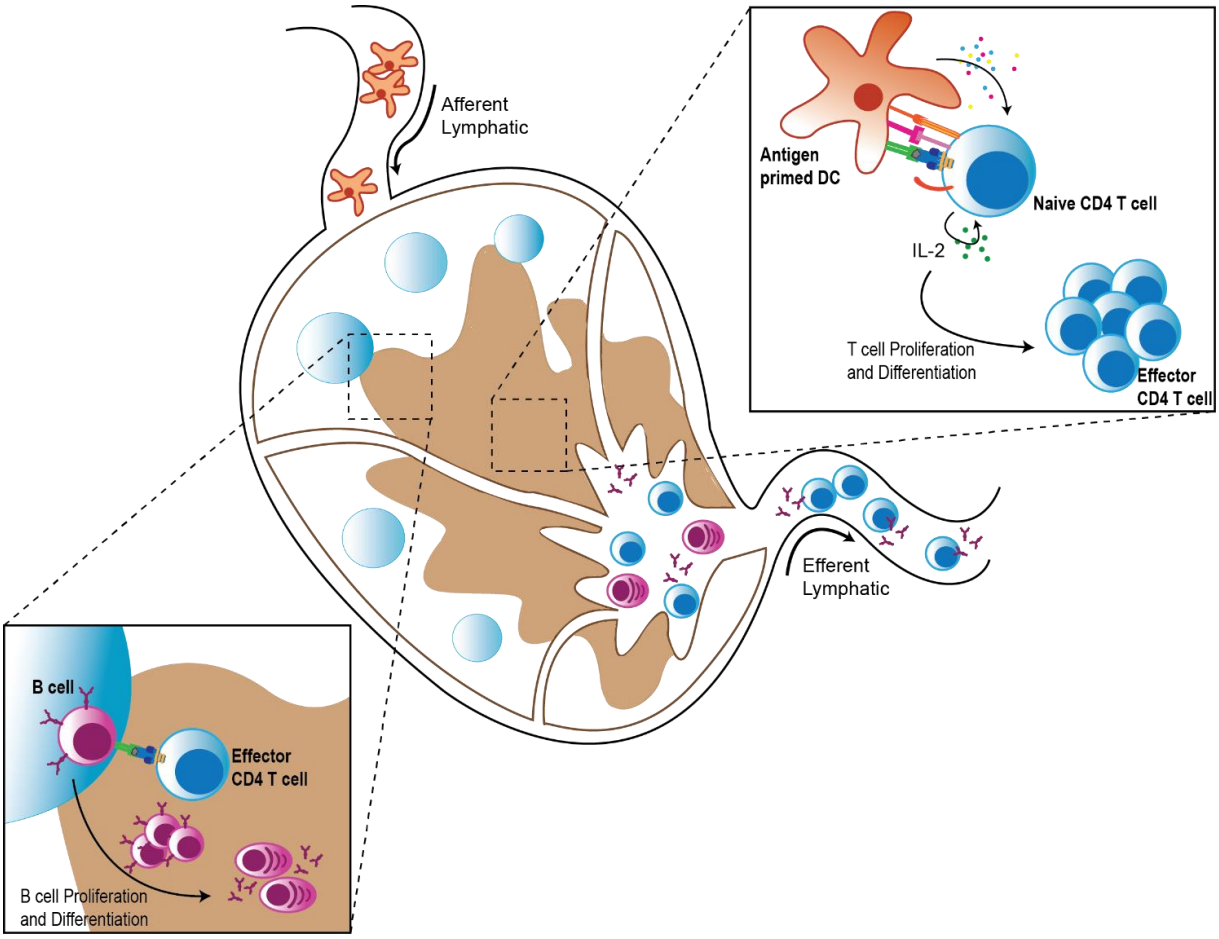


Figure 1.4 – Antigen presentation in the lymph nodes. Based on (Janeway et al. 2001). In the inductive phase of the immune response, antigen primed-DCs migrate into the T-cell-zone in the lymph nodes where they activate naïve CD4 T cells to proliferate and differentiate into a respective Th subset, depending on the antigen and the cytokines produced by the APC. Meanwhile, effector CD4 T cells migrate nearby the B-cell-zone to activate naïve B cells into antibody-secreting plasma cells. Both effector T and B cells migrate to the local of infection through the efferent lymphatic system and blood plasma.

lineage originated in the thymus, and they can modulate the immune response, maintain tolerance to self-antigens preventing autoimmune diseases.

The cytokine environment at the time of antigen presentation, and the nature and quantity of the antigen itself, drive T cell polarization. During this process, $CD4^+$ effector T cells can acquire regulatory features, therefore becoming induced regulatory T cells (iT_{regs}) (Mayne and Williams 2013). iTregs are functional relevant in feto-maternal interfaces (Samstein et al. 2012) and to maintain tolerance to food- and microbiota-derived antigens at mucosal sites (Haribhai et al. 2009).

Each T cell subset has its specific ability to sense different inductive cytokines, and environmental cytokines can arise from the innate response and be produced by other T lymphocytes and APCs. Therefore, $CD4^+$ T cells can be differentiated into a number of cell subsets such as iT_{regs}, helper T cells type 1 (Th1), Th2, Th17 and others. iT_{regs} develop in response to Transforming growth factor beta (TGF-β), Th1 develop in response to IL-12 and Interferon gamma (IFN-γ), Th2 develop in response to IL-4, IL-5 and IL-13, and Th17 develop in response to IL-6, TGF-β and IL-1.

Downstream of TCR and cytokine receptors there is the induction of a network of transcription factors that work alongside to determine the Th lineage (Zhu and Paul 2010). Consequently, each Th

subset has its own master regulator transcription factor: Forkhead box P3 (Foxp3) induces T_{regs}, T-box transcription factor (Tbet) induces Th1 cells, GATA3 induces Th2 cells and RAR-related orphan receptor gamma (RORγT) is the defining transcription factor of Th17 (DuPage and Bluestone 2016).

These subclasses are related with the direction in which the helper T cell pushes the response *(Figure 1.5)*. iT_{regs} secrete the signature cytokines IL-10 (Eberl 2016) and TGFβ, essential for limiting immune responses by regulating T cell priming and function, and they also prevent autoimmunity. Th1 cells are essential for clearing intracellular bacteria and viruses, and activate macrophages. Their signature cytokine is IFN-γ. Th2 cells are highly related with the humoral response, since they are crucial to activate eosinophils and to organize the host defense against extracellular parasites (e.g. helminths), by helping B cells to produce antibodies. They secrete IL-4, IL-5 and IL-13. Th17 cells are higher secretors of IL-17A, IL-17F and IL-21. This subset is related with anti-fungal immunity, and plays a crucial role in maintaining mucosal barriers and clearing pathogens at mucosal surfaces (Hermann-Kleiter and Baier 2010). Besides their protective role, Th17 cells can play a pathogenic role particularly in chronic inflammatory conditions such as Psoriatic arthritis and Psoriasis (Mease 2015). Recent studies dissected the role of IL-17 and Th17 cells in tissue inflammation, autoimmunity and host defence, and demonstrated their contribution to local and systemic aspects of disease pathogenesis. That lead to consider these cells as potential therapeutic target for the treatment of such disorders (Miossec and Kolls 2012). Oppositely to Th1 cells, Th17 cells are very difficult to detect in inflamed tissues due to their

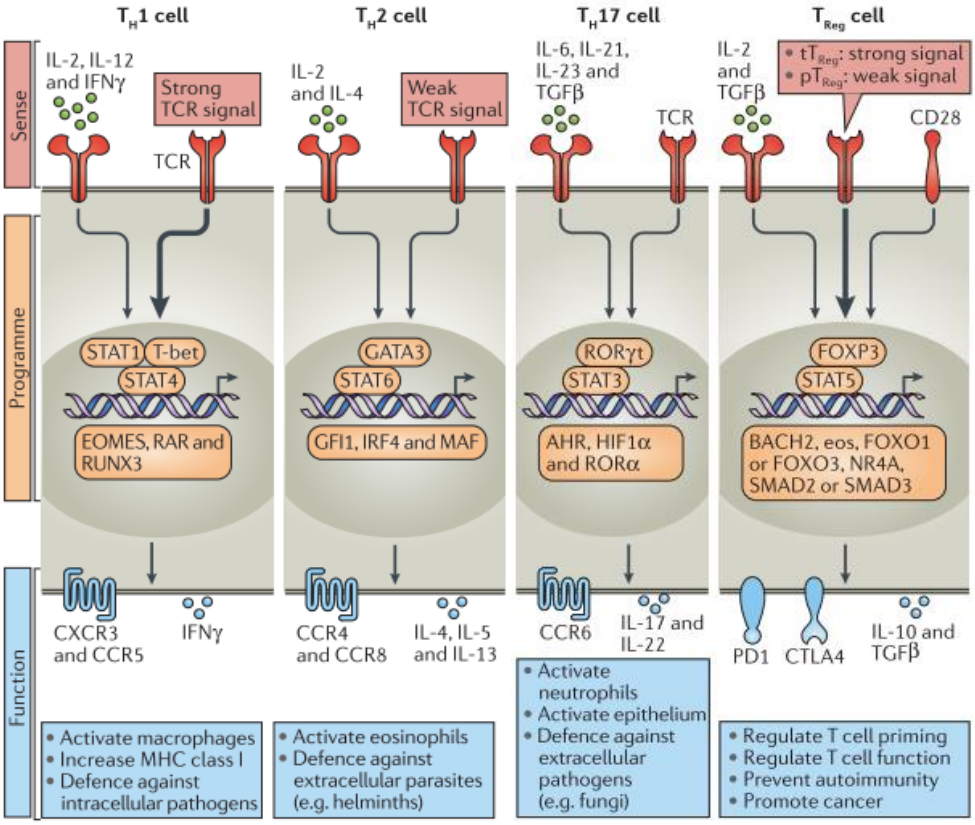


Figure 1.5 – Polarized $CD4^+$ T cell subsets. Adapted from (DuPage and Bluestone 2016). Each $CD4^+$ T cell subset can be defined by their distinct abilities to sense (red), programme (orange) and function (blue) in the control of specific pathogens or immune pathologies. The inductive cytokines, polarizing transcription factors and cytokines or chemokine receptors that are characteristic of each subset are shown, along with their association with specific forms of immune defence.

limited ability to proliferate after TCR engagement, but also for their tendency to shift to a Th1 phenotype in response to different stimuli (Cosmi et al. 2014). As a result from this functional plasticity, those non-classic Th1 have their pathogenic feature increased, thus opening the discussion about which subset should be targeted by therapeutic interventions.

Following the topic of cell plasticity and multifunctionality, even though in a traditional view, naïve T cell activation, proliferation and differentiation are all considered as simultaneous processes, differentiated T cells can be reactivated in the local of infection/trauma or even modifying and reshaping from their "primary" function (DuPage and Bluestone 2016). Indeed, the hallmark feature of Th17 cells is their plasticity, i.e. their ability to express cytokines typical of other lineages in response to distinct microenvironments (Hirota et al. 2011), (Annunziato et al. 2007). As discussed previously, Th17 are considered to be the main population of pathogenic T cells driving autoimmunity (Hirota et al. 2007), (Langrish et al. 2005), poised to develop strong tissue-destructive properties. However, recent evidence has shown that they can adopt an IL-10 producing tolerogenic phenotype and contribute to resolution of inflammation in the central nervous system (Heinemann et al. 2014) and in the gut (Gagliani et al. 2015), (Esplugues et al. 2011). This plasticity of Th17 cells could be considered as a novel therapeutic opportunity, whereby pre-committed pro-inflammatory cells can adopt an anti-inflammatory fate.

A recent study (Heinemann et al. 2014) found that formerly Th17 cells respond to IL-27 and IL-12 by up-regulating Blimp1 and adopt a T regulatory type 1 (T_R1) phenotype characterized by IL-10 production. While T_{regs} are characterized by the major transcription factor Foxp3, T_R1 cells secrete huge amounts of anti-inflammatory IL-10 and express cell-surface markers CD49b and LAG-3 (Lymphocyte-activation gene 3) (Gagliani et al. 2013). Furthermore, the same group (Gagliani et al. 2015) constructed a new mouse fate-mapping mouse model to prove that $CD4^+$ T cells formerly expressing *Il17a* go on to acquire an anti-inflammatory phenotype. This "transdifferentiation" of Th17 cells into T_R1 cells comprises a change in their signature transcription profile and the acquisition of potent regulatory capacities.

Finally, a recent study (Yang et al. 2015) characterized a microbiota-induced hybrid population of T cells expressing both Foxp3 and RORγT and showing both transcriptional and epigenetic profiles of Th17 cells and T_{regs}. They proved that their specific molecular pattern provide them with stable and unique functional properties of effector T_{regs} that allow them to efficiently supress gut-specific inflammatory responses. Despite intense interest, the cellular and molecular cues that confer tissue-protective properties to Th17 cells have remained poorly defined.

It is crucial to understand the general cellular and molecular mechanisms that might explain how a pro-inflammatory Th17 response, which is beneficial in clearing infection, but immunopathogenic in excess, can be controlled by the tissue microenvironment.

Those mechanisms for counter-regulation of Th17 pathogenicity might be exploited to develop new and more effective therapies that restore tolerance in chronic inflammatory/autoimmune diseases without the deleterious side effects of current therapies.

1.3. Toll-like receptors

TLRs are a family of receptors that can initiate innate immunity and inflammation in response to danger signals in the form of infection or tissue damage. The discovery of TLRs furthered our

understanding of how the host rapidly responds to invading pathogens. As described above, TLRs are the best known innate immune receptors which allow the activation of the second-line action against the invading pathogen. When cells encounter a pathogen, a microbial product and/or “stress signal”, TLR activation initiates signal transduction pathways that end up in a potent transcriptional response that culminates in inflammatory cytokine production (Barton and Kagan 2009).

They can be classified and divided depending on their ligands and subcellular localization. TLR 1, 2, 6 and 5 are localized on the cell surface, thus recognizing accessible molecules in the membrane of most pathogens. TLR 3, 7, 8 and 9 are localized in intracellular compartments, recognizing microbial nucleic acids. TLR4 is unique among the TLRs as it is expressed both at the plasma membrane and endosomal compartments. In fact, TLR4 subcellular localization determines the distinct signalling pathways engaged and the consequent cellular cytokine profile (Barton and Kagan 2009).

TLR signal transduction has been increasingly studied through recent years, and it is now known that TLRs can be found in a huge variety of mammalian cell types and that their localization and trafficking patterns are crucial for their signalling functions (Kagan 2010).

Although those families of TLRs diverge in their distinct ligands, localization and functional relevance, most of them share a common signalling pathway to induce innate and adaptive immunity. For over 15 years studies have been focused on the downstream signalling pathways that are activated by the different TLRs in mammalian cells *(Figure 1.6)*. TLR signalling is initiated by ligand-induced dimerization of receptors. It initiates with the activation of the adaptor protein: MyD88 (Myeloid differentiation primary response protein 88) or TRIF (TIR-domain-containing adapter-inducing interferon-β). These adaptor proteins are recruited to the Toll/interleukin-1 receptor (TIR) -domain, a cytosolic tail present in all TLR receptors (O’Neill, Golenbock, and Bowie 2013).

Both the TLRs expressed on the plasma membrane and endosomal compartments, lead to the downstream cascade events involving interactions between IL-1R-associated kinases (IRAKs) and the adaptor molecules TNF receptor-associated factors (TRAFs), and that lead to the activation of the mitogen-activated protein kinases (MAPKs) JUN N-terminal kinase (JNK) and p38. Finally that will lead to the activation of the transcription factors NF-kB (Nuclear factor kappa B), CREB (cyclic AMP-responsive element-binding protein) and AP1 (activator protein 1). This cascade induces a pro-inflammatory response.

In addition, endosomal TLRs (3, 7, 8, 9 and 13) and the TLR4, due to its bipartite distribution between the plasma membrane and the endosomes, can activate a different cascade of signalling molecules that turn on an anti-inflammatory phenotype. This signalling pathway also initiates with interaction between IRAKs and TRAFs, however it ends up with the activation of different transcription factors: Interferon regulatory factor 3 (IRF3) and IRF7 *(Figure 1.6)*.

Only TLR3 and the endocytosed TLR4 rely on TRIF to activate their downstream signaling cascades. All the other TLRs initiate their signaling pathways by activating MyD88. On both cases, some TLRs require an extra molecule to induce the activation of both MyD88- and TRIF-dependent signalling pathways. Those molecules are called the “sorting adaptors” and they promote the communication between the TLR and the respective adaptor. MAL (MyD88-adapter-like protein) also known as TIRAP (TIR domain-containing adaptor protein) is the sorting adaptor for the MyD88-dependent cascade. And

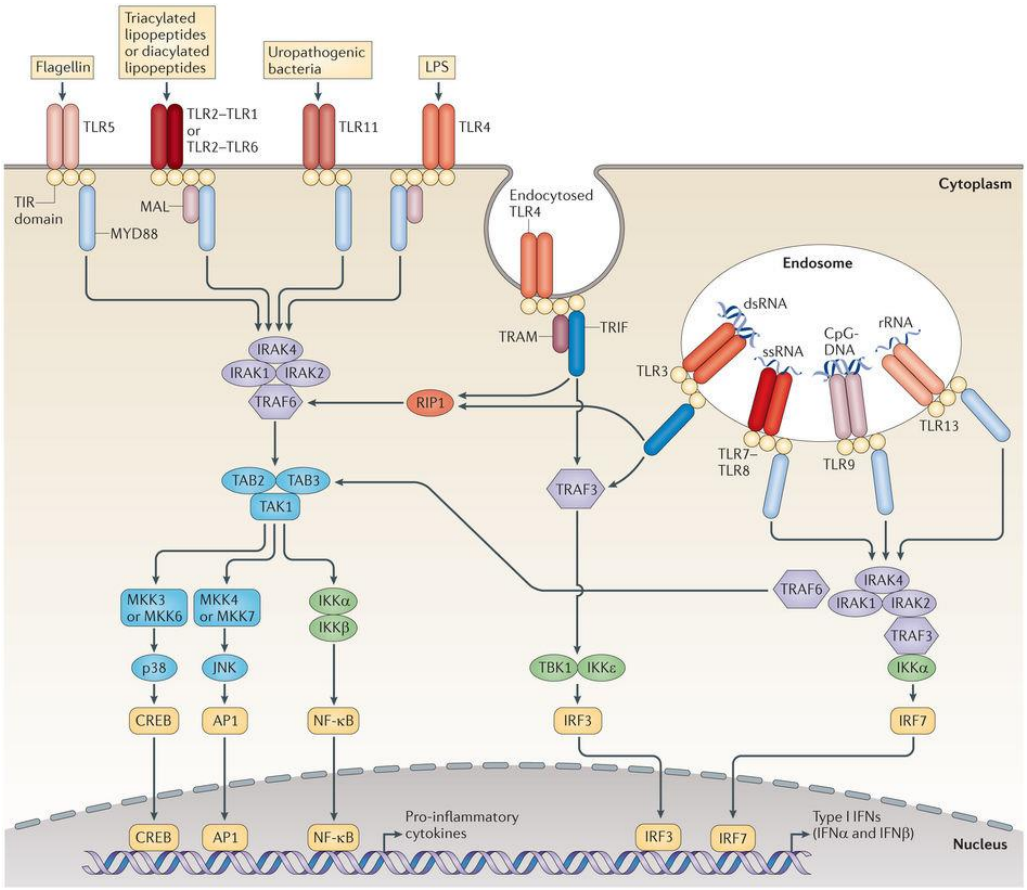


Figure 1.6 - TLR signaling in mammalian antigen presenting cells. From (O'Neill, Golenbock, and Bowie 2013). The TLR signalling pathway is activated by ligand-induced dimerization of receptors, followed by the binding of MYD88 and Mal (for TLR1, TLR2, TLR5, TLR6, TLR7, TLR8 and TLR9) or TRIF and TRAM (for TLR3 and TLR4). TLR4 moves from the plasma membrane to the endosomes to switch signalling from MYD88 to TRIF. Activation of IL-1R-activating kinase (IRAK)-1, IRAK-2, IRAK-4 and TNF-receptor-associated factors (TRAFs) leads to the activation of JNK and p38. Transcription factors such as NF-κB, CREB, AP-1 and IRF proteins induce proinflammatory cytokines. Activation of the endosomal TLRs leads to the production of type I interferons.

TRAM (TRIF related adaptor molecule) works along the TRIF molecule. The reason why some TLRs need only the signalling adaptor when others demand the sorting/signalling adaptor pair is still unknown.

1.3.1. Toll-like receptor 4

TLR4 signalling is triggered by Lipopolysaccharide (LPS), a bacterial component produced by all Gram-negative bacteria (Poltorak et al. 1998). A few years ago, it was found that both TLR3 and TLR4 drive the induction of IFNα and IFNβ through a MyD88-independent pathway that ends up with the activation of the transcription factor IRF3 (Barton and Medzhitov 2003). Therefore, TLR4 was found to rely either on MyD88 or on the adaptor molecule TRIF to support, respectively, a pro- or anti-inflammatory response. Reinforcing these 2 divergent pathways for TLR4 signaling, it was demonstrated that by disrupting receptor endocytosis, MyD88-dependent activation of NF-kB could be enhanced resulting in increased LPS-induced pro-inflammatory outcome (Husebye et al. 2006). Latter, another group (Barton and Kagan 2009) found that TLR4 could be expressed at both plasma membrane and in intracellular compartments, reason why it is able to signal by those 2 distinct pathways *(Figure 1.7)*.

Kagan lab furthered their studies to unravel the mechanism of TRIF/TRAM activation. In 2008 (Kagan et al. 2008) they suggested the new and nowadays accepted model where the TIRAP-MyD88 pathway is induced from the plasma membrane, whereas the TRAM-TRIF pathway is induced from endosomes. They demonstrated that after activation of endocytosed TLR4, the downstream signalling events improved IFN expression and a subsequent regulatory cytokine output (IFNα and IFNβ). This spatiotemporal control of the TLR signalling can be highly related with lipid second messengers incorporated in the different cell membranes. PtdIns(4,5)P_2 can be modified by kinases such as PI3K (Phosphoinositide 3-kinase) to generate PtdIns(3,4,5)P_3 (Phosphatidylinositol 3,4,5-triphosphate) (Li and Rudensky 2016). This depletion in PtdIns(4,5)P_2 leads to a dissociation of the TIRAP/MAL sorting adaptor from the surface TLR4. As the amount of PtdIns(3,4,5)P_3 arises, the TLR4 can be internalized where it will associate with the TRIF/TRAM adaptors. This TRIF-TRAM signalling pathway activates TBK1 (TANK-binding kinase 1) and IKKε (IκB kinase ε) which are responsible for type I IFN production, driving and defining the regulatory or anti-inflammatory states that will help to curtail inflammation and avoid disease (Wall et al. 2017).

Husebye lab extended their work to TLR4 trafficking (Husebye et al. 2010) and they suggested a role for the small GTPase Rab11a to promote TLR4 internalization into recycling endosomes. Rab11a was also found to regulate the recruitment of TLR4 from these recycling endosomes to bacteria-containing phagosomes, where both TRAM and IRF3 are localized, promoting TRIF-dependent TLR4 signaling and the expression of type I IFN.

