Joana Patrícia Fernandes Moura Guedes

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"Whithout the scientist there is no future. The handsome and the beautiful may earn the admiration of society, but all of the wondrous inventions of the future are a by-product of the unsung, anonymous scientists."

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ABSTRACT

The innate immune response is triggered upon the recognition of pathogen-associated molecular patterns (PAMPs) by pattern-recognition receptors (PRRs), such as toll-like receptors (TLRs). Upon PRR activation, downstream signalling cascades are activated in innate immune cells, resulting in the induction of cytokine production, including of interleukin (IL)-10. This anti-inflammatory cytokine regulates cellular activation and inflammatory cytokine production, thus promoting immune homeostasis and preventing tissue damage upon infection.

Here, we aimed at investigating the mechanisms underlying the IL-10 expression and production upon PRR stimulation of macrophages and dendritic cells (DCs). Upon These cells play distinct roles upon infection, as macrophages try to contain infection in situ and DCs will present antigen peptides to naïve T cells at the secondary lymphoid organs, thus activating the acquired immune response. We also investigated the mechanisms that underlie the differential regulation of cytokines of the IL-12 family in macrophages and DCs upon TLR stimulation and its correlation with the differential IL-10 regulation in these cells.

We show that macrophages and DCs vary significantly in the mechanisms that regulate their IL-10 production. Contrary to macrophages, TLR-stimulated DCs did not produce considerable amounts of IL-10. However, Dectin-1 stimulation induced higher IL-10 production by these cells as compared to macrophages. In all, both macrophages and DCs can produce IL-10, but do so in response to different stimuli. Our results suggest that differential induction of the MAPK ERK and p38 in PRR-stimulated macrophages and DCs likely explains the differences observed for IL-10 production. Additionally, we found that Dectin-1/TLR2-stimulation led to a reduction in IL-10 expression by DCs, an unexpected finding that requires further investigation. In the second part of this work, we show that macrophages are limited in their capacity of producing IL-12, IL-23 and IL-27 upon TLR-stimulation, which is partly due to their high IL-10 production in response to TLR triggering. Although IL-10 and IL-12 family members are reciprocally regulated in TLR-stimulated macrophages and DCs, this regulation is more pronounced in macrophages. Furthermore, this regulation of IL-12 family cytokines by IL-10 occurs at the transcriptional level.

In summary, this thesis shows that IL-10 expression is tightly regulated, cell type specific and dependent on the cell stimulus. This tight regulation of IL-10 has implications on the expression of IL-12 family members, and can thus impact cellular activation and the differential priming of Tcells.

RESUMO

A resposta imunológica inata é ativada após o reconhecimento de padrões moleculares associados a agentes patogénicos (PAMPs), por recetores de reconhecimento desses padrões (PRRs). A ativação de PRRs, induz a iniciação cascatas de sinalização intracelular que resulta na indução de citoquinas, como a interleucina (IL)-10. A IL-10 é uma citoquina anti-inflamatória que regula a ativação celular e a produção de citoquinas pró-inflamatórias, como a IL-16, TNF ou a IL-12, promovendo assim homeostasia imunológica e prevenindo danos tecidulares após infecção.

O nosso objetivo era investigar os mecanismos de expressão e regulação da IL-10 produzida por macrófagos e células dendríticas (DCs), após ativação por PRRs. Em contexto de infeção estas células desempenham funções distintas: enquanto que os macrófagos permanecem *in situ* de forma a combater a infeção, as DCs migram até aos órgãos linfóides secundários onde apresentam péptidos antigénicos, ativando as células T naïve. Foram também explorados os mecanismos que estão na base da regulação diferencial da citoquinas da família da IL-12, em macrófagos e DCs ativadas via TLR, e qual a sua correlação com a regulação diferencial de IL-10 nestas células. Neste trabalho mostramos que os mecanismos de regulação da IL-10 em macrófagos e DCs são distintos. Ao contrário dos macrófagos, as DCs ativadas via TLR não produzem níveis consideráveis de IL-10. Contudo, estimulação via Dectin-1 induziu uma grande produção de IL-10 nestas células, comparado com macrófagos. Em suma, ambos os macrófagos e as DCs são capazes de produzir IL-10 em resposta a diferentes estímulos. Os nossos resultados sugerem que a indução diferencial das MAPK ERK e p38 poderá explicar as diferenças observadas na produção de IL-10. Adicionalmente, observamos que a co-estimulação por Dectin-1/TLR2 induz uma redução na expressão de IL-10 em DCs, um resultado inesperado que requer uma investigação mais aprofundada. Na segunda parte deste trabalho, mostramos que os macrófagos estimulados via TLR são limitados na sua capacidade de produzir IL-12, IL-23 e IL-27 devidos à sua elevada expressão de IL-10 em resposta a TLRs. Apesar de os membros da família da IL-12 serem reciprocamente reguladas pela IL-10 em macrófagos e DCs, este mecanismo é mais pronunciado em macrófagos. Mostramos também que esta regulação