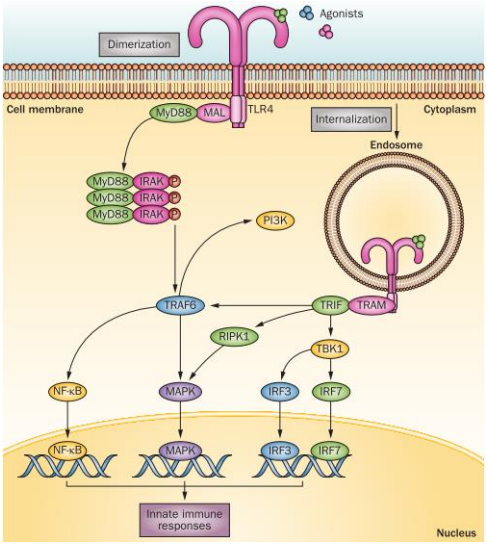


Figure 1.7 - TLR4 signalling in antigen presenting cells. From (Gómez et al. 2014). Upon ligand-induced TLR4 dimerization, the MyD88-dependent signalling pathway is activated. TLR4 can also internalize into endosomes and signal by the MyD88-independent pathway. TLR4 signalling activates multiple transcription factors, including MAPK, interferon regulatory factors and NF-κB, which promote innate immune responses.

Actually, in addition to the release of pro-inflammatory mediators, TLRs activation also leads to the production of IL-10. This *Il10* expression is strictly regulated to ensure an effective immune response, while preventing chronic infection and tissue destruction (Teixeira-Coelho et al. 2014). Interestingly, Saraiva and collaborators (Teixeira-Coelho et al. 2014) found that the TRIF pathway previously stated regulates IL-10 production at the post-transcriptional level, discriminating between TLR2 and TLR4 activation on macrophages. In fact, they show that, unlike TLR2, TLR4 signals protected *Il10* mRNA from degradation, not only due to the activation of TRIF but also due to enhanced p38 signaling. Other studies (Saraiva and O'Garra 2010), (Gabrysova et al. 2014) mention the crucial role of extracellular signal–regulated kinase (ERK) activation in the expression of IL-10 in different cells, from macrophages and DCs to Th cells. In fact, in the previously stated paper (Teixeira-Coelho et al. 2014) they showed that inhibition of ERK does not affect *Il-10* expression post-transcriptionally, but reduced the amount of IL-10 secreted after LPS-induced TLR4 activation in macrophages. Thus, ERK plays a major role in transcriptionally regulating IL-10.

Overall, this strict regulation of TLR4 signaling through compartmentalization leads to a transition between two distinct pathways related with contrasting cytokine outputs. This capacity to rapidly block one pathway and activate the other drives and defines the regulatory or anti-inflammatory state that will help to curtail inflammation and avoid disease.

1.4. Expression of TLRs in T cells

T cells are key players in the adaptive immunity. They must be activated as a response to environmental cues which initialize a complex signaling cascade and the production of immune mediators responsible for disease resolution. T cell full activation requires 3 signals, as illustrated in *Figure 1.8*. Even though TLRs are well-known to be expressed on innate immune cells, it was found that T cells can also express a variety of TLRs, being capable to directly respond to different TLR agonists. Thus, we wondered if TLRs could be an emerging 4th signal for not only helper T cell activation, but also for their function.

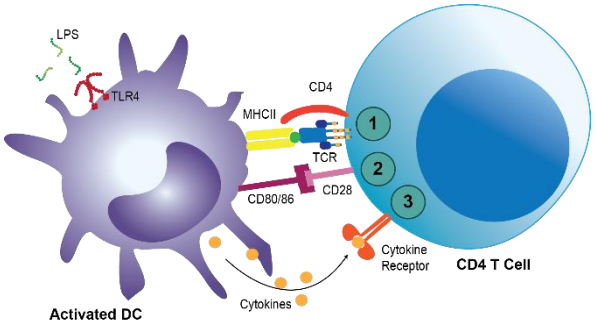


Figure 1.8 – Three required signals for T cell full activation. Based on (Janeway et al. 2001). To be activated, T cells require 3 signals: 1st – The recognition of foreign antigens by the TCR in the context of the MHC; 2nd – The activation of costimulatory signals provided by the interaction of co-receptors, such as CD28 and CD40L; 3rd – The response to environmental cytokines.

In human peripheral T cells isolated with >95% purity, low expression of TLR1-10 mRNA was detected by qPCR (Real-time polymerase chain reaction) (Hornung et al. 2002). Even though whether CD4$^+$ T cells can express TLRs at the protein level remains controversial, T cells were found to express MyD88, the main adaptor protein that initiates TLR signaling, and its deletion affects T cell functions (Fukata et al. 2008). Studies in mouse model indicate that TCR activation and/or the presence of cytokines such as IL-2 and IFN-α upregulate TLR expression (Reba et al. 2014), (Komai-Koma et al. 2004). Moreover, these studies found that TLR ligands directly promote activated CD4$^+$ T cell survival and/or proliferation in murine cells (Gelman et al. 2004), (Reynolds et al. 2012), (Imanishi et al. 2007), (Reynolds et al. 2010), (Zheng et al. 2008) and in human cells (Komai-Koma et al. 2004), (X. Chen et al. 2009), (Babu et al. 2006). When stimulated with Pam3Cys, a TLR2 agonist, CD4$^+$ T cells display improved cytokine production and proliferation (Komai-Koma et al. 2004). Also, it was proved that TLR2 works as a co-stimulatory receptor for human T cells, enhancing the TCR-induced production of the cytokines, IFN-γ, IL-10, IL-13, Granulocyte-macrophage colony-stimulating factor (GM-CSF) and Vascular endothelial growth factor (VEGF), and the TCR-mediated secretion of chemokines CXCL10 and CCL5 (Chapman et al. 2013). Other co-stimulatory effects were observed with other TLR ligands. Human memory (CD45RO$^+$) T cells, when challenged with TLR5 and TLR7/8 ligands, flagelin and resiquimod respectively, have their proliferation improved and also an increased production of IFNγ (Jeannin et al. 2005).

Naïve $CD4^+$ T cells differentiate into distinct subsets depending on the nature of the environment during TCR activation. Whether TLR signalling has a direct effect on T cell differentiation or function has remained a matter of debate.

T_{regs} play a crucial role in the maintenance of immune tolerance and in the control of cellular immune responses. As the effector T cells, T_{regs} can also express different TLRs and be directly affected by them. It was previously stated that TLR2 and TLR8 stimulation can reduce the suppressive activity of T_{regs} (Liu et al. 2006), (Peng et al. 2005) and oppositely, TLR2 and TLR5 were able to enhance their suppressive activity (Zanin-Zhorov et al. 2006), (Crellin et al. 2005). Future studies are required to resolve the apparent discrepancies about the TLR2-induced effect on T_{regs}. Interestingly, a recent study (Gerriets et al. 2016) demonstrate that T_{reg} cell metabolism was dynamically regulated by TLR signals and Foxp3. Whereas TLR signals promote T_{reg} proliferation by increasing PI3K-AKT(protein kinase-B)-mTORC1(mammalian target of rapamycin C1) signaling and glycolysis, Foxp3 opposed PI3K-AKT-mTORC1 signaling to diminish glycolysis and anabolic metabolism while increasing oxidative and catabolic metabolism required for a suitable suppressive activity of T_{regs}.

Finally, it was reported that engagement of TLR7 in $CD4^+$ T cells activates an anergic gene-expression program, preventing cell cycle, secretion of pro-inflammatory cytokines after stimulation and a final unresponsiveness state of $CD4^+$ T cells (Dominguez-Villar et al. 2014).

As stated previously, TLRs are crucial to initiate the adaptive immunity as they mediate the recognition of the pathogen by DCs and their subsequent maturation and activation, leading to an improved antigen presentation, to polarization of T cell responses and reversal of suppressive T_{reg} activity (Iwasaki and Medzhitov 2004). Additionally, T cell functions can be directly influenced by TLRs expressed on T cells. How TLRs can influence these functions still remain unknown, as they can lead to direct effects and cellular responses or their signaling could intersect the TCR pathway, modulating T cell responses triggered by TCR stimulation.

1.4.1. TLR4 on T cells

TLR4 has been detected at the mRNA level in CD4 T cells (Hornung et al. 2002), (Gelman et al. 2004), (Tomita et al. 2008), (Fukata et al. 2008), (Reynolds et al. 2010), (González-Navajas et al. 2010). In those studies, they definitely proved that TLR4 can modulate the development and/or function of certain Th cell subsets. However in some they used mice models, and in others they used polarizing conditions during T cell stimulation, preventing the acquisition of reliable results regarding the role of TLR4 on T cell differentiation. That is one of the reasons why there are conflicting reports about TLR4 effects on T cell functions.

Studies found that when T cells were stimulated with LPS, the bacterial ligand for TLR4, the release of the hallmark $CD4^+$ Th2 cell cytokine, IL-4, was reduced (Watanabe et al. 1999), (Matsuguchi et al. 2000).

Furthermore, human $CD4^+CD28^-$ T cells from Ankylosing Spondylitis patients show improved perforin production after LPS-induced TLR4 activation (Raffeiner et al. 2005). Following the same effect, Reynolds and collaborators demonstrated that TLR4 promotes T cell survival in a mouse model of experimental autoimmune encephalomyelitis (EAE) (Reynolds et al. 2012). Albeit LPS did not promote

IL-17 production, loss of TLR4 solely in $CD4^+$ T cells almost completely abrogated disease symptoms. Implying a role for TLR4 in the survival of Th17 cells.

However, the putative role of TLR4 expression in autoimmunity has yielded contradictory reports. In another autoimmune disease model, the transfer colitis model (González-Navajas et al. 2010), TLR4 has been shown to have a tonic inhibitory role on subsequent TCR-dependent $CD4^+$ T cell responses. In fact, TLR4-deficient T cells exacerbated disease. Importantly, in this model, LPS treatment causes $CD4^+$ T cells to lose IFNγ but upregulate IL-17 expression, promoting disease resolution. Interestingly, other studies also demonstrated a protective role from colitis in a $MyD88^{-/-}$ model after LPS engagement (Fukata et al. 2008), (Tomita et al. 2008). This suggests that TLR4 in a $MyD88^{-/-}$ model keeps signaling in a TRIF-dependent way providing a protective role associated with IL-10 production, as what happen in innate immune cells.

The apparent incongruity of these two reports might be a result of differential TLR ligand availability in the gut versus the sterile environment of the central nervous system. $CD4^+$ T cells in the gut are continuously exposed to TLR stimuli from the microbiota, while $CD4^+$ T cells in the central nervous system would probably be responding to host-derived danger signals.

This can be connected with the previous discussed issue of the impact of the environment on T cell differentiation. Knowing that T cells also express and respond directly through TLRs, it is easier to explain for instance, how the microbiota can influence $CD4^+$ T cell phenotype. Thus, it is possible that, in addition to cytokines, other tissue microenvironment factors, such as LPS-bearing commensal and pathogenic bacteria can directly affect T cells through TLRs, driving a Th17 reprogramming into IL-10 secreting tolerogenic cells (Gagliani et al. 2015), (Esplugues et al. 2011), or even redirecting T_{regs} to a $Foxp3^+RORγT^+$ phenotype with a stable and unique functional suppressive activity during gut-specific inflammatory responses (Yang et al. 2015). I propose that this immunoregulatory mechanism might be particularly important in the microbe rich gut environment.

It is of great importance to explore in more detail the potential of defined TLR ligands to modulate adaptive immune responses in infectious diseases, cancer and autoimmunity. To manipulate T cell functions through TLRs it is required a full understanding of the molecular pathways that orchestrate the intersection between the TCR and TLRs which will depend on the T cell subset and its activation status.

1.5. Aims

Determine how TLR4 engagement imparts on T cell function

Unravel the crosstalk between TCR and TLR4 signaling in T cells

Determine how LPS might counter-regulate T cell inflammatory response

2 MATERIALS AND METHODS

Reagents

- Biocoll separating solution (Biochrom)
- Bovine Serum Albumin (BSA) (HyClone, Thermo Scientific)
- Brefeldin A (BFA, 5 mg/mL) (Penicillium brefeldianum) (Sigma- Aldrich)
- CellTrace™ Far Red (Thermo Fisher)
- Chlorpromazine (CPZ) (10 mM)
- Dimethyl sulfoxide (DMSO) (Sigma)
- EBioscience™ Foxp3/Transcription Factor (Invitrogen, Thermo Fisher)
- Fetal Bovine Serum (FBS) (Biochrom)
- Fixable Viability Dye eFluor 780 (Affymetrix, Thermo Fisher)
- Fluoromount-G (SouthernBiotech)
- Interleukin-2 (IL-2) (NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH from Dr. Maurice Gately, Hoffmann - La Roche Inc.)
- Ionomycin (Calcium salt, Streptomyces conglobatus, 2.5 mg/mL) (Merck Millipore)
- L-Glutamine (Gibco, Thermo Fisher)
- Lipopolysaccharide (LPS) (Salmonella enterica serotype Minnesota, purified by gel-filtration chromatography) (Sigma-Aldrich)
- LIVE/DEAD™ Fixable Aqua Dead Cell Stain (Thermo Fisher)
- Paraformaldehyde (PFA) (Sigma-Aldrich)
- Penicillin/Streptomycin (Pen/Strep) (Gibco, Thermo Fisher)
- Phorbol 12-myristate 13-acetate (PMA, 2.5 mg/mL) (Sigma-Aldrich)
- Phosphate buffered saline (PBS) (Sigma-Aldrich)
- Poly-L-Lysine hydrobromide (PLL, 0.1 mg/mL) (Sigma-Aldrich)
- RPMI (Roswell Park Memorial Institute) Medium 1640 (Gibco, Thermo Fisher)
- Saponin 5% (Carl Roth)
- UltraComp eBeads (Invitrogen, Thermo Fisher)

2.1.1. Antibodies

- αCD28 *[CD28.2]*– 1 mg/mL (BioLegend)
- αCD45 *[GAP 8.3]* – 2,5 mg/mL (American Type Culture Collection)
- αRab11a *[EPR7587(B)]*– 0,3 mg/mL (Abcam)
- αTCR *[UCHT1]*– 0,5 mg/mL (BioLegend)
- αTLR4 *[76B357.1]*– 1 mg/mL (Abcam)
- Alexa 488 αCD4 *[OKT4]* – 0,5 mg/mL (BioLegend)
- Alexa 488 Anti-FITC [5D6.2] – 1 mg/mL (Merck Millipore)
- Alexa 488 Anti-mouse IgG2b – 2 mg/mL (Thermo Fisher)
- Alexa 568 Anti-mouse IgG2a – 2 mg/mL (Thermo Fisher)
- Alexa 568 Anti-mouse IgG2b - 2 mg/mL (Thermo Fisher)

- Alexa 647 αIL10 *[JES3-9D7]* – 20 µg/mL (BioLegend)
- Alexa 647 Anti-mouse IgG2b - 2 mg/mL (Life technologies, Thermo Fisher)
- Alexa 647 Anti-rabbit – 2 mg/mL (Thermo Fisher)
- Anti-mouse IgG1 (*crosslinking antibody*) *[RMG1-1]*– 0,5 mg/mL (BioLegend)
- APC-Cy7 αIL17-A *[BL168]* – 0,5 mg/mL (BioLegend)
- APC-Cy7 anti-human CD38 [HIT2] – 0,4 mg/mL (Biolegend)
- Brilliant Violet 421 anti-human CD279 (PD-1) *[EH12.2H7]* – 50 µg/mL (Biolegend)
- FITC anti-mouse IgG1 – 1 mg/mL (Abcam)
- Mouse IgM αTCR *[MEM-93]* – 1 mg/mL (Sigma-Aldrich)
- Pacific Blue αIFNγ *[4S.B3]* - 0,5 mg/mL (BioLegend)
- Pe/Cy7 anti-human CD45RO *[UCHL1]* – 1 mg/mL (Biolegend)
- Phospho-AKT (Ser473) *[Poly6490]* – 0,11 mg/mL (BioLegend)
- Phospho-p38 MAPK (Thr180/Tyr182) – 44 µg/mL (Cell Signaling)
- Phospho-p38 MAPK (Tyr322) – 1 mg/mL (EnoGene)
- Phospho-PKCθ(Protein kinase C-theta) (Ser676) – 1 mg/mL (Sigma-Aldrich)
- Phospho-ZAP70(Zeta chain of T cell receptor associated protein kinase 70kDa) *(Tyr319)*/ Syk(Spleen tyrosine kinase) *(Tyr352)* – 50 µg/mL (Cell Signaling)
- Rat αCD28 *[low endotoxin]* – 1 mg/mL (Raybiotech, Inc.)

2.2. Isolation of human peripheral blood mononuclear cells

Whole blood was collected from healthy donors by the certified CEDOC staff at the NOVA Medical School of Lisbon, in accordance with stipulated by the ethical committee. These donors provided consent for their blood cells to be used in research studies conducted at CEDOC.

Human peripheral blood mononuclear cells (PBMCs) were isolated from the blood by Ficoll density centrifugation(Fuss et al. 2009). PBMCs were frozen in FBS containing 10% DMSO, kept at −80°C for 24 hours and then stored at −150°C until use.

2.3. Sorting and purification of $CD4^+$ T cell

PBMCs were thawed and cultured overnight in complete RPMI 1640 medium (RPMI 1640, 10% FBS, 2 mM L-glutamine, 1% Pen/Strep) with IL-2 (5 IU/mL).

PBMCs were then washed with FACS buffer (PBS 1X + 2% FBS), labelled with αCD4 antibody A488 (1 µg/mL) and sorted in BD FACS Aria II. Cells were gated into the Lymphocyte and $CD4^+$ population *(Figure 2.1)*.

This step of purification is critical to obtain highly pure $CD4^+$ T cells and exclude any possibility of contamination by professional APCs. $CD4^+$ T cells were isolated with a purity of ~99,756%.

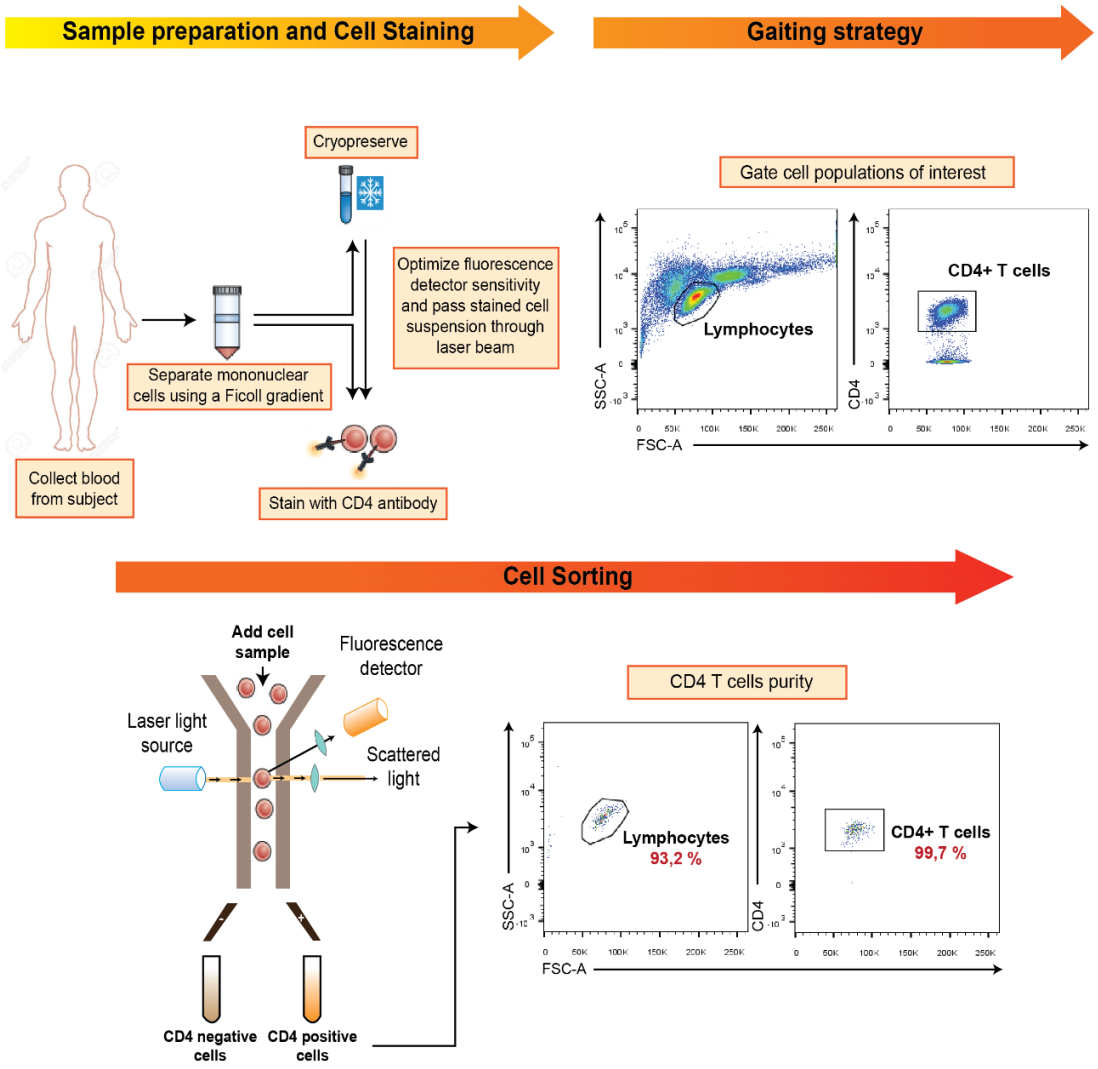


Figure 2.1 - PBMCs Isolation and Cell Sorting strategy. Adapted from (Maecker 2012). PBMCs were collected from blood by Ficoll gradient. Sometimes, sample preparation involves cryopreservation before staining with fluorescent antibodies. Cells were stained with αCD4 antibody A488 and passed through a laser beam to record the fluorescence emission and gate cell population of interest. Cells were then sorted in BD FACS Aria II with a purity of ~99,756%.

2.4. Culture and stimulation assays of CD4⁺ T cells

After purification, sorted cells were washed and cultured at $2x10^6$ cells/mL for 5 days in complete RPMI 1640 medium and IL-2 (20 IU/mL) in 96 round U-bottomed plates coated only with PLL or with PLL, αTCR (10 µg/mL) and αCD28 (2 µg/mL).

$CD4^+$ T cells were left untreated or treated with LPS, the TLR4 ligand, at 1.8 µg/mL.

2.5. T cell survival assay

After 5-days in culture, unstimulated and stimulated cells were washed twice with PBS 1x and stained with either LIVE/DEAD™ Fixable Aqua Dead Cell Stain or Fixable Viability Dye eFluor 780 for 20 min at 4ºC. Cell survival was assessed by gating on lymphocytes and unlabelled cells for the dye by flow cytometry (BD FACS Canto II).

2.6. Immunofluorescence Microscopy

TLR4 surface and total expression

Coverslips were coated with PLL and each one was placed on a 24-well plate. $CD4^+$ T cells were resuspended at the concentration of 1-2x10^6 cells/mL in ~50 µL RPMI/coverslip and dropped at the centre of each coverslip. Spin the 24-well plate at 300 rpm, RT. Cells were fixed in 2% PFA for 30 min, room temperature (RT).

To assess TLR4 surface expression, coverslips were washed with blocking buffer (PBS 1x + BSA 1%) and surface labelled with primary antibodies for CD45 (10 µg/mL) and TLR4 (10 µg/mL) for 1 hour at RT, and with fluorescently conjugated secondary antibodies (A488 Anti-mouse IgG2b 10 µg/mL; A568 Anti-mouse IgG2a 10 µg/mL) for 45 min at RT.

To assess TLR4 intracellular expression, after surface staining only for CD45, cells were washed with permeabilization buffer (Blocking buffer + 0,05% Saponin) and labelled intracellularly with primary antibody against TLR4 (10 µg/mL) and with fluorescently conjugated secondary antibody A488 Anti-mouse IgG2b 10 µg/mL. Incubation periods were identical to the ones performed in the surface staining.

TLR4 expression at the synaptic membrane after TCR stimulation

Coverslips were coated with only PLL or PLL, mouse IgM αTCR (10 µg/mL) and rat αCD28 (2 µg/mL). Coverslip were placed on a 24-well plate and incubated in a water-bath at 37ºC. $CD4^+$ T cells were resuspended at the concentration of 1-2x10^6 cells/mL in ~50 µL RPMI/coverslip and dropped at the centre of each coverslip. After re-stimulation at 37ºC in two condition (5 or 20 minutes of stimulation with soluble LPS 1.8 µg/mL and coated antibodies for TCR and CD28), cells were fixed in 2% PFA for 30 min, RT.

Coverslips were washed with blocking buffer (PBS 1x + BSA 1%) and surface labelled with primary antibodies for CD3 (*UCHT1*; mouse IgG1, 5 µg/mL) and TLR4 (10 µg/mL) for 1 hour at RT, and with fluorescently conjugated secondary antibodies (FITC Anti-mouse IgG1 5 µg/mL; A568 Anti-mouse IgG2b 10 µg/mL) for 45 min at RT. Since the 2ary antibody for CD3 is conjugated with FITC, another staining is required using the 3ary antibody A488 anti-FITC (10 µg/mL), and incubate for just 30 minutes.

ZAP70 intracellular expression

Coverslips were coated with PLL, mouse IgM αTCR (10 µg/mL) and rat αCD28 (2 µg/mL). Coverslips were placed on a 24-well plate and incubated in a water-bath at 37ºC. $CD4^+$ T cells were resuspended at the concentration of 1-2x10^6 cells/mL in ~50 µL RPMI/coverslip and dropped at the centre of each coverslip. After re-stimulation at 37ºC in two condition (30 minutes of stimulation with soluble LPS 1.8 µg/mL prior to 5 or 10 minutes of stimulation with coated antibodies for TCR and CD28), cells were fixed in 2% PFA for 30 min, RT.

Coverslips were washed with blocking buffer (PBS 1x + BSA 1%) and surface labelled with primary antibody against CD45 (10 µg/mL) for 1 hour at RT, and with fluorescently conjugated secondary antibodies (A568 Anti-mouse IgG2a 10 µg/mL) for 45 min at RT.

After surface staining, cells were washed with permeabilization buffer (Blocking buffer + 0,05% Saponin) and labelled intracellularly with primary antibodies against TLR4 (10 µg/mL) and $ZAP70^{Y319}$ (1

µg/mL), and with fluorescently conjugated secondary antibodies (A488 Anti-mouse IgG2b 10 µg/mL; A647 Anti-rabbit 10 µg/mL) diluted in the same buffer. Incubation periods were identical to the ones performed in the surface staining.

Rab11 intracellular expression

Coverslips were coated with only PLL or PLL, mouse IgM αTCR (10 µg/mL) and rat αCD28 (2 µg/mL). Coverslip were placed on a 24-well plate and incubated in a water-bath at 37ºC. $CD4^+$ T cells were resuspended at the concentration of 1-2x10^6 cells/mL in ~50 µL RPMI/coverslip and dropped at the centre of each coverslip. After re-stimulation at 37ºC in one condition (30 minutes of stimulation with soluble LPS 1.8 µg/mL prior to 20 minutes of stimulation with coated antibodies for TCR and CD28), cells were fixed in 2% PFA for 30 min, RT.

Coverslips were washed with blocking buffer (PBS 1x + BSA 1%) and surface labelled with primary antibody against CD45 (10 µg/mL) for 1 hour at RT, and with fluorescently conjugated secondary antibodies (A568 Anti-mouse IgG2a 10 µg/mL) for 45 min at RT.

After surface staining, cells were washed with permeabilization buffer (Blocking buffer + 0,05% Saponin) and labelled intracellularly with primary antibodies against TLR4 (10 µg/mL) and Rab11a (3 µg/mL), and with fluorescently conjugated secondary antibodies (A488 Anti-mouse IgG2b 10 µg/mL; A647 Anti-rabbit 10 µg/mL) diluted in the same buffer. Incubation periods were identical to the ones performed in the surface staining.

All the images were analysed by confocal microscopy using the LSM710 microscope system with ZEN 2010 software (Carl Zeiss) and 63x oil immersion objective (Carl Zeiss).

2.7. T cell Proliferation assay

Cells were cultured in complete RPMI medium supplemented with IL-2 (20 IU/mL), stimulated with αTCR 10 µg/mL + αCD28 2 µg/mL, with or without LPS 1.8 µg/mL, and labelled with CellTrace™ Far Red. After 6 days in culture, cell proliferation was assessed by flow cytometry (BD FACS Canto II).

2.8. Phenotyping of $CD4^+$ T cells

Sorted CD4+ T cells were cultured for 5 days in complete RPMI medium supplemented with IL-2 (20 IU/mL) and stimulated with αTCR 10 µg/mL + αCD28 2 µg/mL, with or without LPS 1.8 µg/mL.

Cells were washed with PBS 1x and incubated with LIVE/DEAD™ Fixable Aqua Dead Cell Stain 20 min 4ºC. Cells were washed once with PBS 1x and then with FACS buffer. Then, they were surface labelled with fluorescently conjugated primary antibodies for PD-1 (1 µg/mL), CD45RO (5 µg/mL) and CD38 (8 µg/mL) for 20 min at RT. Cells were quickly fixed with 2% PFA for 10 min RT and washed twice with FACS buffer. Only then we performed the surface staining for TLR4, since the αTLR4 antibody only work on PFA fixed cells. Cells were surface labelled with the primary antibody for TLR4 (1,33 µg/mL) for 20 min at RT, and with fluorescently conjugated secondary antibody A647 Anti-mouse IgG2b 2,67 µg/mL for 20 min at RT.

2.9. Cytokine production

After 4 days in culture, cells were treated with PMA (50 ng/ml), Ionomycin (500 ng/ml) and BFA (2 µg/ml), a protein transport inhibitor, for ~15 hours.

Cells were washed with PBS 1x and incubated with LIVE/DEAD™ Fixable Aqua Dead Cell Stain 20 min 4ºC.

The intracellular staining was performed using the permeabilization kit/protocol (EBioscience™ Foxp3/Transcription Factor). For cytokine production we performed 30 min RT of permeabilization. The antibodies against IL17A (3,33 µg/mL), IFNγ (10 µg/mL) and IL10 (0,4 µg/mL) diluted in permeabilization buffer from the kit were incubated for 30 min at RT. Cells were washed and analysed by flow cytometry (BD FACS Canto II).

2.10. Cell Signalling protocol – Phospho-protein analysis

After 5 days in culture with complete RPMI medium and IL-2 (20 IU/mL), cells were transferred into eppendorfs and incubated in a water-bath at 37ºC and treated, for the indicated times, with:

(i) CPZ 30 µM and/or

(ii) LPS 1.8 µg/mL and/or

(iii) αTCR 10 µg/mL + αCD28 2 µg/mL + "*crosslinking-antibody*" Anti-mouse IgG1 10 µg/mL

Each signaling molecule has its own time of stimulation in agreement with its position in the TCR signalling cascade (pAKT^{S473}, pMAPK^{Y182}, pPKCθ^{S676} – 15 min; pMAPK^{Y323} – 60 min).

After stimulation, eppendorfs were immediately placed in ice, and 500 µL of cold PBS 1x were added to each one. Cells were washed with PBS 1x and incubated either with LIVE/DEAD™ Fixable Aqua Dead Cell Stain or Fixable Viability Dye eFluor 780 for 20 min at 4ºC.

The intracellular staining was performed using the permeabilization kit/protocol (EBioscience™ Foxp3/Transcription Factor) and, for phospho-molecule detection, cells were permeabilized for 1 hour at RT. The antibodies pAKT^{S473} (2,2 µg/mL), pMAPK^{Y182} (0,9 µg/mL), pPKCθ^{S676} (20 µg/mL) and pMAPK^{Y323} (20 µg/mL) diluted in permeabilization buffer from the kit were incubated for 30 min at RT. Also, this time of incubation was applied for the secondary antibody staining (Alexa 647 Anti-rabbit 4 µg/mL).Cells were washed and analysed by flow cytometry (BD FACS Canto II).

2.11. Statistical Analysis

Mann-Whitney *U*-test was used for comparison between two groups, with a *P* value <0,05 considered statistically significant. Comparison among three groups were performed using a Kruskal-Wallis one-way ANOVA test. All statistical analysis and graphic preparation were performed using Graph Pad Prism (Graph Pad software version 6, Graph Pad software Inc., CA).

3 RESULTS AND DISCUSSION

3.1. Expression of TLR4 on activated human CD4⁺ T cells

TLRs are highly expressed on APCs, such as macrophages and DCs, however TLRs were also found to be expressed on T lymphocytes. Few years ago it was demonstrated the mRNA expression of TLR1-10 in human peripheral T cells (Hornung et al. 2002). More specifically, mRNA expression of TLR4 was found in several studies (Gelman et al. 2004), (Tomita et al. 2008), (Fukata et al. 2008), (Reynolds et al. 2010), (González-Navajas et al. 2010). Furthermore, cell surface expression of TLR4 was also found in human CD4⁺ T cells (Komai-Koma et al. 2004). However it was determined in the presence of considerable amounts of polarizing cytokines such as IFN-α.

Nonetheless, TLRs subcellular distribution, signaling specificities and their respective contributions for human T lymphocyte function remains poorly understood.

We mimicked non-polarizing T cell stimulation to evaluate whether TLR4 could be expressed in human peripheral CD4⁺ T cells. For that purpose, we sorted CD4⁺ T cells with ~99,765% purity to exclude any possible contamination with APCs and we cultured cells for 5 days with TCR+CD28 stimulation and 20 IU/mL of IL-2, the required amount to regulate the rates of cell death and promote CD4⁺ T cell survival (Ganusov, Milutinović, and De Boer 2007).

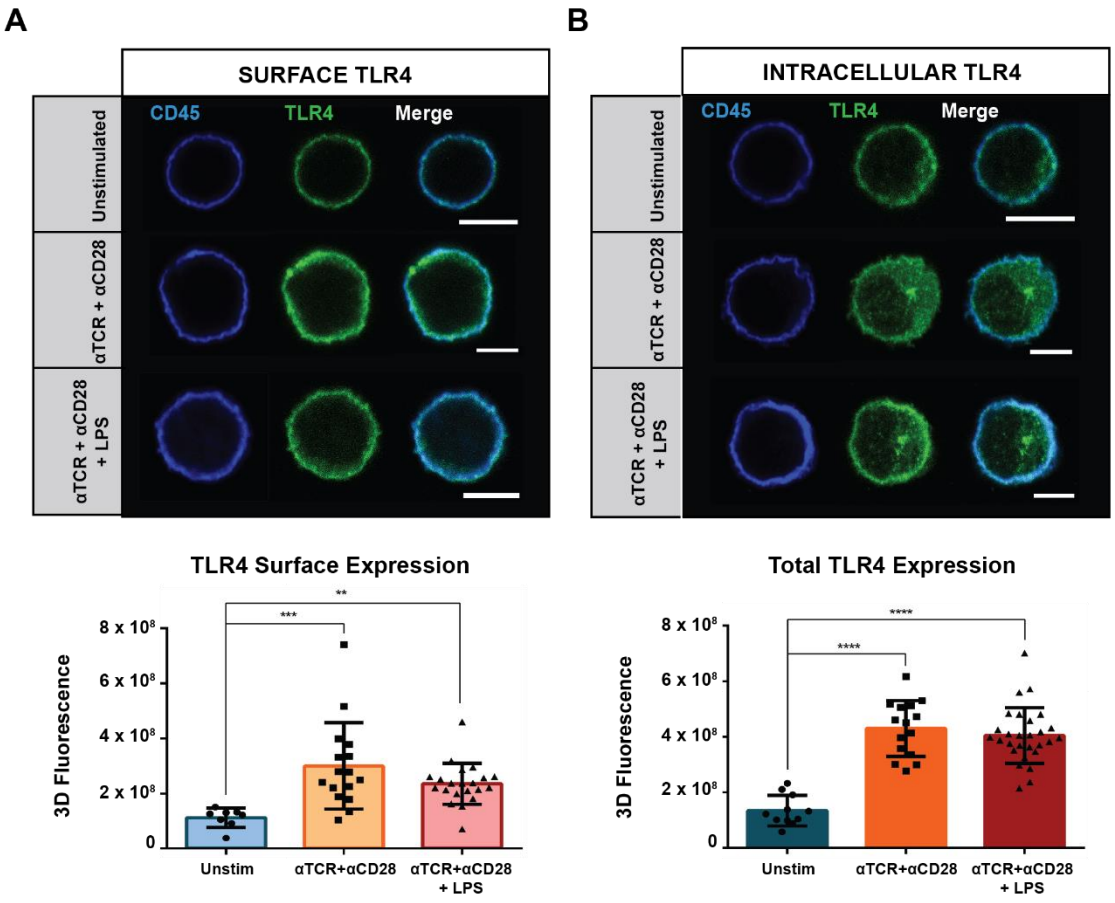
After 5 days in culture, we detected TLR4 surface expression on human CD4⁺ T cells by confocal microscopy. We also found that CD4⁺ T cells improved TLR4 expression in response to TCR stimulation, both in the absence or presence of LPS, the TLR4 ligand *(Figure 3.1.A)*.

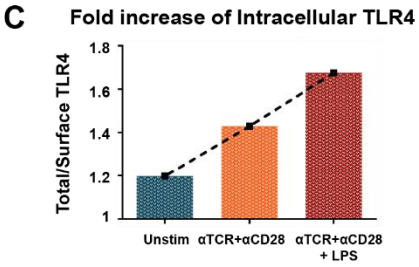
3.2. Chronic engagement of TLR4 improves intracellular expression of TLR4

In innate immune cells, TLRs are expressed in different subcellular localizations. Some TLRs are expressed at the plasma membrane as they recognize accessible molecules in the membrane of most pathogens, however others are localized in intracellular compartments, recognizing microbial nucleic acids. In macrophages, TLR4 is unique as it can be expressed on both plasma membrane and intracellular compartments. In fact, TLR4 can be internalized after its activation where it triggers a distinct signalling pathway, changing the subsequent cytokine profile (Gómez et al. 2014), (Husebye et al. 2006). While TLR4 activation at the plasma membrane of macrophages induces a pro-inflammatory response, the intracellular signaling pathway improves the IFN expression related with a regulatory cytokine output (IFNα, IFNβ and IL-10) (Kagan et al. 2008), (Wall et al. 2017), (Teixeira-Coelho et al. 2014).

This strict regulation of TLR4 signaling through compartmentalization reflects the capacity to quickly block one pathway and activate the other, transitioning from a pro-inflammatory to a regulatory state that help to curtail inflammation and avoid disease. However, whether this spatiotemporal regulation is also present in T cells remains unknown. To clarify that issue, we assessed the TLR4 subcellular distribution, to confirm if it is also expressed in an intracellular compartment. Indeed, we found that TLR4 partitions between the plasma membrane and an intracellular compartments in CD4⁺ T cells *(Figure 3.1.B)*. We also found that chronic engagement of TLR4 with LPS increases TLR4 intracellular expression, promoting its vesicular distribution in CD4⁺ T cells *(Figure 3.1.C and D)*. Those results

reinforce the hypothesis that TLR4 might signal mostly from the intracellular compartment after chronic engagement with LPS.





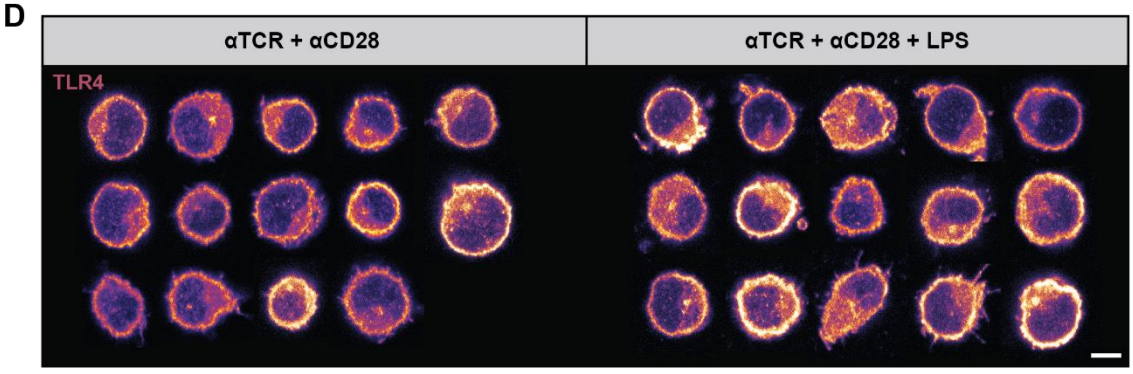


Figure 3.*1* *(in previous page)* **- TCR engagement upregulates TLR4 surface expression in CD4 T cells and chronic TLR4 engagement improves intracellular TLR4.** Highly purified CD4⁺ T cells were cultured for 5 days either unstimulated or stimulated with αTCR 10 µg/mL and αCD28 2 µg/mL or in combination with LPS 1.8 µg/mL. **A -** Sorted CD4⁺ T cells were surface labelled with primary antibodies for CD45 (10 µg/mL) and TLR4 (10 µg/mL), followed by secondary antibodies A488 Anti-mouse IgG2b 10 µg/mL and A568 Anti-mouse IgG2a 10 µg/mL. Population analysis quantifying the 3D fluorescent intensity of surface TLR4 on unstimulated T cells (n=8) and stimulated cells in the absence (n=16) or presence (n=21) of LPS. **B -** Sorted CD4⁺ T cells were surface labelled with the same primary antibody for CD45 and secondary A568 Anti-mouse IgG2a, and labelled intracellularly with primary antibody against TLR4 and secondary A488 Anti-mouse IgG2b. Population analysis quantifying the 3D fluorescent intensity of total TLR4 on unstimulated T cells (n=11) and stimulated cells in the absence (n=15) or presence (n=28) of LPS. **C –** Fold increase of intracellular TLR4 was determined by relating the total expression of TLR4 with the surface expression in the same condition. **D –** TLR4 total expression in stimulated cells in the absence and presence of LPS. Data are representative of one experiment. TLR4 and CD45 expression were determined by confocal microscopy using LSM710 microscope system with ZEN 2010 software (Carl Zeiss) and 63x oil immersion objective. Scale bar = 5 µm. Statistical analysis to compare among the three groups was performed using Kruskal-Wallis one-way ANOVA test **p<0,01, ***p<0.001, ****p<0.0001.

Even though it seems that, in CD4⁺ T cells, TLR4 can also be regulated through compartmentalization, the mechanisms that orchestrate this modulation might be different from the ones occurring in APCs.

Moreover, studies unrevealing the different TLR4 signalling pathways in human CD4⁺ T cells are still remote, and it remains to be addressed whether TLR4 signaling can modulate their function.

3.3. LPS increases human CD4⁺ T cell survival, but doesn't seem to affect proliferation

To investigate how TLR4 engagement imparts on T cell function, we first evaluated T cell survival and proliferation after chronic engagement of TLR4.

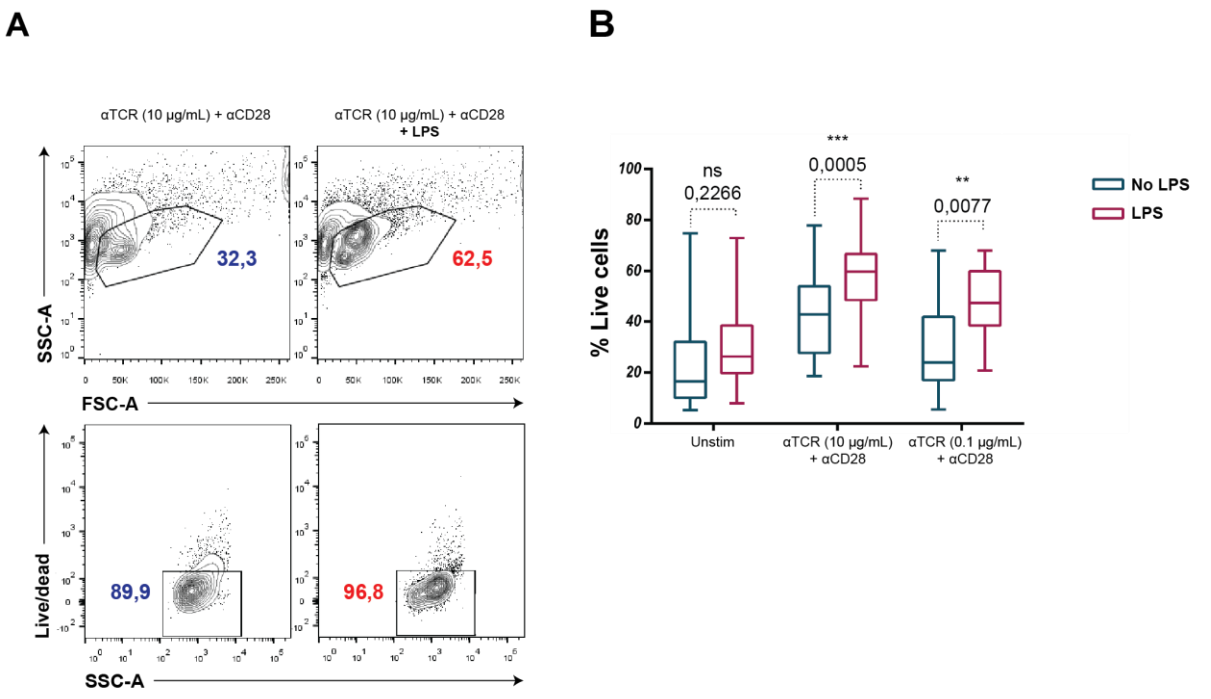


Figure 3.2 *(in previous page)* **- TLR4 engagement on CD4 T cells promotes their survival.** Sorted CD4⁺ T cells were cultured for 5 days either unstimulated or stimulated with αTCR 10 µg/mL / 0.1 µg/mL and αCD28 2 µg/mL or in combination with LPS 1.8 µg/mL. **A -** Representative plots of flow cytometry analysis, demonstrating the effect of LPS on survival of CD4⁺ T cells stimulated with αTCR 10 µg/mL and αCD28 2 µg/mL. Live/Dead staining on CD4⁺ T cells was performed using LIVE/DEAD™ Fixable Aqua Dead Cell Stain or Fixable Viability Dye eFluor 780. **B -** Population analysis of T cell survival in cells treated as in A. Pooled data from 29 independent experiments. Mann-Whitney *U*-test was used for comparison between two groups **p<0.01, ***p<0.001.

Even though most studies unravelling the impact of TLR4 on CD4 T cells have some limitations regarding its impact on T cell differentiation, all of them consensually demonstrated that TLR4 engagement improved T cell survival.

In the experimental design of this project we had in mind that APCs and B cells are highly sensitive to LPS, as they express high levels of TLR4. Therefore in all the experiments performed we sorted CD4⁺ T cells by flow cytometry, excluding the possibility that LPS effects could be due to contamination with other types of immune cells, as they were sorted with a ~99,765% of purity *(Figure 3.1)*. Using primary human CD4⁺ T cells we observed that chronic engagement of TLR4 with LPS for 5 days improved cell survival *(Figure 3.2 A)*. That effect is pronounced in stimulated cells *(Figure 3.2 B)*, which is in line with our previous results showing that TCR engagement enhances TLR4 expression.

Although LPS affects T cell survival, T cell proliferation doesn't seem to be affected *(Figure 3.3)*. However, we only have one preliminary result, which do not allow us to conclude about TLR4 effect in this case, especially as we are working with primary human samples whose results do not follow a normal distribution. In fact, survival and proliferation are mostly associated with the same signaling pathways, suggesting the hypothesis that LPS would also affect T cell proliferation. Yet, if cells chronically stimulated with LPS might in fact have the same proliferation rate, the improved survival might be related with less apoptosis. For that, we propose determining expression of anti-apoptotic molecules (Bcl2, Bcl-xL) and measure apoptosis at different cell cycle stages by propidium iodide flow cytometry assay.

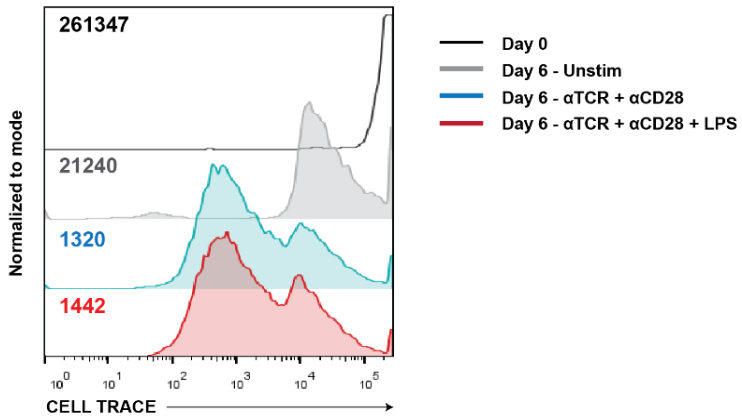


Figure 3.3 - TLR4 engagement on CD4 T cells does not affect their proliferation. Highly purified CD4⁺ T cells were cultured for 6 days either unstimulated or stimulated with αTCR 10 µg/mL and αCD28 2 µg/mL or in combination with LPS 1.8 µg/mL. At day 0, cells were labelled with CellTrace™ Far Red and, at day 6, they were stained for LIVE/DEAD™ Fixable Aqua Dead Cell Stain. Live cells of each condition were analysed by BD FACS Canto II. Data are representative of one experiment. Median values acquired by analysis in BD FACS Canto II.

3.4. CD4⁺TLR4⁺ T cells are double positive for PD1 and CD38

After confirming the TLR4 expression in CD4⁺ T cells by confocal microscopy, we resorted to flow cytometry to characterize TLR4⁺ cells. We assessed 3 cell markers: PD-1, as an exhaustion marker it is part of the costimulatory pathway (PD-1 - PD-1 ligands) that controls multiple tolerance checkpoint

to prevent autoimmunity. This pathway is involved in delivering inhibitory signals that regulate the balance among T-cell activation, tolerance, and immune-mediated tissue damage (Francisco, Sage, and Sharpe 2010); CD38, an activation marker, tightly expressed in activated, larger and more granular T cells (Sandoval-Montes and Santos-Argumedo 2005); and CD45RO, an isoform of the CD45 phosphatase, considered as a memory marker based on the fact that cells expressing CD45RO isoform are highly efficient in respond against a previously encountered antigen (Mackay 1999).

Even though $CD4^+TLR4^+$ T cells can be both $CD45RO^+$ or $CD45RO^-$, when plotted for PD-1 versus CD38, most of them (77,2%) are double positive for those last 2 markers *(Figure 3.4)*. After gating live $CD4^+$ T cells we can note that $TLR4^+$ cells (*red*) are larger and more complex, with greater values of FSC-A and SSC-A, when compared with total $CD4^+$ T lymphocytes (*blue*). We can conclude that 5-day-stimulated $CD4^+TLR4^+$ cells are phenotypically characterized by the expression of activation and exhaustion markers.

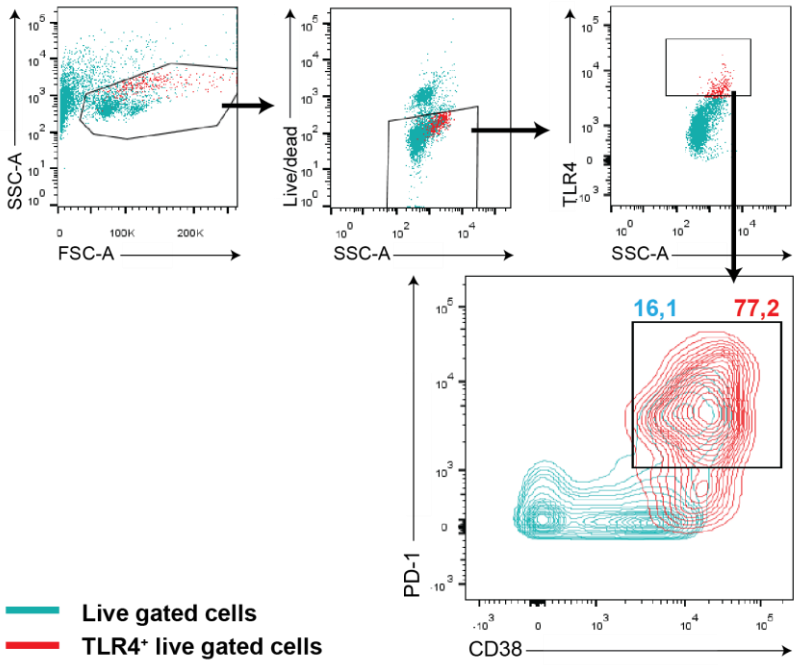


Figure 3.4 - $CD4^+TLR4^+$ T cells are double positive for PD1 and CD38. Highly purified $CD4^+$ T cells were cultured for 5 days either unstimulated or stimulated with αTCR 10 µg/mL and αCD28 2 µg/mL. Cells were stained for LIVE/DEAD™ Fixable Aqua Dead Cell Stain and surface labelled with αTLR4 (1.33 µg/mL), αCD38 (8 µg/mL) and αPD-1 (1 µg/mL). Analysis of live $CD4^+$ T cells and $CD4^+TLR4^+$ cells for both activation and exhaustion markers, CD38 and PD1 respectively. The percentages of double positive $PD1^+CD38^+$ cells were evaluated by flow cytometry in BD FACS Canto II. Data are representative of 2 experiments.

3.5. TLR4 engagement upregulates CD38 and CD45RO

TLR4 engagement with soluble LPS improved the expression of intracellular TLR4 and T cell survival. We were interested in determining the expression levels of surface markers in stimulated cells either in the presence or absence of soluble LPS. We found that TLR4 engagement improved the expression of CD38 and CD45RO, activation and memory markers, on $CD4^+TLR4^+$ cells. CD38 improved expression reflects the enriched activation state of $CD4^+TLR4^+$ T cells. $CD4^+TLR4^-$ cells appear to undergo bystander activation, since there is also an overall improvement in CD45RO and CD38 expression. Differently, LPS seems to decrease PD-1 expression directly in $CD4^+TLR4^+$ cells and to increase the expression of PD-1, an exhaustion marker, on bystander $CD4^+TLR4^-$ cells *(Figure 3.5)*.

LPS signals might be capable to decline PD-1 expression on CD4⁺TLR4⁺ cells, making them unwilling

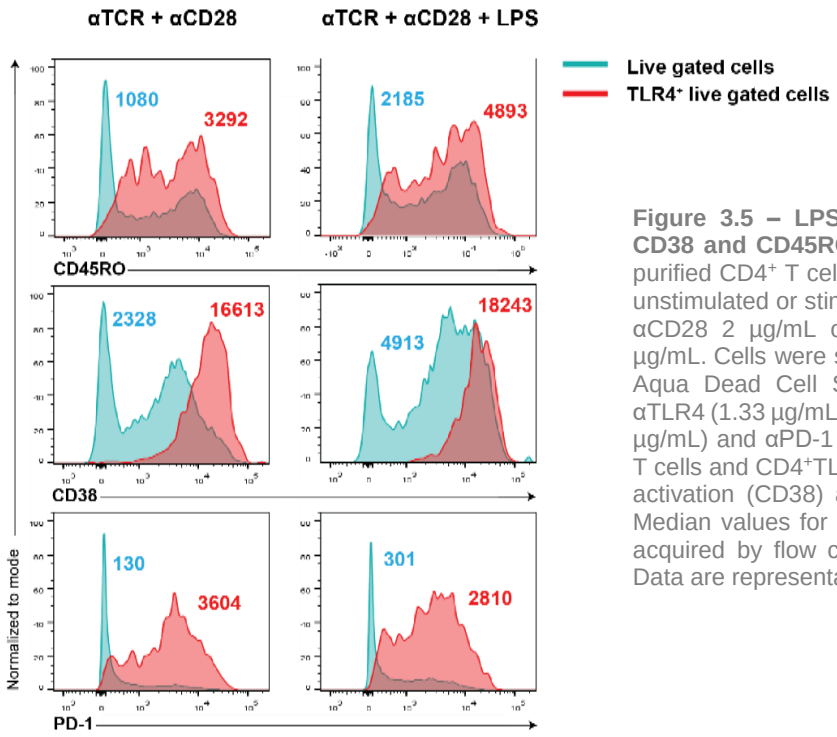


Figure 3.5 – LPS promotes the expression of CD38 and CD45RO in CD4⁺TLR4⁺ T cells. Highly purified CD4⁺ T cells were cultured for 5 days either unstimulated or stimulated with αTCR 10 µg/mL and αCD28 2 µg/mL or in combination with LPS 1,8 µg/mL. Cells were stained for LIVE/DEAD™ Fixable Aqua Dead Cell Stain and surface labelled with αTLR4 (1.33 µg/mL), αCD45RO (5 µg/mL), αCD38 (8 µg/mL) and αPD-1 (1 µg/mL). Analysis of live CD4⁺ T cells and CD4⁺TLR4⁺ cells for memory (CD45RO), activation (CD38) and exhaustion (PD-1) markers. Median values for expression of each marker were acquired by flow cytometry in BD FACS Canto II. Data are representative of 2 experiments.

to receive inhibitory signals.

3.6. Chronic engagement of TLR4 improved AKT^{S473}, p38 MAPK^{Y182} and PKCθ^{S676} activation

Next, we were determined to unravel which T cell pathways could be affected by TLR4 signaling, influencing CD4⁺ T cell activation and differentiation. We inspected the activation state of 3 signaling molecules after the chronic incubation with LPS. At day 5 we re-stimulated cells with soluble antibodies against TCR and CD28, stained them for the respective phosphomolecules and analysed them by flow cytometry.

After chronic engagement with LPS, all 3 signaling molecules AKT^{S473}, PKCθ^{S676} and p38 MAPK^{Y182} were remarkably activated in comparison with TCR+CD28 stimulated CD4⁺ T cells *(Figure 3.6)*.

AKT, along with PI3K and mTOR, is a conserved kinase that regulates cell growth and metabolism in response to environmental cues (Wullschleger, Loewith, and Hall 2006). AKT is mainly known to protect cells from death and it is capable to phosphorylate a variety of components of cell death machinery. AKT inhibits the activity of pro-death proteases like caspase-9; it restores the anti-apoptotic function of the survival factor BCL-X_L (B-cell lymphoma extra-large) by blocking its interaction with the pro-apoptotic molecule BAD (BCL-2 associated death promoter); and, apart from other functions, it can also phosphorylate FOXO1 (Forkhead box protein O1) preventing its nuclear translocation and the activation of FOXO1 gene targets which include several pro-apoptotic proteins like FAS ligand (Vivanco and Sawyers 2002).

pMAPK^{Y182} is the conserved phosphorylated form of p38 MAPK. p38 MAPK is capable to regulate

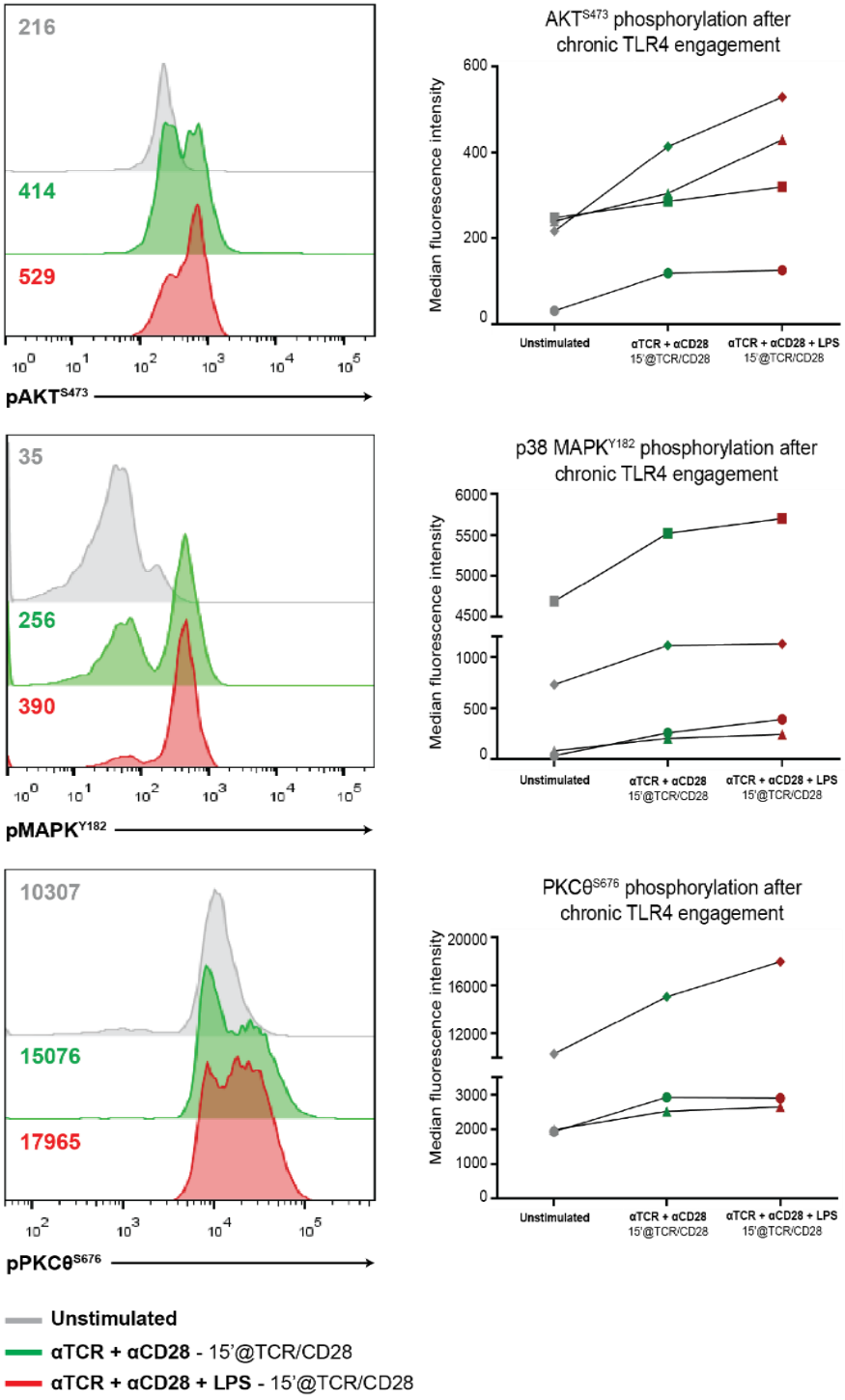


Figure 3.6 - Chronic engagement of TLR4 improved AKT^{S473}, p38 MAPK^{Y182} and PKCθ^{S676} activation. Cells were cultured for 5 days (αTCR 10 µg/mL; αCD28 2 µg/mL; LPS 1,8 µg/mL). Cells were re-stimulated depending on the time of stimulation of each signaling molecule in agreement with its position in the TCR signalling cascade (pAKT^{S473}, pMAPK^{Y182}, pPKCθ^{S676} – 15 min). Re-stimulation was performed by adding soluble antibodies (αTCR 10 µg/mL + αCD28 2 µg/mL) plus a secondary crosslinking antibody Anti-mouse IgG1 10 µg/mL. Cells were stained for LIVE/DEAD™ Fixable Aqua Dead Cell Stain or Fixable Viability Dye eFluor 780, permeabilized using eBioscience™ Foxp3 / Transcription Factor Fixation/Permeabilization kit and stained for the respective phospho-molecules (Antibodies for: pAKT^{S473} 2,2 µg/mL, pMAPK^{Y182} 0,9 µg/mL, pPKCθ^{S676} 20 µg/mL). Data are representative of 4 independent experiments for AKT^{S473} and MAPK^{Y182}, and 3 independent experiments for PKCθ^{S676}. Median fluorescence intensities acquired by analysis in BD FACS Canto II.

the activity of specific transcription factors and to regulate transcription and/or mRNA stability of inflammatory cytokine genes (Taro Kawai and Akira 2009). The enzymatic activity of p38 MAPK^{Y182} is highly influenced by extracellular events such as osmotic and heat shock, and ultraviolet radiation. In response to those stress-inducing stimuli, p38 MAPK has been involved in mediating cell death and/or survival (Noubade et al. 2014). This residue is known to respond to pro-inflammatory cytokines through cytokine receptors and, in macrophages, to some TLR ligands, like LPS (Ashwell 2006).

PKCθ is the first described PKC family member to be recruited to the immunological synapse through CD28 mediated recruitment (Arendt et al. 2002). Upon T cell stimulation, PKCθ is recruited to the plasma membrane where it is phosphorylated, allowing its catalytic activation and its downstream signaling functions. We were interested in this phospho-molecule as it is required for several crucial T cell functions: survival, proliferation and homeostasis (Brezar, Tu, and Seddiki 2015).

After cells being chronically stimulated with LPS, all the 3 signaling molecules were more extensively activated *(Figure 3.6)*. They are all related with T cell crucial functions of survival, activation and differentiation, in agreement to what we conclude so far: chronic engagement of TLR4 improves survival, increases TLR4 intracellular expression and improves the expression of CD38, an indicator of activation and complexity, and the memory marker CD45RO.

Those improved states of activation can be a result of either an additional signal from the TLR4 (AKT^{S473} and p38 MAPK^{Y182}) or a crosstalk between TLR4 and TCR pathways (PKCθ^{S676}). In both cases, TLR4 is likely to be functioning as a co-stimulatory molecule, amplifying TCR signaling.

3.7. LPS directly activates AKT^{S473} and p38 MAPK^{Y182}, but not PKCθ^{S676} in CD4^{+} T cells

We were interested in evaluate whether the acute TLR4 engagement *per se* affects T cell signaling independently from TCR. We look at the 3 signaling molecules that we have already shown to be affected by the TLR4 signaling. For the phosphoflow protocol, we first stimulated cells for 5 days to improve TLR4 expression. Only then we re-stimulated cells with soluble LPS for 15 minutes.

In agreement to what is observed in other cell types, both AKT^{Ser473} and MAPK^{Y182} can be activated solely via TLR4 engagement. Curiously, PKCθ^{S676} cannot be activated only via TLR4 *(Figure 3.7)*.

First, this suggests that TLR4 pathway directly activates AKT^{S473}, contributing to the increment of cell survival after chronic stimulation with LPS *(Figure 3.2)*.

Second, MAPK^{Y182} activation by TLR4 might also be associated with the improved survival, but we definitely can't exclude that it can also impacts on T cell differentiation since p38 MAPK is capable to regulate the transcription and mRNA stability of inflammatory cytokine genes (Taro Kawai and Akira 2009).

Finally, PKCθ, upon T cell stimulation, is recruited to the immunological synapse through CD28 mediated recruitment. Together with our results, we concluded that PKCθ^{S676} indeed requires CD28 engagement to be activated and it cannot be activated solely via TLR4. However, with these experiments we did not explore the possibility that TLR4 could be acting as a co-stimulatory molecule amplifying TCR signaling.

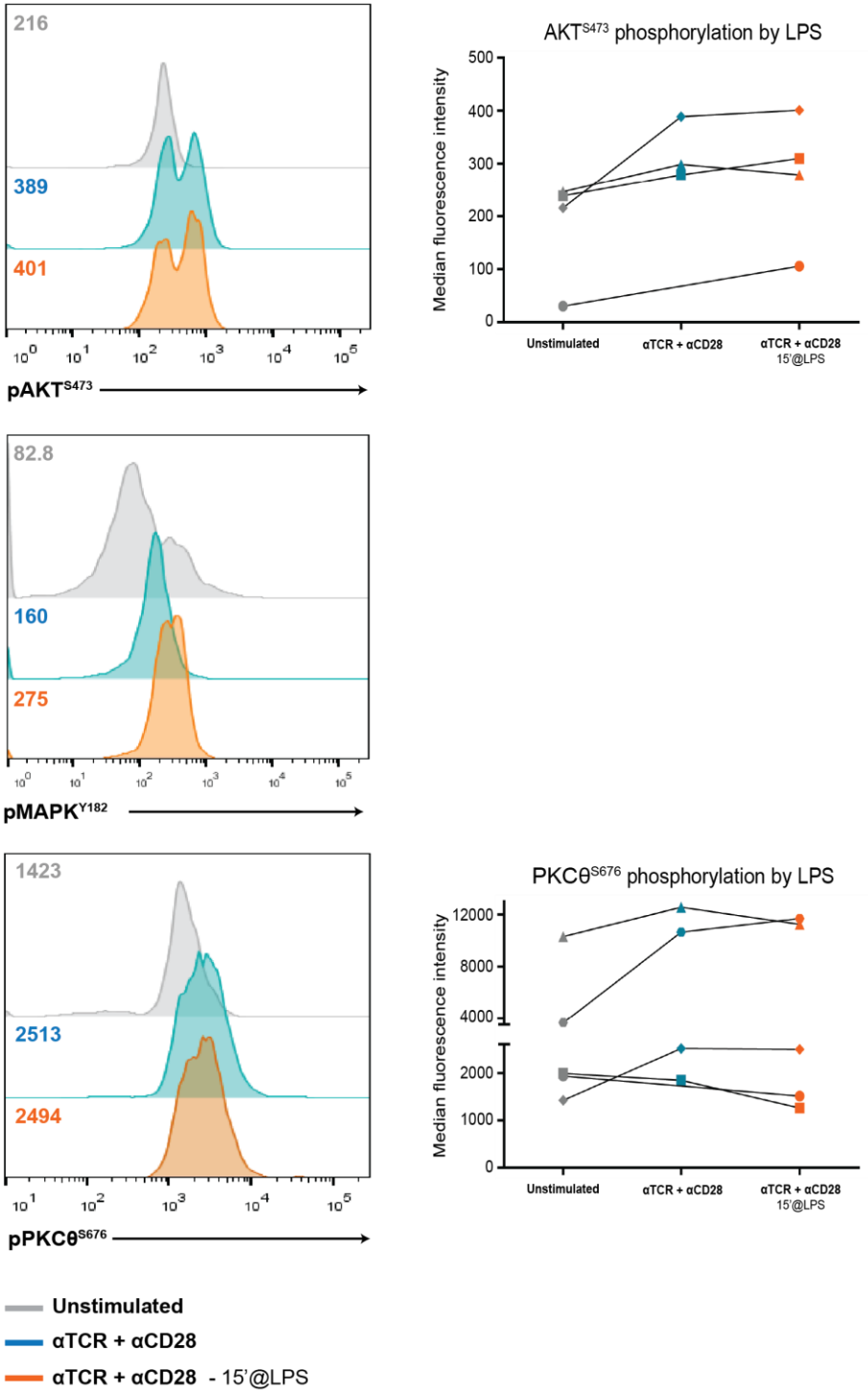


Figure 3.7 - LPS can activate directly AKT^{S473} and p38 MAPK^{Y182}, but not PKCθ^{S676}. Cells were cultured for 5 days in the indicated conditions (αTCR 10 µg/mL; αCD28 2 µg/mL). Cells were re-stimulated depending on the time of stimulation of each signaling molecule in agreement with its position in the TCR signalling cascade (pAKT^{S473}, pMAPK^{Y182}, pPKCθ^{S676} – 15 min). Re-stimulation was performed by adding LPS 1.8 µg/mL. Cells were stained for LIVE/DEAD™ Fixable Aqua Dead Cell Stain or Fixable Viability Dye eFluor 780, permeabilized using eBioscience™ Foxp3 / Transcription Factor Fixation/Permeabilization kit and stained for the respective phospho-molecules (Antibodies for: pAKT^{S473} 2,2 µg/mL, pMAPK^{Y182} 0,9 µg/mL, pPKCθ^{S676} 20 µg/mL). Data are representative of 5 independent experiments for pPKCθ^{S676}, 4 independent experiments for pAKT^{S473} and 1 experiment for MAPK^{Y182}. Median fluorescence intensities acquired by analysis in BD FACS Canto II.

3.8. TLR4 internalization decreased the activation of AKT^{S473}, PKCθ^{S676} and p38 MAPK^{Y182}

We have shown that AKT^{S473}, PKCθ^{S676} and p38 MAPK^{Y182} activation is modulated by TLR4 signaling *(Figure 3.6)*. Yet, when we directly activated CD4^{+} T cells solely via TLR4, we saw that PKCθ^{S676} required CD28 to be activated *(Figure 3.7)*. It remains to be addressed the possibility that TLR4 could be cross-talking with TCR pathway, still acting as a co-stimulatory molecule amplifying TCR signaling. Hence, we assessed the activation of AKT^{S473}, p38 MAPK^{Y182} and PKCθ^{S676} in response to a combinatory activation of both TCR and TLR4.

We have already discussed that, in macrophages, TLR4 signaling ensues two different pathways, culminating in 2 different cytokine outputs. One pathway is seeded at the plasma membrane and leads to the production of inflammatory cytokines, while the other is nucleated in an endosomal compartment and drives the production of the immunomodulatory cytokines IL-10 and type I interferons. Similarly, we found that TLR4 subcellular distribution in CD4^{+} T cells partitions between the plasma membrane and a vesicular compartment *(Figure 3.1)*.

How TLR4 signalling is conveyed in T cells is still unexplored and it is not known whether it is similarly regulated by TLR4 subcellular distribution. To address this key question, we first devised a simple kinetic experiment to determine if TLR4 engagement from the plasma membrane or from the endosomes would impinge differently on the TLR4 cross-talk with TCR signalling. After having stimulated T cells for 5 days to upregulate TLR4 expression, we then stimulated cells with soluble LPS for 30 min and, only then, we re-stimulated them with soluble antibodies for TCR and CD28. This protocol relies on previous studies showing that 30 minutes of LPS-induced stimulation is the enough time to promote TLR4 internalization (Husebye et al. 2006).

We found out that engaging the TLR4 from the endosomes decreases its ability to directly activate AKT^{S473} and p38 MAPK^{Y182} and also TLR4 cross-talk with the TCR signalling machinery measured by PKCθ^{S676} phosphorylation *(Figure 3.8)*. These results indicate that TLR4 activates the AKT^{S473} and p38 pathways from its plasma membrane localization. Similarly, TLR4 and TCR signalling cross-talk occurs at the plasma membrane.

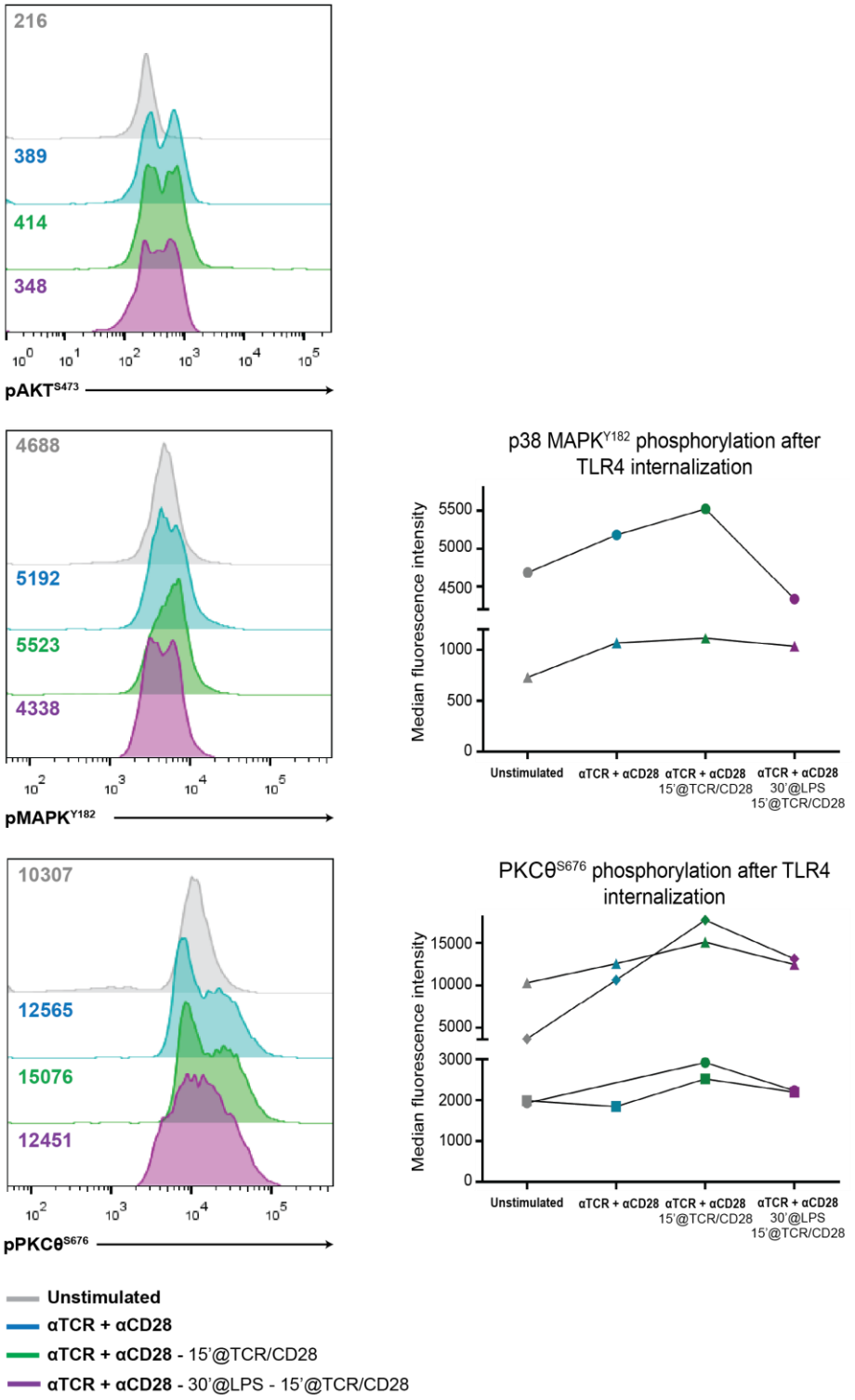


Figure 3.8 - TLR4 internalization decreased the activation of AKT^{S473}, PKCθ^{S676} and p38 MAPK^{Y182}. Cells were cultured for 5 days (αTCR 10 µg/mL; αCD28 2 µg/mL). Cells were re-stimulated depending on the time of stimulation of each signaling molecule in agreement with its position in the TCR signalling cascade (pAKT^{S473}, pMAPK^{Y182}, pPKCθ^{S676} – 15 min). Re-stimulation was performed by adding soluble antibodies (αTCR 10 µg/mL + αCD28 2 µg/mL) plus a secondary crosslinking antibody Anti-mouse IgG1 10 µg/mL. Also, for the acute TLR4 engagement, LPS was added 30 minutes before the TCR+CD28 re-stimulation. Cells were stained for LIVE/DEAD™ Fixable Aqua Dead Cell Stain or Fixable Viability Dye eFluor 780, permeabilized using eBioscience™ Foxp3 / Transcription Factor Fixation/Permeabilization kit and stained for the respective phospho-molecules (Antibodies for: pAKT^{S473} 2,2 µg/mL, pMAPK^{Y182} 0,9 µg/mL, pPKCθ^{S676} 20 µg/mL). Data are representative of 4 independent experiments for PKCθ^{S67}, 2 independent experiments for MAPK^{Y182} and 1 experiment for AKT^{S473}. Median fluorescence intensities acquired by analysis in BD FACS Canto II.

3.9. LPS is capable to impinge on TCR signaling by activating p38 MAPK^{Y323}

While in macrophages TLR4, and other pattern recognition receptors, are the main receptors driving macrophages activation and differentiation, in T cells, TLRs are not by far as relevant. In T cells, as it name suggests, the T Cell Receptor (TCR) is the main driver of T cell activation, proliferation and differentiation. That suggests that most of TLR4 impacts on T cells likely result from the crosstalk between the TCR and the TLR4 pathways. For that we thought about looking at a highly specific residue from T cells, the Tyrosine 323 residue from the p38 MAPK.

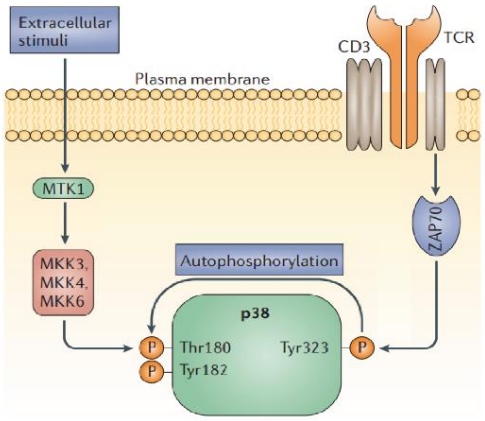


Figure 3.9 – Alternative p38 activation (p38 MAPK^{Y323}) pathway in T cells. Adapted from (Ashwell 2006). Stimulation through the TCR results in sequential activation of LCK and ZAP70. ZAP70 phosphorylates p38α/p38β on Tyr323, which induces autophosphorylation and activity towards third-party substrates.

All cell types share a canonical pathway to activate p38 MAPK on the tyrosine 182 residue. In additional to this canonical pathway, T cells also have an alternative pathway, until now thought to be under the exclusive control of the TCR. On T cells TCR engagement leads to a specific phosphorylation of p38 MAPK on the residue tyrosine 323 *(Figure 3.9)*.

This alternative p38 activation pathway directs cytokine production (Salvador et al. 2005). A recent study (Alam et al. 2015) have shown that inhibition of p38 alternative pathway reduced the production of TNF-α, IL-17A and IL-10 and protected from pancreatic cancer progression.

Despite p38 MAPK^{Y323} having been previously described to be under the exclusive control of TCR engagement, we observed that TLR4 ligation *per se* leads to the phosphorylation of tyrosine 323 *(Figure 3.10)*. This suggests that LPS can affect T cell differentiation, inducing an alteration on the outcomes (i.e cytokine production) after T cell activation.

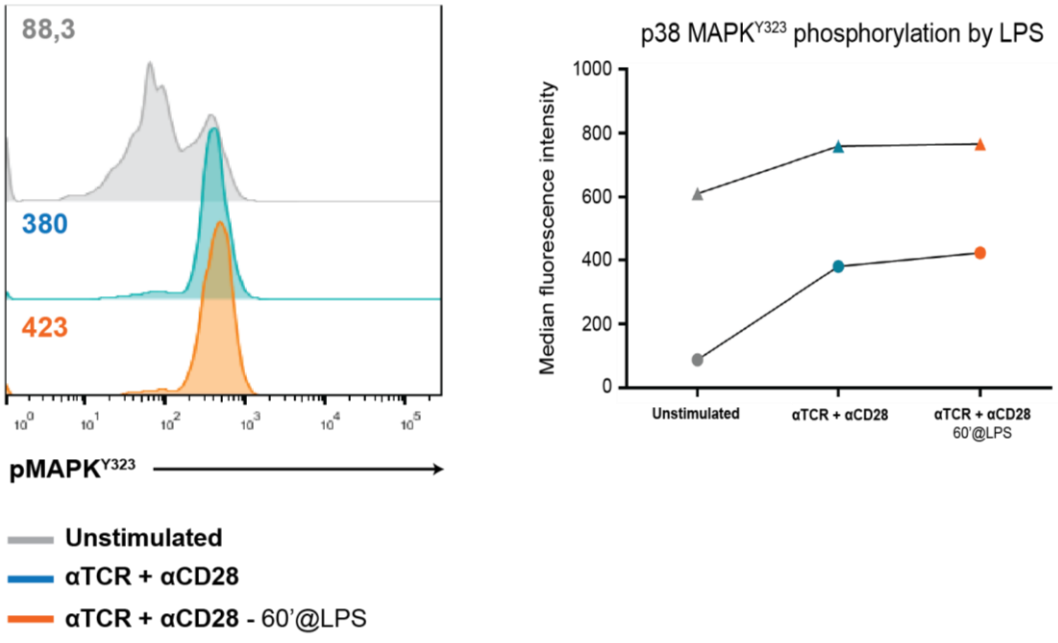


Figure 3.10 *(in previous page)* **- LPS is capable to impinge TCR signaling by activating p38 MAPK^{Y323} on CD4^{+} T cells**. Cells were cultured for 5 days (αTCR 10 µg/mL; αCD28 2 µg/mL). Cells were re-stimulated depending on the time of stimulation of each signaling molecule in agreement with its position in the TCR signalling cascade (pMAPK^{Y323} – 60 min). Re-stimulation was performed by adding LPS 1.8 µg/mL. Cells were stained for LIVE/DEAD™ Fixable Aqua Dead Cell Stain or Fixable Viability Dye eFluor 780, permeabilized using eBioscience™ Foxp3 / Transcription Factor Fixation/Permeabilization kit and stained with antibody against pMAPK^{Y323} 20 µg/mL. Data are representative of 2 independent experiments. Median fluorescence intensities acquired by analysis in BD FACS Canto II.

3.10. TLR4 internalization improved the activation of p38 MAPK^{Y323}

When we promoted TLR4 internalization, by incubating with LPS for 30 minutes, prior to TCR and CD28 crosslinking, we observed that contrary to AKT^{S473}, MAPK^{Y182} and PKCθ^{S676}, TLR4 internalization increases p38 MAPK phosphorylation on residue tyrosine 323 *(Figure 3.11)*. Meaning that activation of p38 MAPK^{Y323} likely requires TLR4 signaling from the endocytic compartment.

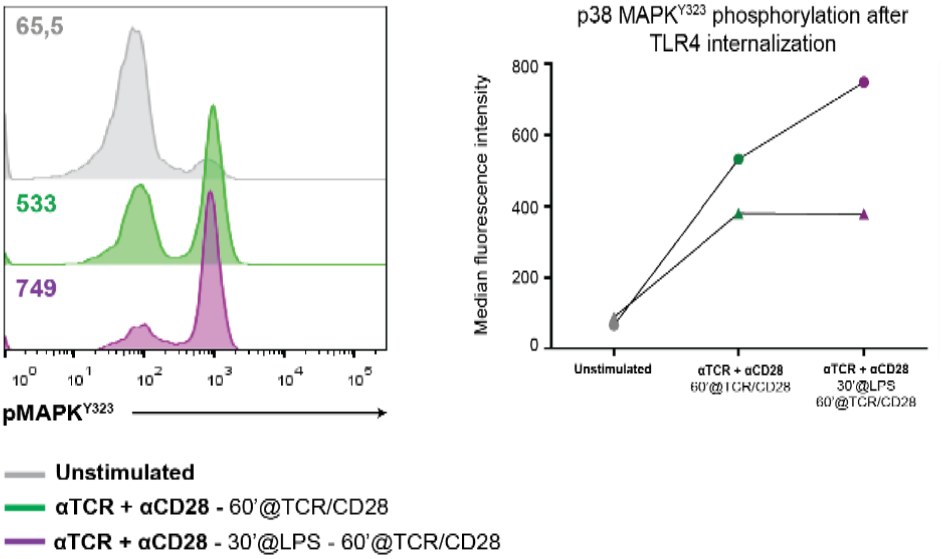


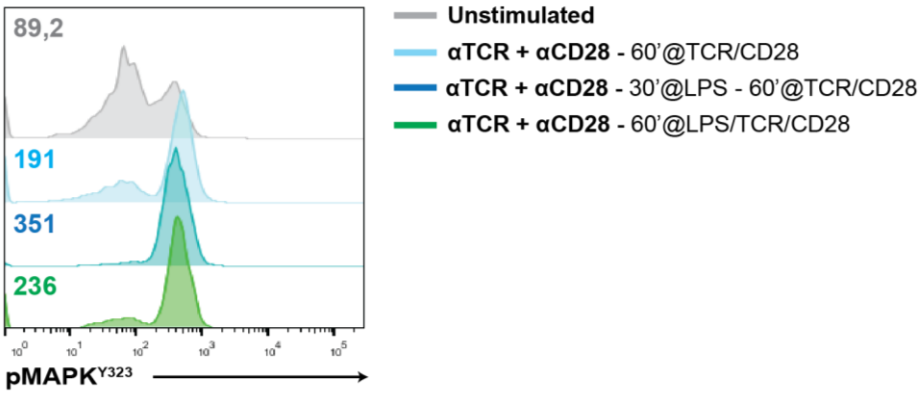
Figure 3.11 - TLR4 internalization improved the activation of p38 MAPK^{Y323}. Cells were cultured for 5 days (αTCR 10 µg/mL; αCD28 2 µg/mL). Cells were re-stimulated depending on the time of stimulation of each signaling molecule in agreement with its position in the TCR signalling cascade (pMAPK^{Y323} – 60 min). Re-stimulation was performed by adding soluble antibodies (αTCR 10 µg/mL + αCD28 2 µg/mL) plus a secondary crosslinking antibody Anti-mouse IgG1 10 µg/mL. Also LPS 1.8 µg/mL was added 30 minutes before the TCR+CD28 re-stimulation. Cells were stained for LIVE/DEAD™ Fixable Aqua Dead Cell Stain or Fixable Viability Dye eFluor 780, permeabilized using eBioscience™ Foxp3 / Transcription Factor Fixation/Permeabilization kit and stained with antibody against pMAPK^{Y323} 20 µg/mL. Data are representative of 2 independent experiments. Median fluorescence intensities acquired by analysis in BD FACS Canto II.

3.11. TLR4 internalization is highly required for a full activation of p38 MAPK^{Y323}

To confirm the fact that p38 MAPK^{Y323} requires TLR4 internalization, while other molecules rely on TLR4 signaling from the plasma membrane, we performed an experiment where we acutely activated TLR4 with soluble LPS: in the 1st condition we added LPS 30 minutes before the TCR+CD28 re-stimulation; in the 2nd condition we added LPS at the same time as we added the soluble antibodies against TCR and CD28. The 1st condition involves an initial TLR4 internalization, while the 2nd one imply

an acute and simultaneous activation of plasma-membrane TLR4, TCR and CD28. In the control condition, we only re-stimulate cells with TCR and CD28.

We normalized the median values of the 1st and 2nd condition by subtracting from each one the control median value. We concluded that TLR4 internalization is highly required for a full MAPK^{Y323} activation *(Figure 3.12)*. Indeed, the 30 minutes of LPS-induced stimulation required for TLR4 internalization improves p38 MAPK^{Y323} activation in comparison with the simultaneous stimulation of membrane TLR4, TCR and CD28.



	Δ Internalized TLR4 = Δ(30'@LPS + @TCR/CD28) - (@TCR/CD28)	Δ Surface TLR4 = Δ(15'or 60'@LPS/TCR/CD28) - (@TCR/CD28)	Δ Internalized TLR4 / Δ Surface TLR4
pMAPK^{Y323}	351-191=160	236 -191=45	160/45 = **3,5**

Figure 3.12 - TLR4 internalization is highly required for a full activation of p38 MAPK^{Y323}. Cells were cultured for 5 days (αTCR 10 µg/mL; αCD28 2 µg/mL). Cells were re-stimulated depending on the time of stimulation of each signaling molecule in agreement with its position in the TCR signalling cascade (pMAPK^{Y323} – 60 min). Re-stimulation was performed by adding soluble antibodies (αTCR 10 µg/mL + αCD28 2 µg/mL) plus a secondary crosslinking antibody Anti-mouse IgG1 10 µg/mL. In one condition, LPS 1.8 µg/mL was added at the same time as the antibodies for TCR and CD28, while in the other condition LPS was added 30 minutes before the TCR+CD28 re-stimulation. Cells were stained for LIVE/DEAD™ Fixable Aqua Dead Cell Stain or Fixable Viability Dye eFluor 780, permeabilized using eBioscience™ Foxp3 / Transcription Factor Fixation/Permeabilization kit and stained for pMAPK^{Y323} (20 µg/mL). Data are representative of one experiment. Median values acquired by analysis in BD FACS Canto II.

3.12. Inhibition of TLR4 internalization with Chlorpromazine improves the activation of AKT^{S473}, PKCθ^{S676} and MAPK^{Y182}, while inhibiting the phosphorylation of p38 MAPK^{Y323}

To determine how the inhibition of TLR4 internalization affects the activation of those four phosphomolecules, we resorted to Chlorpromazine (CPZ) an inhibitor of clathrin dependent endocytosis (Wang, Rothberg, and Anderson 1993). We choose this inhibitor since TLR4, along with LPS, was showed to be endocytosed by a receptor-mediated mechanism dependent on dynamin and clathrin (Husebye et al. 2006). The same study demonstrated that inhibition of endocytosis improves LPS signaling from the membrane, resulting in amplified NF-kB activation.

Even though TCR is mainly internalized also by a clathrin-dependent mechanism, there are also clathrin-independent mechanisms for TCR endocytosis depending on other proteins like dynamin and other small GTPases (Martínez-Martín et al. 2011), (Willinger et al. 2015). Thus, we can support the idea that CPZ isn't affecting all the TCR signaling. Even though the TCR signaling might by slightly

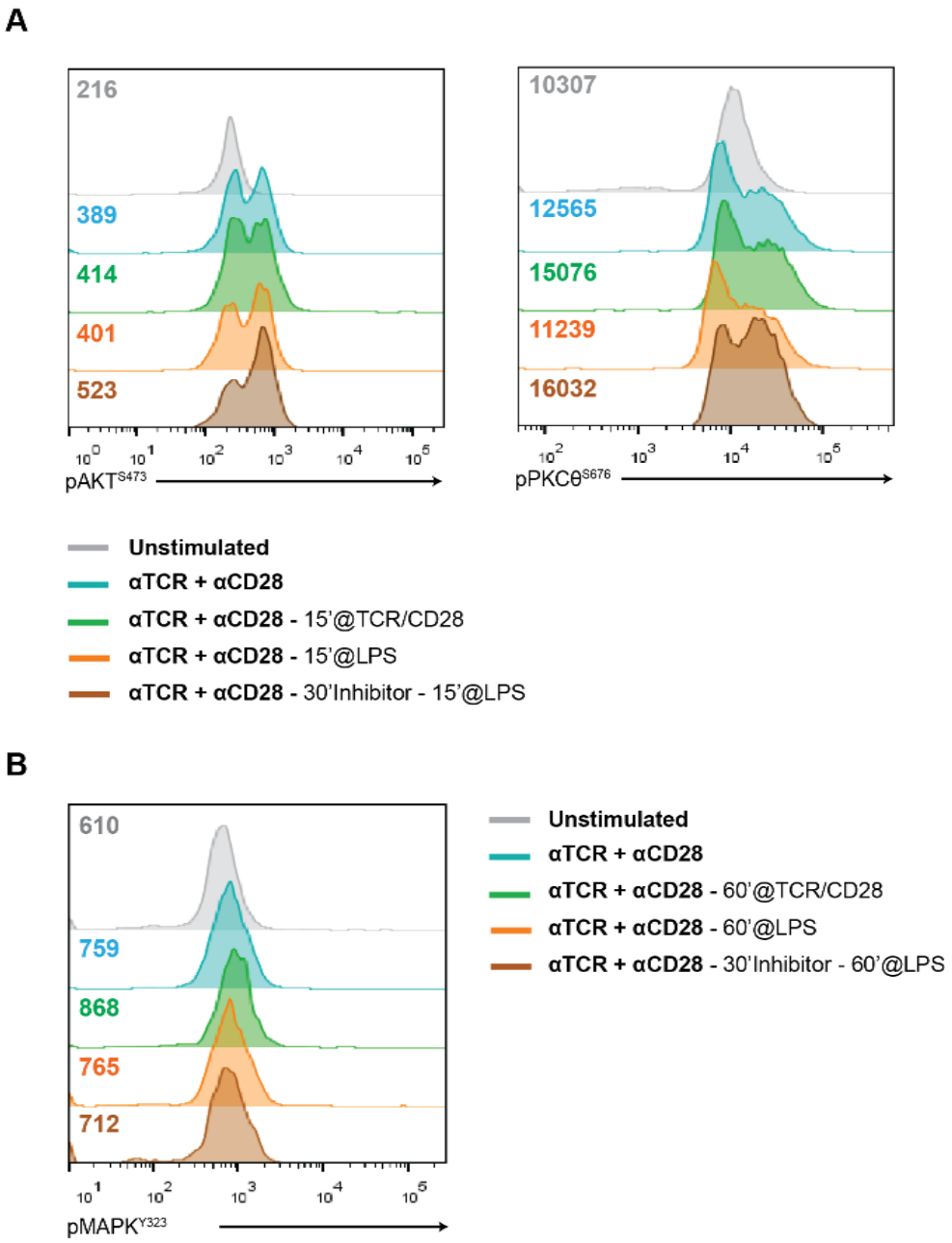


Figure 3.13 - Inhibition of TLR4 internalization with Chlorpromazine improves the activation of AKT^{S473} and PKCθ^{S676}, while inhibits the p38 MAPK^{Y323}. Cells were cultured for 5 days (αTCR 10 µg/mL; αCD28 2 µg/mL). Cells were re-stimulated depending on the time of stimulation of each signaling molecule in agreement with its position in the TCR signalling cascade (pAKT^{S473}, pPKCθ^{S676} – 15 min; pMAPK^{Y323} – 60 min). Re-stimulation was performed applying at least one of the following mentioned, as described in the figure: **(i)** soluble antibodies (αTCR 10 µg/mL + αCD28 2 µg/mL) plus a secondary crosslinking antibody Anti-mouse IgG1 10 µg/mL; **(ii)** Inhibitor CPZ 30 µM; **(iii)** LPS 1.8 µg/mL. Cells were stained for LIVE/DEAD™ Fixable Aqua Dead Cell Stain or Fixable Viability Dye eFluor 780, permeabilized using eBioscience™ Foxp3 / Transcription Factor Fixation/Permeabilization kit and stained for the respective phospho-molecules (Antibodies for: pAKT^{S473} 2,2 µg/mL, pPKCθ^{S676} 20 µg/mL, pMAPK^{Y323} 20 µg/mL). Data are representative of 1 experiment for each molecule. Median fluorescence intensities acquired by analysis in BD FACS Canto II.

affected by CPZ it is important to notify that we are stimulating cells with fixed antibodies, preventing TCR molecules to be as affected as TLR4.

When we inhibited endocytosis for 30 minutes prior to TLR4 engagement, AKT^{S473} and PKCθ^{S676} activation improves *(Figure 3.13 A)*. These results reinforce the idea that the activation of those two signaling molecules rely on TLR4 signaling from the plasma membrane of CD4^{+} T cells. Curiously, after inhibition of TLR4 internalization, LPS *per se* starts to be capable of activating PKCθ^{S676} independently from TCR and CD28 signaling. Earlier we showed that PKCθ^{S676} can't be activated solely via TLR4 *(Figure 3.7)* since it requires CD28 mediated recruitment to the immunological synapse after TCR stimulation. In this case we can see that, after CPZ incubation and LPS engagement, PKCθ^{S676} in CD4^{+} T cells is even more activated than after TCR+CD28 re-stimulation. One possible explanation might be related with the fact that CPZ, as an antipsychotic agent, binds direct and selectively to membrane components, important intermediates in a number of receptor-mediated cell signaling pathways (J. Y. Chen et al. 2003), (Staub et al. 2006). PKCθ localization and catalytic function are dictated by intramolecular interactions, the availability of specific membrane lipids, reversible phosphorylation events, and dynamic interactions with other protein factors (Arendt et al. 2002). The influence on the membrane phospholipids by CPZ might affect PKCθ localization, allowing its activation, even in the absence of TCR engagement.

When I looked at MAPK^{Y323}, CPZ intervention resulted in a decreased activation of the signaling molecule *(Figure 3.13 B)*, indicating a reliance on TLR4 signaling from endosomal compartments.

I also assessed the effect of CPZ prior to a combinatory activation of both TLR4 and TCR, and we observed that both PKCθ^{S676} and MAPK^{Y182} showed improved activation when TLR4 internalization is blocked *(Figure 3.14)*.

We intend to titrate this CPZ drug at most indicated concentrations to use with primary human cells, since we are applying suggested concentrations used in cell lines. That way we might be able to use this inhibitor also in chronic conditions to see the effect regarding cytokine production.

To close this chapter we propose a model *(Figure 3.15)* with a schematic representation of the different signaling molecules and their likely activation pathways resulting from co-stimulation or cross-talk between the TCR and TLR4 signaling. Some molecules require TLR4 signaling from the plasma membrane while others rely on the endocytic compartments of TLR4. AKT^{S473} and MAPK^{Y182} are activated after TLR4 engagement at the plasma membrane of CD4+ T cells, while PKCθ^{S676} requires TCR+CD28 activation to be recruited to the plasma membrane where TLR4 and TCR pathways cross-

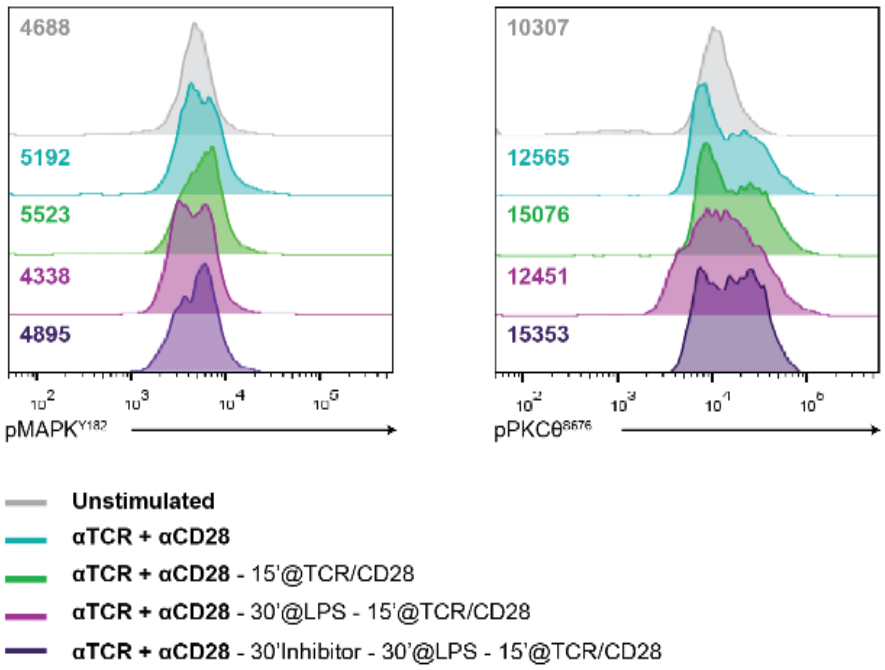


Figure 3.14 - Inhibition of TLR4 internalization with Chlorpromazine improves the activation of PKCθ^{S676} and MAPK^{Y182}. Cells were cultured for 5 days (αTCR 10 µg/mL; αCD28 2 µg/mL). Cells were re-stimulated depending on the time of stimulation of each signaling molecule in agreement with its position in the TCR signalling cascade (pMAPK^{Y182}, pPKCθ^{S676} – 15 min). Re-stimulation was performed applying at least one of the following mentioned, as described in the figure: **(i)** soluble antibodies (αTCR 10 µg/mL + αCD28 2 µg/mL) plus a secondary crosslinking antibody Anti-mouse IgG1 10 µg/mL; **(ii)** Inhibitor CPZ 30 µM; **(iii)** LPS 1.8 µg/mL. Cells were stained for LIVE/DEAD™ Fixable Aqua Dead Cell Stain or Fixable Viability Dye eFluor 780, permeabilized using eBioscience™ Foxp3 / Transcription Factor Fixation/Permeabilization kit and stained for the respective phospho-molecules (Antibodies for: pMAPK^{Y182} 0,9 µg/mL and pPKCθ^{S676} 20 µg/mL). Data are representative of 1 experiment for each molecule. Median fluorescence intensities acquired by analysis in BD FACS Canto II.

talk. We proved this cross-talk occurs mostly at the plasma membrane as once we block TLR4 internalization with CPZ we improved PKCθ^{S676} activation *(Figure 3.13 and 3.14)*.

To reinforce the fact that TLR4 impact on T cells is most likely a result from a crosstalk between TLR4 and TCR pathways, we assessed a highly specific molecule from the TCR activation cascade, the p38 MAPK^{Y323}. We proved that it can be activated by TLR4 nucleated at the endocytic compartment. We can't still conclude if this activation happens directly from the TLR4 or not. However, since this residue is known to be activated only through ZAP70, we suggest that TLR4 signaling from the endocytic compartment might intersect with the TCR pathway at ZAP70. ZAP70 would work as a mediating molecule for p38 MAPK^{Y323} activation. Also, a recent study (Schweighoffer et al. 2017) reinforce this idea demonstrating a crucial role for SYK in the TLR4 impacts on B cell survival, proliferation and functions. Likewise SYK is required for TLR4 signaling in B cells, ZAP70 – a SYK-related protein in T cells – can also be required for TLR4 signaling from the compartment.

In the very first results, I saw that chronic engagement of TLR4 improved its intracellular expression *(Figure 3.1)*. This means that chronic encounter with LPS acts by promoting TLR4 signaling from the endocytic compartments and consequently raising MAPK^{Y323} activation in CD4^{+} T cells.

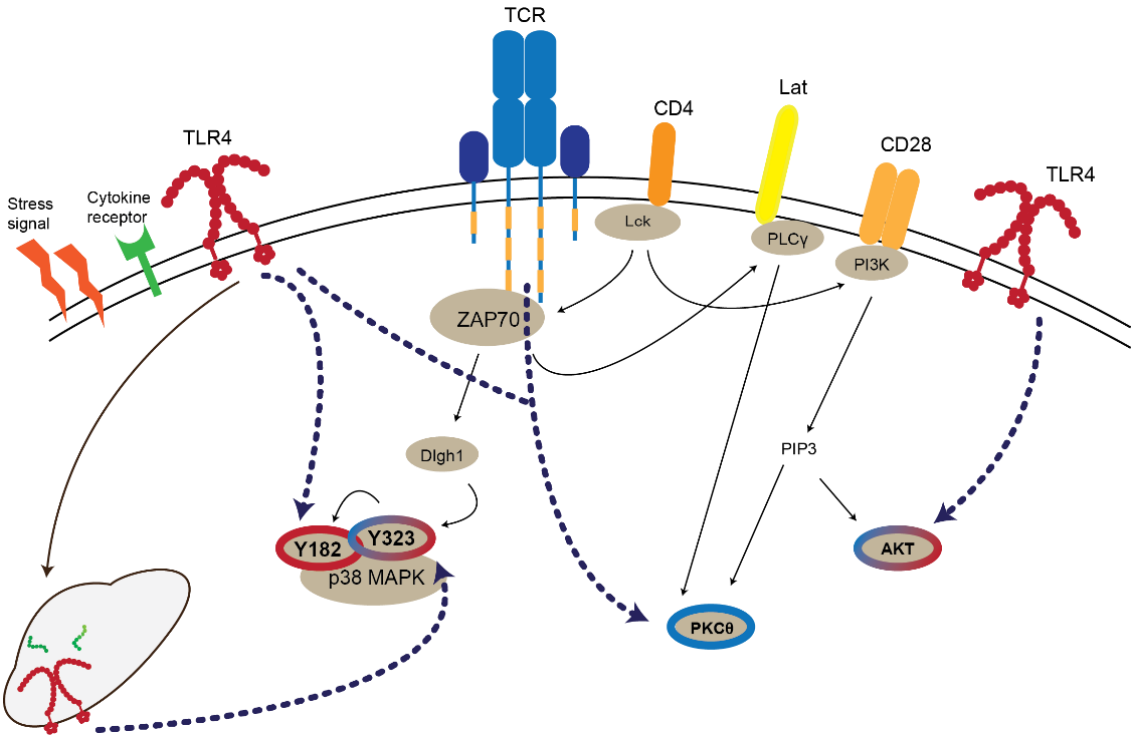


Figure 3.15 – TLR4 activates AKT^{S473} and p38 MAPK^{Y182} from the plasma membrane. Similarly TLR4 and TCR signaling cross-talk occurs at the plasma membrane. Surprisingly, TLR4 engagement is capable to impinge on TCR signaling by activating p38 MAPK^{Y323}, a residue until now thought to be under exclusive control of TCR. The activation of p38 MAPK^{Y323} likely requires TLR4 signaling from the endocytic compartment.

3.13. TLR4 is recruited to the synaptic membrane after TCR stimulation in CD4$^+$ T cells

To confirm the crosstalk between TCR and TLR4 pathways we carried out an experiment where we stimulated CD4$^+$ T cells for 5 and 20 minutes with soluble LPS and coverslip-coated antibodies against TCR and CD28. Then, we assessed TCR and TLR4 expression by confocal microscopy.

As CD4$^+$ T cells were stimulated in the coverslip, they were forming a surrogate immunological synapse, and we could see the formation of TCR projections along the coverslip *(Figure 3.16)*. This specialized interface is formed after TCR triggering as a natural mechanism that favors T cells to closely interact with APCs allowing a sustained antigen recognition and the formation of a signaling supramolecular scaffold, nucleating various signaling complexes involved in remodelling the T cell cytoskeleton, T cell development and activation (Silva et al. 2016).

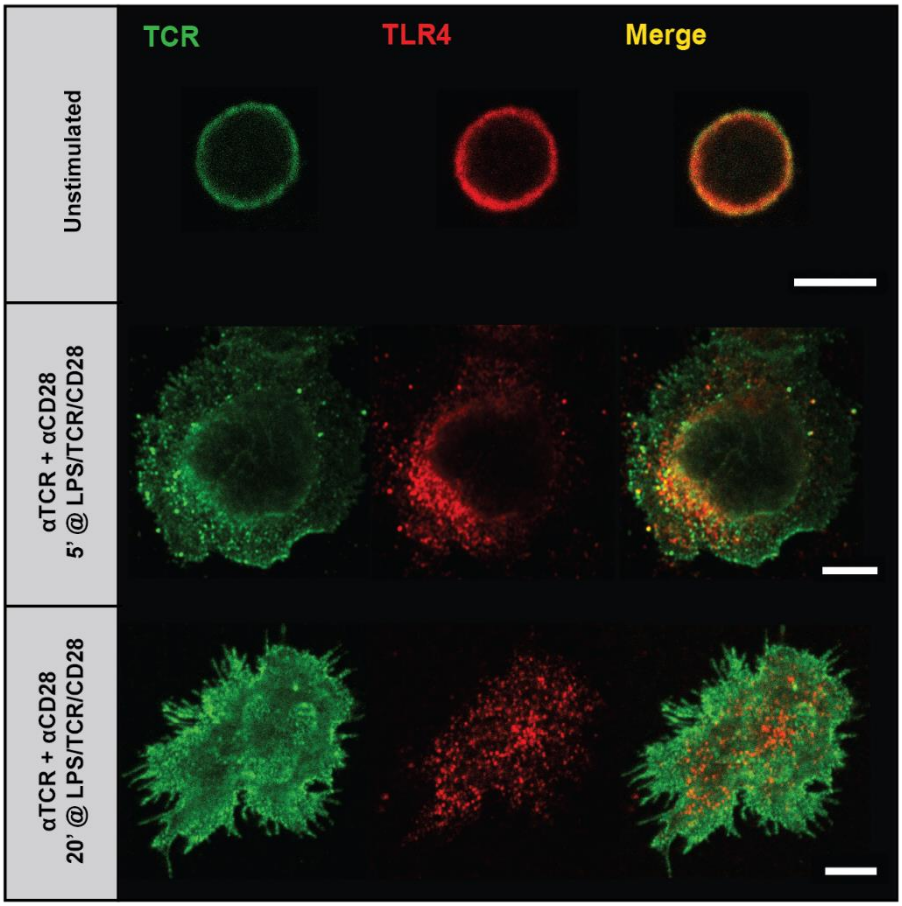


Figure 3.16 – TLR4 is recruited to the synaptic membrane after TCR stimulation. At 20 minutes of stimulation, TLR4 converge to the cSMAC while being endocytosed. Highly purified $CD4^+$ T cells were cultured for 5 days either unstimulated or stimulated with αTCR 10 µg/mL and αCD28 2 µg/mL. Stimulated cells were re-stimulated at 37ºC for 5 or 20 minutes with soluble LPS and coated antibodies for TCR and CD28. Sorted $CD4^+$ T cells were surface labelled with primary antibodies for CD3 (*UCHT1*; mouse IgG1, 5 µg/mL) and TLR4 (10 µg/mL), and with fluorescently conjugated secondary antibodies (FITC Anti-mouse IgG1 5 µg/mL; A568 Anti-mouse IgG2b 10 µg/mL). Finally, cells were stained with a 3ary antibody A488 anti-FITC (10 µg/mL). Data are representative of one experiment. TCR and TLR4 expression were determined by confocal microscopy using LSM710 microscope system with ZEN 2010 software (Carl Zeiss) and 63x oil immersion objective. Overlapping regions between TCR and TLR4 appear as yellow in the overlay panels. Scale bar = 5 µm.

TLR4 molecules are also expressed along those membrane projections even though they are predominantly organized in clusters. After 5 minutes of stimulation, clusters are polarized, and even though we should not conclude TCR and TLR4 are interacting, we can at least assume, by their proximity, that their pathways probably interplay with each other. After 20 minutes of stimulation, we can clearly observe that TLR4 clusters are converging to a specific location called the central supramolecular activation cluster (cSMAC), while they are being internalized into the cell. We are not capable to see the same reaction for TCR clusters since they are mostly attached to coverslip-coated antibodies against TCR used for stimulation. In the case of TLR4 since we used soluble LPS, we can see its movement along the cell.

3.14. ZAP70 as a candidate to crosstalk between TLR4 and TCR pathways

TCR activation induces the recruitment and activation of the kinases Lck and ZAP70, which initiate a network of molecular pathways required for the remodelling of T cell cytoskeleton, T cell development and activation (Silva et al. 2016). A recent study developed a project in B cells were they studied how TLR4 signaling in B cells contributes to antibody-mediated immunity and autoimmunity (Schweighoffer et al. 2017). As ZAP70 is rapidly recruited and activated after TCR engagement, the same happens for SYK in B cells. In fact, ZAP70 is a SYK-related molecule whose expression is mostly confined to the T and NK cell lineages (Mócsai, Ruland, and Tybulewicz 2010). The SYK tyrosine kinase is essential for signaling from the B cell antigen receptor (BCR), and thus for antibody responses. Schweighoffer and collaborators demonstrated that SYK is required for B cell survival, proliferation, and cytokine secretion in response to signaling through TLR4. They conclude that SYK transduces TLR4 signals to activate ERK and AKT, but not NF-kB whose activation is dependent on MyD88 signaling.

Likewise SYK is required for TLR4 signaling in B cells through BCR activation, we wondered whether ZAP70 can also impact on the cross-talk between TLR4 and TCR. Therefore, we restimulated $CD4^+$ T cells with soluble LPS for 30 minutes followed by a TCR/CD28 restimulation for 5 or 10 minutes at 37 degrees. Although $pZAP70^{Y319}$ seems to be slightly expressed nearby the intracellular structures of TLR4, it is predominantly expressed closer to the plasma membrane *(Figure 3.17)* where it might act as an intermediate of TLR4-induced T cell activation.

As a work in progress, we propose that $pZAP70^{Y319}$ activation should be also assessed by flow cytometry in order to confirm if this residue can be directly activated by TLR4 engagement, and if its activation depends on TLR4 internalization.

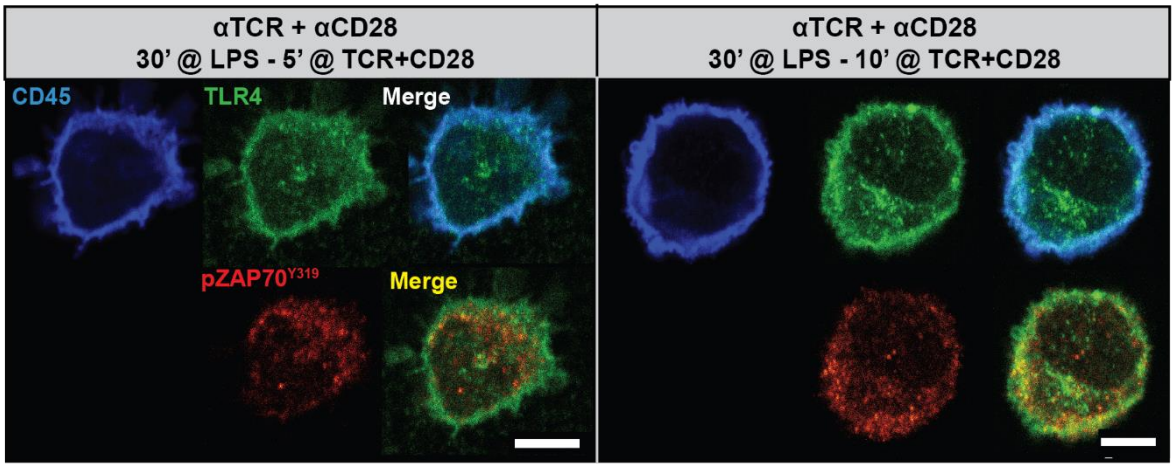


Figure 3.17 - $pZAP70^{Y319}$ is predominantly expressed closer to the plasma membrane, being a candidate signaling molecule to cross-talk between TLR4 and TCR pathway. Highly purified $CD4^+$ T cells were cultured for 5 days either unstimulated or stimulated with αTCR 10 µg/mL and αCD28 2 µg/mL. Stimulated cells were re-stimulated at 37ºC for 30 minutes with soluble LPS prior to 5 or 10 minutes of stimulation with coated antibodies for TCR and CD28. Sorted $CD4^+$ T cells were surface labelled with primary antibody for CD45 (10 µg/mL) and secondary A568 Anti-mouse IgG2a 10 µg/mL, and labelled intracellularly with primary antibodies against TLR4 (10 µg/mL) and $ZAP70^{Y319}$ (1 µg/mL), and with fluorescently conjugated secondary antibodies (A488 Anti-mouse IgG2b 10 µg/mL; A647 Anti-rabbit 10 µg/mL). Data are representative of one experiment. TLR4, CD45 and $pZAP70^{Y319}$ expression were determined by confocal microscopy using LSM710 microscope system with ZEN 2010 software (Carl Zeiss) and 63x oil immersion objective. Overlapping regions between $pZAP70^{Y319}$ and TLR4 appear as yellow in the overlay panels. Scale bar = 5 µm.

3.15. Rab11a-positive intracellular compartments colocalize with internalized TLR4

Previous studies focused on the TLR4 trafficking in APCs, investigating which small GTPases were affecting TLR4 translocation from the plasma membrane to the intracellular compartments.

Few years ago it was suggested that the small GTPases Rab11a promotes TLR4 internalization into recycling endosomes and also found that it regulates the recruitment of TLR4 from the recycling endosomes to the bacteria-containing phagosome, where TRAM and IRF3 are localized, promoting a TRIF-dependent TLR4 signaling and the expression of type I IFN (Husebye et al. 2010).

Also, other demonstrated that Rab8a interacts directly with PI3Ky to modulate TLR4-driven AKT/mTOR pathway in macrophages (Luo et al. 2014). Recently, the same group deepen their study for TLR4 and extended it for other TLRs (Wall et al. 2017). They found that PI3Ky is recruited by Rab8a to dorsal ruffle membranes and early micropinosomes, where it phosphorylates PtdIns(4,5)P_2 to PtdIns(3,4,5)P_3 for attraction of signaling kinases such as AKT and the downstream molecule mTOR. Also, this depletion of PtdIns(4,5)P_2 leads to a dissociation of the TIRAP/MAL sorting adaptors from the

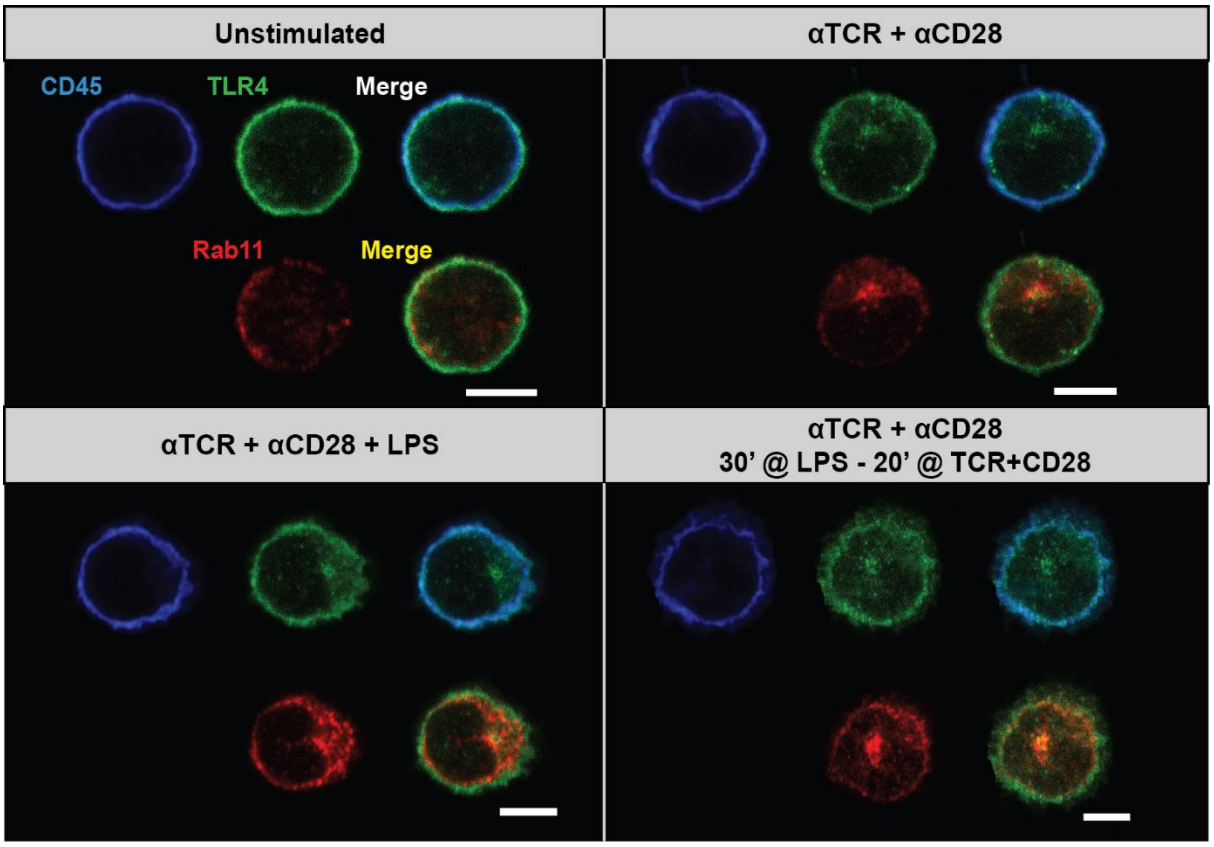


Figure 3.18 - Rab11a-positive intracellular compartments colocalize with internalized TLR4. Highly purified CD4+ T cells were cultured for 5 days either unstimulated or stimulated with αTCR 10 µg/mL and αCD28 2 µg/mL or in combination with LPS 1.8 µg/mL. For the last condition, stimulated cells were re-stimulated at 37ºC for 30 minutes with LPS and 20 minutes with coated antibodies for TCR and CD28. Sorted CD4+ T cells were surface labelled with primary antibody for CD45 (10 µg/mL) and secondary A568 Anti-mouse IgG2a 10 µg/mL, and labelled intracellularly with primary antibodies against TLR4 (10 µg/mL) and Rab11a (3 µg/mL), and with fluorescently conjugated secondary antibodies (A488 Anti-mouse IgG2b 10 µg/mL; A647 Anti-rabbit 10 µg/mL). Data are representative of one experiment. TLR4, CD45 and Rab11 expression were determined by confocal microscopy using LSM710 microscope system with ZEN 2010 software (Carl Zeiss) and 63x oil immersion objective. Overlapping regions between Rab11a and TLR4 appear as yellow in the overlay panels. Scale bar = 5 µm.

surface TLR4. As PtdIns(3,4,5)P_3 arise, TLR4 is internalized and associates with TRIF/TRAM adaptors. This transition positively regulates anti-inflammatory cytokines such as IL-10, curtailing inflammation.

We analyse the intracellular expression of TLR4 and Rab11a. We observed that Rab11a colocalizes partially with intracellular compartments of TLR4 *(Figure 3.18)*. Moreover, the most important result is the one where we re-stimulated cells for 30 minutes with LPS inducing TLR4 internalization, and 20 minutes with the antibodies αTCR and αCD28. In this last case, as expected the intracellular compartment of TLR4 become more defined and, interestingly the colocalization between TLR4 and Rab11a is increased significantly. Those results suggest that Rab11a might be one of the small GTPases involved in TLR4 internalization.

3.16. LPS causes CD4$^+$ T cell to decrease IFNγ but to upregulate IL17A production

With this work we intend to unravel the effect of LPS, as the main component of bacteria, on human CD4$^+$ T cell activation and differentiation.

Cells incubated with LPS have improved TLR4 intracellular expression, resulting in improved TLR signaling from the endocytic compartment and raised p38 MAPKY323 activation. This tyrosine residue 323 takes part of the T cell alternative activation pathway for p38 MAPK and is highly involved in T cell differentiation. Its phosphorylation is known to redirect CD4 T cells into a Th17 phenotype. For that reason we were interested in assessing how this specific activation pathway would affect T cell functions regarding cytokine production.

After showing the expression of TLR4 in T cells, we proceeded to demonstrate that it can modulate T cell survival and activation. Regarding CD4$^+$ T cells, some reports have focused on how TLRs can influence on the modulation and plasticity of some Th subsets. However, this role of TLR4 has yielded conflicting reports. Some found that TLR4 engagement promotes perforin production and exacerbated symptoms on Ankylosing Spondylitis patients (Raffeiner et al. 2005). Also, others proved in a mouse model of EAE that TLR4 is required to ease Th17 and, to a lesser degree, Th1 responses (Reynolds et al. 2012). However, contrasting studies were performed in a transfer colitis model where they found TLR4 have an inhibitory role on subsequent TCR-dependent CD4$^+$ T cell responses by abrogating IFNγ production while improving IL17A, which lead to disease resolution (González-Navajas et al. 2010). It is presumed that those two contradictory reports are related with the differential TLR ligand availability in the gut versus the sterile environment of the central nervous system. In the gut, CD4$^+$ T cells are constantly encountered with TLR stimuli from the microbiota, while in the central nervous system the slightest signal is probably perceived as a host-derived danger molecule.

In this work, as we cultured highly purified CD4$^+$ T cells for 5 days in a constant contact with LPS, we believed it can mimic a chronic encounter with the TLR ligand as what happens in the gut. In fact, when we assessed cytokine production after 5 days of LPS stimulation we detect an alteration on the cytokine profile. LPS was found to promote the production of IL17A while impairing IFNγ *(Figure 3.19)*.

Previously, we saw that chronic engagement of LPS improves intracellular expression of TLR4 *(Figure 3.1 C and D)*. Also, we found that p38 MAPK^{Y323} highly relies on TLR4 signaling from the

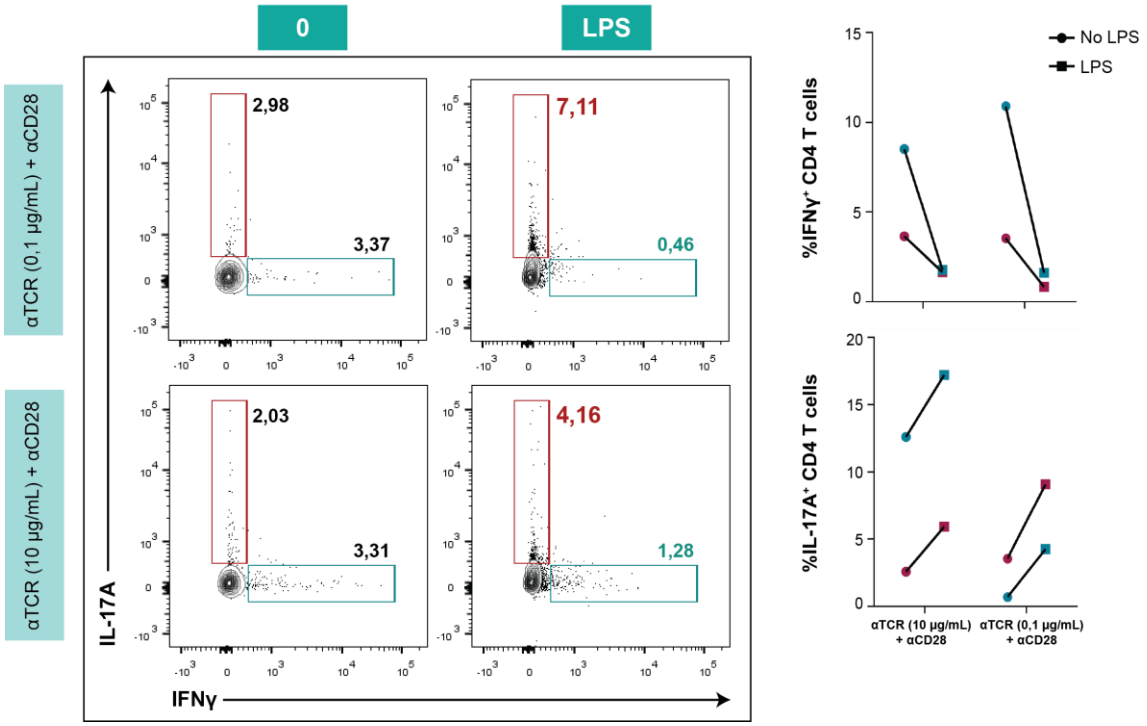


Figure 3.19 - Direct recognition of LPS promotes IL-17 production while inhibits IFNγ. Sorted CD4⁺ T cells were cultured for 5 days stimulated with αCD3 10 µg/mL and αCD28 2 µg/mL, or in combination with LPS 1,8 µg/mL. At day 4, cells were treated with phorbol 12-myristate 13-acetate (PMA), Ionomycin and protein transport inhibitor Brefeldin A (BFA) for ~15 hours. Cells were stained for LIVE/DEAD™ Fixable Aqua Dead Cell Stain or Fixable Viability Dye eFluor 780, permeabilized using eBioscience™ Foxp3 / Transcription Factor Fixation/Permeabilization kit and stained for IL17A (3.33 µg/mL) and IFNγ (10 µg/mL) and analysed by flow cytometry. Population analysis was performed by flow cytometry in BD FACS Canto II and representative plots illustrate the percentages of IL17A- and IFNγ-producing CD4⁺ T cells. Data are representative of 2 independent experiments.

endocytic compartment *(Figure 3.11, 3.12, 3.13)*. Altogether, those results suggest that MAPK^{Y323} activation likely raises after chronic encounter with LPS. MAPK^{Y323} is an alternative p38 activation pathway in T cells that directs cytokine production (Salvador et al. 2005). Recent studies found that activation of p38 MAPK signaling in CD4 T cells plays an important role in Th17 cell function by regulating IL-17 production at the transcriptional level (Noubade et al. 2014). Even more, it has been showed that a specific inhibition of p38 MAPK^{Y323} reduced the production of IL17A (Alam et al. 2015). Hence, we can hypothesize that IL17A enhanced production results from the improved activation of p38 MAPK^{Y323} signaling pathway.

Even though naïve T cell activation, proliferation and differentiation are mostly considered as simultaneous processes, differentiated T cells can be reactivated and reshaped to a different function during the course of some infection/trauma. Th17 cells are considered to be the main population of pathogenic T cells driving autoimmunity (Hirota et al. 2007), (Langrish et al. 2005). However, a defining feature of Th17 cells is their plasticity, i.e. their ability to express cytokines typical of other lineages in response to distinct microenvironments (Hirota et al. 2011), (Annunziato et al. 2007). Hence, Th17 function depends on the balance between pro- and anti-inflammatory cytokines. Since co-production of IFNγ turns Th17 into pathogenic autoreactive cells, our results showing a decreased production of IFNγ, allow us to speculate that a chronic engagement of CD4⁺ T cells with LPS redirects them to a more tolerogenic Th17 phenotype.

3.17. Direct recognition of LPS promotes IL-10 production, suggesting a redirection of Th17 cells to a tolerogenic profile

We know that in innate immune cells, there are two distinct pathways for TLR4 activation, the one from the plasma membrane and the other from the intracellular compartments (O'Neill, Golenbock, and Bowie 2013), (Husebye et al. 2006), (Gómez et al. 2014). The first one is initiated with the activation of the adaptor protein MyD88, while the second depends on TRIF (Barton and Kagan 2009). Each one promotes a divergent route for cytokine production: TLR4 activation from the plasma membrane induces a pro-inflammatory phenotype (Husebye et al. 2006); the intracellular signaling pathway improves the IFN expression and a consequent regulatory cytokine output (IFNα, IFNβ, IL10) (Kagan et al. 2008), (Wall et al. 2017), (Teixeira-Coelho et al. 2014).

Until this point, we not only proved that TLR4 is in fact expressed on $CD4^+$ T cells as it is expressed intracellularly in confined vesicular compartments *(Figure 3.1 A and B)*. We already related IL17A improved production with the improved TLR4 signaling from the vesicular compartments, namely induced $MAPK^{Y323}$ activation. However, we questioned if $CD4^+$ T cells might behave as innate immune cells, relying on a TRIF-dependent signal from intracellular compartments that could trigger an anti-inflammatory response with the production of IFNα, IFNβ and/or IL-10. Indeed, we found that chronic engagement with LPS improves IL10 production, and most IL10-producing CD4 T cells are also positive for IL-17A production *(Figure 3.20)*.

In fact, after chronic engagement with LPS, $CD4^+$ T cells once again, survive more and produce more IL-17. To reinforce previous results we show that after gating IL-17-producing cells, 54.6% of them

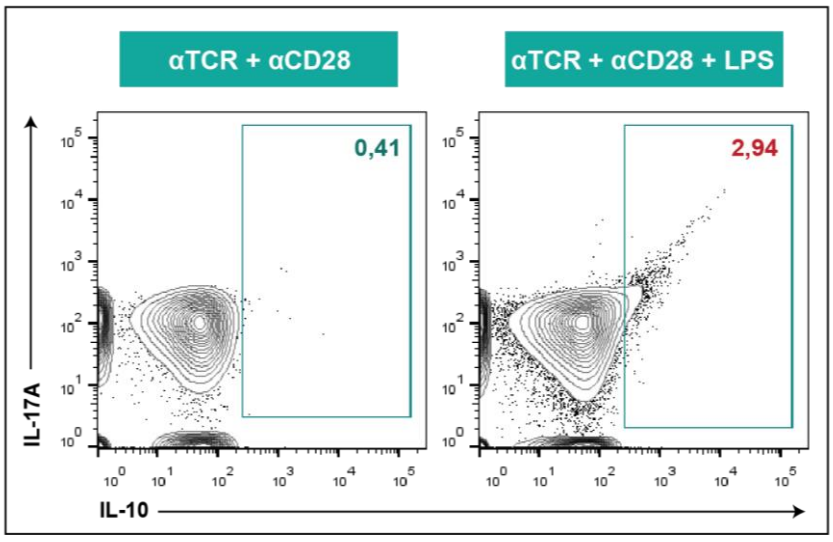


Figure 3.20 – Direct recognition of LPS promotes IL-10 production mostly on IL-17A-producing CD4 T cells. Sorted $CD4^+$ T cells were cultured for 5 days stimulated with αCD3 10 µg/mL and αCD28 2 µg/mL, or in combination with LPS 1,8 µg/mL. At day 4, cells were treated with phorbol 12-myristate 13-acetate (PMA), Ionomycin and protein transport inhibitor Brefeldin A (BFA) for ~15 hours. Cells were stained for LIVE/DEAD™ Fixable Aqua Dead Cell Stain, permeabilized using eBioscience™ Foxp3 / Transcription Factor Fixation/Permeabilization kit and stained for IL17A (3.33 µg/mL and IL10 (0.4 µg/mL). Population analysis was performed by flow cytometry in BD FACS Canto II and representative plots illustrate the percentages of IL10-producing $CD4^+$ T cells. Data are representative of 1 experiment.

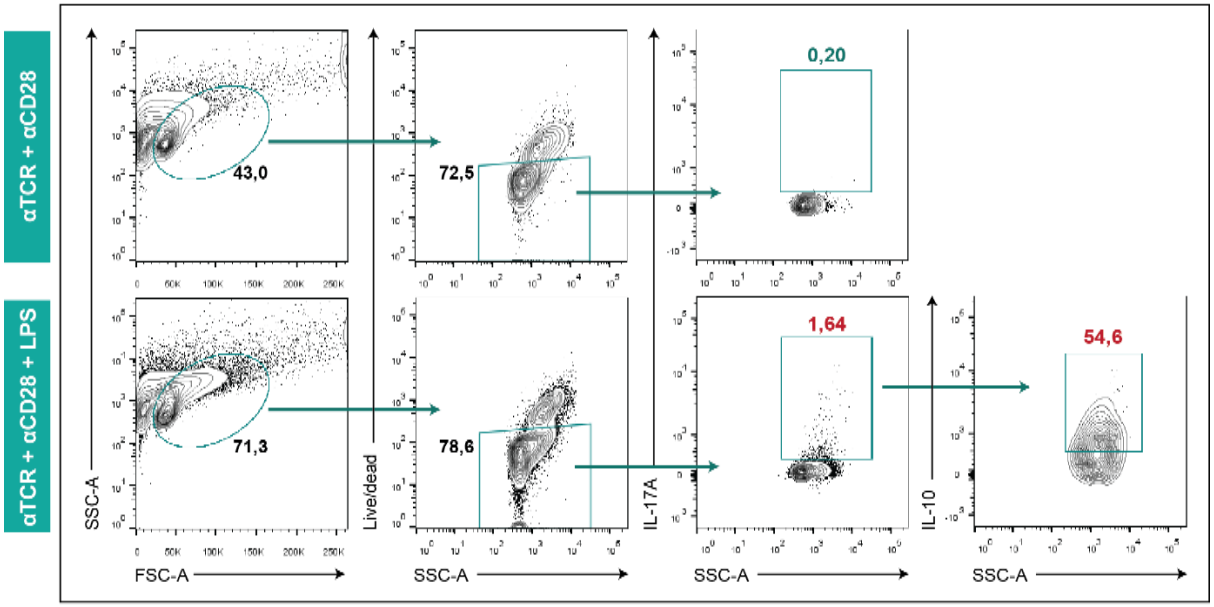


Figure 3.21 - Direct recognition of LPS promotes IL-17 and IL-10 production, suggesting a redirection of CD4⁺ T cells to a tolerogenic phenotype. Sorted CD4⁺ T cells were cultured for 5 days stimulated with αCD3 10 µg/mL and αCD28 2 µg/mL, or in combination with LPS 1,8 µg/mL. At day 4, cells were treated with phorbol 12-myristate 13-acetate (PMA), Ionomycin and protein transport inhibitor Brefeldin A (BFA) for ~15 hours. Cells were stained for LIVE/DEAD™ Fixable Aqua Dead Cell Stain or Fixable Viability Dye eFluor 780, permeabilized using eBioscience™ Foxp3 / Transcription Factor Fixation/Permeabilization kit and stained for IL17A (3.33 µg/mL and IL10 (0.4 µg/mL) and analysed by flow cytometry. Population analysis was performed by flow cytometry in BD FACS Canto II and representative plots illustrate the percentages of IL17A- and IL10- producing CD4⁺ T cells. Data are representative of 1 experiment.

are also producing IL-10 *(Figure 3.21)*. Altogether, we can hypothesize that chronic TLR4 activation seems to redirect Th17-like T cells to a tolerogenic-like phenotype, producing both IL17A and IL10, and ceasing IFNγ production.

Although Th17 are poised to develop strong tissue-destructive properties, recent evidence has shown they can also adopt an IL-10 producing tolerogenic phenotype and contribute to resolution of inflammation in the gut and in the central nervous system (Heinemann et al. 2014), (Gagliani et al. 2015). Altogether, with our results we suggest that TLR4 is likely involved in modulating Th cell differentiation.

A recent study showed that inhibition of the alternative p38 pathway in T cells abolish IL17A and IL10 production (Alam et al. 2015). These data suggest that p38 MAPK^{Y323} improved activation after TLR4 engagement might also be related with IL10 enhanced production.

This time in macrophages, others demonstrated that downstream kinases of p38 and ERK1/2 - Mitogen- and stress-activated kinase (MSK) 1 and MSK2 - are capable to phosphorylate CREB to induce the anti-inflammatory cytokine IL-10 to limit inflammation (Ananieva et al. 2008). This should made us consider that MAPK^{Y182}, whose activation also improves after direct engagement of TLR4 in the plasma membrane, can also promote IL10 production in CD4⁺ T cells. However, we are more inclined to believe that, in this context of chronic inflammation, MAPK^{Y182} activation possibly influence IFNγ production since it is capable to regulate transcription and/or mRNA stability of inflammatory cytokine genes (Taro Kawai and Akira 2009). However, in regards to ERK, despite the fact that we still have not evaluated how its activation is affected by TLR4 signaling, we think that it is likely to be involved in this improved IL10 production. Together with Ananieva's results, other studies mention ERK crucial role in the

expression of IL10 in different cells, from macrophages to Th cells (Saraiva and O'Garra 2010), (Gabrysova et al. 2014). Saraiva lab (Teixeira-Coelho et al. 2014) showed how ERK inhibition reduced the amount of IL-10 secreted after LPS-induced TLR4 activation in macrophages.

4 CONCLUSIONS AND FUTURE PERSPECTIVES

TLR4, among the other TLRs, is mainly expressed on innate immune cells like macrophages and dendritic cells. However, it has also been detected on activated human and mice CD4 T cells at the mRNA level (Hornung et al. 2002), (Gelman et al. 2004), (Tomita et al. 2008), (Fukata et al. 2008), (González-Navajas et al. 2010), (Reynolds et al. 2012). Also, TLRs expression was also achieved through the activation of human T lymphocytes in the presence of considerable amounts of polarizing cytokines like IFN-α (Komai-Koma et al. 2004). This suggested that T cells can directly respond to bacteria without the need of the intermediate innate immune cell. Besides those studies work with both mice and human cells, they all found that TLR4 is capable to improve CD4 T cell survival. However, we found some limitation on their protocols: First of all they were only capable to detect TLR4 expression at the mRNA level and also, to improve that expression they use "artificial" polarizing conditions by using considerable amounts of cytokines; In some of the studies they focus specifically on phenotypically induced mice models diseases, so divergent on the tissue microenvironment, from the CNS to the gut environment. Those factors prevented the acquisition of reliable results regarding the role of TLR4 on T cell differentiation, since they were looking to previously polarized/differentiated CD4 T cells; Last, but most importantly, they only focus on the outcomes after TLR4 engagement (T cell survival, differentiation...), and not on the molecular basis of TLR4 on CD4 T cells.

Our project is different from the previous studies since we rely on primary human T cells, which gives it an elevated translational potential. Most of all, we are focused on the molecular mechanisms of TLR4 on human CD4 T cells: How TLR4 is expressed; which are its subcellular localizations; how it is internalized into the cell; its signaling specificities; how its pathway might intersect other pathways on T cells, namely the TCR pathway; which signaling molecules are affected; how it affects T cell phenotype; and its physiological functions on CD4 T cells.

In this work we mimicked non-polarizing T cell stimulation and showed for the first time that TLR4 is expressed in human CD4 T cells, both at the plasma membrane and in intracellular compartments. Also, we found that chronic LPS stimulation for 5 days increased TLR4 intracellular expression and promotes its vesicular distribution in $CD4^+$ T cells *(Figure 3.1)*.

To determine how TLR4 engagement imparts on T cell function, we first assessed T cell survival and proliferation. After chronic encounter with LPS CD4 T cells survive more *(Figure 3.2)*, nevertheless, in one preliminary result, proliferation doesn't seem to be affected *(Figure 3.3)*. This was surprising since survival and proliferation are mostly associated with the same signaling pathways, suggesting the hypothesis that LPS would also affect T cell proliferation. Yet, if cells chronically stimulated with LPS might in fact have the same proliferation rate, the improved survival might be related with less apoptosis. For that, we propose determining expression of anti-apoptotic molecules (Bcl2, Bcl-xL) and measure apoptosis at different cell cycle stages by propidium iodide flow cytometry assay.

To characterize $TLR4^+CD4^+$ T cells, we inspected the expression of 3 cell markers of exhaustion (PD-1), activation (CD38) and memory (CD45RO). $TLR4^+CD4^+$ T cells are larger, more complex, and express higher levels of PD1 and CD38 *(Figure 3.4)*. After engagement with LPS they slightly change their phenotype as they have improved expression of CD38 and CD45RO. Differently, LPS seems to decrease PD-1 expression directly in $CD4^+TLR4^+$ cells. LPS signals might be capable to decline PD-1 expression on $CD4^+TLR4^+$ cells, making them unwilling to receive inhibitory signals *(Figure 3.5)*.

In innate immune cells, TLR4 signaling comprises 2 different pathways that culminate in 2 different cytokine outputs. One pathway is nucleated at the plasma membrane promoting a pro-inflammatory response, while the other is initiated from endosomes and ends up with the production of anti-inflammatory cytokines (Barton and Kagan 2009), (Kagan 2010) *(Figure 1.5)*. We indeed showed that TLR4 is also expressed intracellularly in human CD4 T cells *(Figure 3.1)*, however how TLR4 signaling is conveyed in T lymphocytes is still unexplored and it is not known whether it is similarly regulated by TLR4 subcellular distribution. Together, our results show that TLR4 engagement ensues similarly two distinct signaling pathways driving distinct functional outcomes. At the plasma membrane, TLR4 drives pAKT^{S473} and p38 MAPK^{Y182} activation *(Figure 3.7, 3.8 and 3.15)*, which might offer a mechanistic explanation for increased T cell survival *(Figure 3.2)*. We can't exclude that p38 MAPK^{Y182} improved activation might also impinge alterations in T cell differentiation since p38 MAPK is capable to regulate the transcription and mRNA stability of inflammatory genes (Taro Kawai and Akira 2009). In addition, the plasma membrane is the site for TLR4 and TCR signaling crosstalk manifested by improved PKCθ^{S676} phosphorylation *(Figure 3.7, 3.8 and 3.15)*. Surprisingly, once located to the endocytic compartment, TLR4 intersects TCR signaling by increasing the phosphorylation of a residue that has been described to be under the exclusive control of TCR engagement, p38 MAPK^{Y323} *(Figure 3.10, 3.11, 3.12 and 3.15).* By increasing the activation of p38 MAPK^{Y323} TLR4 might impinge directly on CD4 T cell differentiation, due to the known role of this pathway in directing cytokine production (Salvador et al. 2005) *(Figure 3.15)*.

To reinforce the crosstalk between TCR and TLR4 signaling pathways, we evaluated their expression after CD4 T cell restimulation. We found that, as TCR, TLR4 is also expressed along the membrane projections of the mimicked immunological synapse, even though TLR4 is predominantly organized in clusters. At the beginning of stimulation, TLR4 clusters are polarized, and even though we should not conclude TCR and TLR4 are interacting, we can at least assume, by their proximity, that their pathways likely interplay with each other. After 20 minutes of stimulation, TLR4 clusters converge to the cSMAC while they are being internalized into the cell *(Figure 3.16)*.

The crosstalk manifested by improved PKCθ^{S676} is also nucleated at the plasma membrane, since when we inhibit TLR4 internalization we improved the activation of PKCθ^{S676}. However, PKCθ^{S676} is not directly activated solely via TLR4 engagement *(Figure 3.7)*. PKCθ^{S676} requires TCR+CD28 engagement, and there are some candidates for the crosstalk between TLR4 and TCR signaling. ZAP70 is a SYK-related molecule whose expression is mostly confined to T and NK cells (Mócsai, Ruland, and Tybulewicz 2010), that is recruited after TCR activation and initiates a network of molecular pathways required for T cell development and activation (Silva et al. 2016). We wondered if ZAP70 could impact on the crosstalk between TCR and TLR4. By confocal microscopy, we found that pZAP70^{Y319} seems to be slightly expressed nearby the intracellular structures of TLR4, however it is predominantly expressed closer to the plasma membrane *(Figure 3.17)* where it might act as an intermediate of TLR4-induced T cell activation. In the present I suggest we should evaluate ZAP70^{Y319} activation by flow cytometry as we did for the other phospho-molecules, as the only way to confirm if it is directly affected by TLR4 engagement.

We extended our work to TLR4 trafficking, investigating which small GTPases were affecting TLR4 translocation from the plasma membrane. We relied on Husebye's work in macrophages (Husebye et al. 2010) that suggests GTPase Rab11a promotes TLR4 internalization into recycling endosomes and its recruitment to bacteria-containing phagosomes. When we re-stimulated cells for 30 minutes with LPS inducing TLR4 internalization, and 20 minutes with the antibodies αTCR and αCD28, as expected the intracellular compartment of TLR4 become more defined and, interestingly the colocalization between TLR4 and Rab11a is significantly increased, likely suggesting Rab11a as one of the small GTPases involved in TLR4 internalization.

After showing the expression of TLR4 in T cells, we demonstrated that it can modulate T cell activation. Regarding $CD4^+$ T cells, some reports have focused on how TLRs can influence on the modulation and plasticity of some Th subsets. However, this role of TLR4 was conflictingly reported to both sustain EAE (Reynolds et al. 2012), and to protect from colitis (González-Navajas et al. 2010). This might be related with the fact that in the gut, $CD4^+$ T cells are constantly encountered with TLR stimuli from the microbiota, while in the central nervous system it is probably perceived as a host-derived danger signal. In our lab, we stimulated T cells in non-polarizing conditions for 5 days in the presence of LPS, which we believe can mimic a chronic encounter with the TLR ligand as what happens in the gut. After those 5 days in culture with LPS, we found an alteration in the cytokine profile. LPS was found to promote the production of IL-17A, while inhibiting IFNγ. Chronic engagement with LPS improved intracellular expression of TLR4, and p38 $MAPK^{Y323}$ activation relies on TLR4 signaling from the vesicular compartments. Thus, p38 $MAPK^{Y323}$ activation is presumably improved after LPS engagement which might offer an explanation for the increased IL-17A production, since p38 $MAPK^{Y323}$ is known to direct cytokine production (Salvador et al. 2005) and studies showed that its inhibition reduced the production of IL-17A, TNFα and IL-10 (Alam et al. 2015). Regarding IFNγ downregulation, we believe it can be a promoted by $MAPK^{Y182}$ due to its capacity to regulate translation and mRNA stability of pro-inflammatory genes (Taro Kawai and Akira 2009). Since co-production of IFNγ turns Th17 into pathogenic autoreactive cells (Hirota et al. 2007), (Langrish et al. 2005), our results showing a decreased production of IFNγ, allow us to speculate that a chronic engagement of $CD4^+$ T cells with LPS redirects them to a more tolerogenic Th17 phenotype.

Our results regarding $CD4^+$ T cell signalling showed that, likewise innate immune cells, TLR4 engagement in T cells ensues 2 distinct pathways driving 2 distinct functional outcomes. We questioned if $CD4^+$ T cells might behave as innate immune cells, relying on a TRIF-dependent signal from intracellular compartments and triggering an anti-inflammatory response with the production of IFNα, IFNβ and/or IL-10. Indeed, we found that chronic engagement with LPS improves IL10 production in IL17A-producing $CD4^+$ T cells *(Figure 3.20 and 3.21)*. We hypothesize that chronic TLR4 activation induces Th17-like T cells to be redirected to a tolerogenic phenotype, producing both IL17A and IL10, and ceasing IFNγ production. As we discussed previously, $MAPK^{Y323}$ pathway might also be the one improving IL-10 production after chronic TLR4 activation. However ERK is a suitable candidate, as it was reported as crucial in the expression of IL-10 in different cells, from macrophages to Th cells (Saraiva and O'Garra 2010), (Gabrysova et al. 2014). Saraiva lab (Teixeira-Coelho et al. 2014) showed that ERK inhibition reduced IL-10 production after LPS-induced TLR4 activation in macrophages.

After determining how TLR4 engagement imparts on T cell function, and after shed some lights on TLR4 signaling specificities in CD4 T cells, we intend to continue unravelling how TLR4 activation conveys in T lymphocytes and to assess how TLR4 counter-regulates Th17 pathogenicity.

First we need to do a kinetics for CD4 T cell stimulation, regarding for instance cytokine production, since the low levels of cytokine production might be a result from short time of differentiation (5 days).

We are going to determine the production of other immunosuppressive cytokine, such as TGFβ by ELISA and also confirm by this technique the improved production of the other cytokines IL17-A, IL10 and IFNγ. More importantly, to make sure about the eventual tolerogenic phenotype of our CD4 T cells after chronic LPS stimulation we need to do some functional assays: in a co-culture assay we are going to culture our stimulated cells with CD4 effector cells, in this case we will evaluate the tolerogenic capacity of our cells if they are capable to inhibit the proliferation of the effector CD4 T cells; also we might culture our cells with cytotoxic CD8 T cells evaluating if our cells are capable to block the production of cytotoxic mediators like pro-inflammatory cytokines, perforins and granzymes by CD8 T cells as a reflect of their tolerogenic capacity.

After using CPZ as an inhibitor of TLR4 internalization, we optimally expect to selectively suppress some signalling molecules to unravel how they are affecting the phenotype of $CD4^+$ T cells. In this aim, we hypothesize that by suppressing TLR4 signaling from intracellular endosomes, we might clarify that it is crucial to trigger the anti-inflammatory response. Since we related p38 $MAPK^{Y323}$ activation from the endocytic compartment as the main responsible for IL-17A production and eventual IL-10 production, we might act by blocking selectively the p38 MAPK. Even more, since we believe TLR4 intracellular signaling might function as in innate immune cells, its suppression can be carried out by inhibiting TRIF-dependent signaling.

Furthermore, we are interested in assessing how TLR4 is capable to counteract Th17 pathogenicity by driving a genetic and/or metabolic reprogramming. For that, we will compare the transcriptomic profile driven by TLR4 in human $CD4^+$ T cells with the transcriptomes from conventional Th17 cells and T_{regs}. Alternatively, in a more focused approach, we will assess for the differential expression of the master-regulators of T cell differentiation (RORγT, Foxp3) and the transcription factors known to facilitate T cell polarization into IL-10-producing cells (cMaf, Blimp-1). Metabolic reprogramming and T cell inflammatory capability are tightly interconnected (Buck, O'Sullivan, and Pearce 2015), (Gerriets et al. 2016). I will determine how TLR4 impinges on T cell metabolism by evaluating mitochondrial potential, Glut1, pS6 and mTOR levels by flow cytometry and oxygen consumption and acidification rate by seahorse.

Th17 functions depend on the balance between pro- and anti-inflammatory cytokines: co-production of IFNγ turns them into pathogenic autoreactive cells, while IL-10 co-production confers them tissue-protective properties. This developing project might elucidate how LPS-bearing bacteria can instruct formerly pro-inflammatory Th17 cells, which are beneficial in clearing infection but immunopathogenic in excess, to adopt a tolerogenic phenotype. Curiously, it was found that the tissue microenvironment in the intestine is crucial in converting Th17 pathogenic cells into tolerogenic IL-10-producing Th17 cells (Esplugues et al. 2011). Nevertheless, the molecular determinants for limiting this

Th17 pathogenicity in the bacteria rich intestine microenvironment have remained unknown. In this context of our preliminary data TLR4 stands out as a likely candidate. Overall, with the results we have so far, we propose a model in which TLR4 can be involved in modulating Th cell differentiation into tolerogenic phenotypes, through immunoregulatory mechanisms particularly important in the microbe rich environment *(Figure 4.1)*.

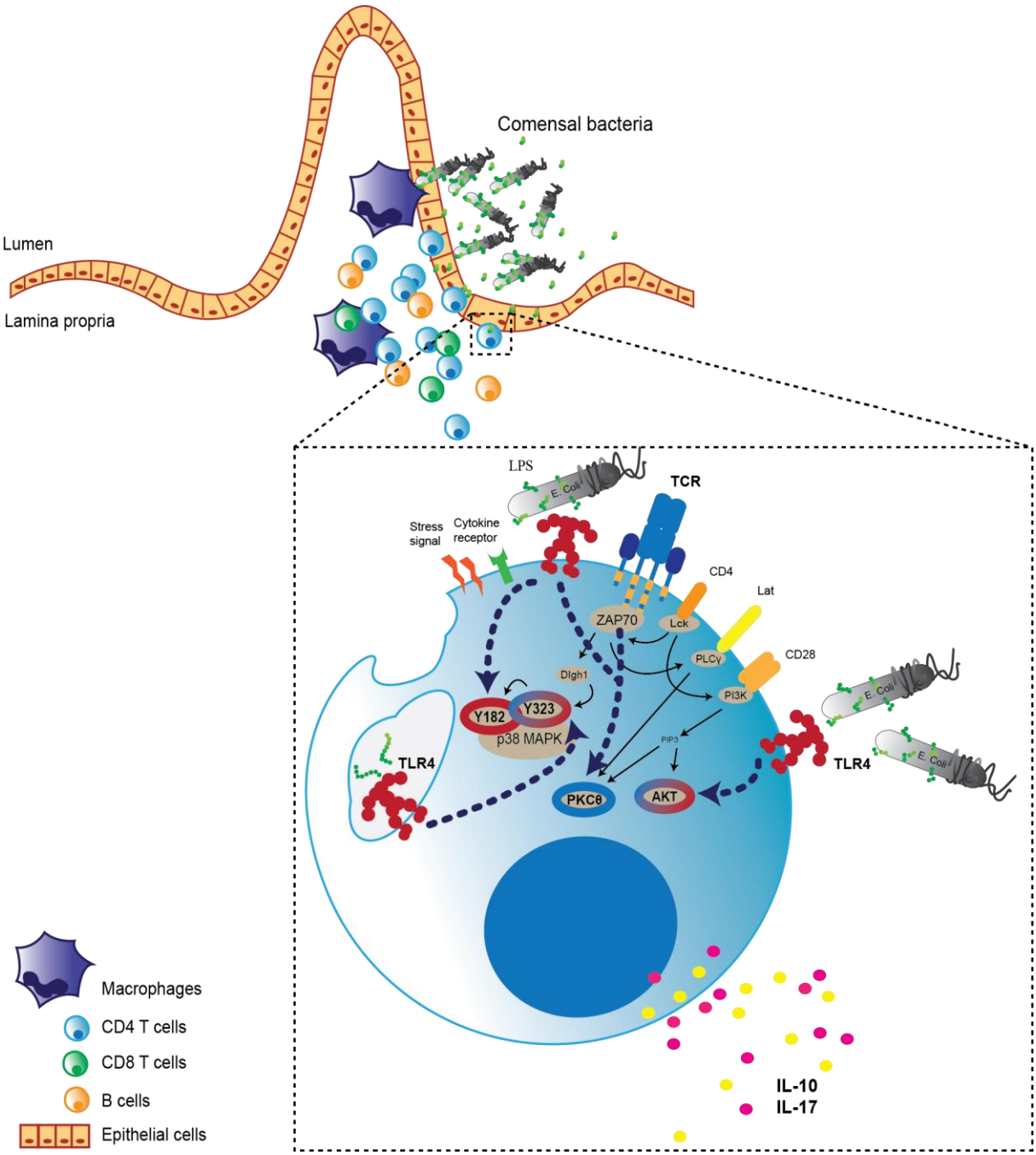


Figure 4.1 – Proposed model for the role of TLR4 in T cells, particularly in microbe rich environments. Based on (Tamburini et al. 2016). We hypothesize that in addition to cytokines, other microenvironment factors like LPS-bearing commensal bacteria can impact on human T cell phenotype through TLR4 signaling. We found that chronic encounter with LPS molecules improved the production of IL17A and IL10 while ceasing the production of IFNγ by CD4 T cells. Thus, cells are being redirected to a tolerogenic phenotype that help to curtail chronic inflammation particularly in microbe rich gut environments.

This counter-regulation of Th17 pathogenicity might have physiological relevance in mediating tissue protection in a specific context of chronic encounter with TLR ligands, as what happens in the gut. Unravelling how this counter-regulation happens in CD4 T cells is crucial to provide new targets for developing novel and more effective therapies that restore immune tolerance in chronic inflammatory/autoimmune diseases in the gut without the deleterious side effects of current therapies.

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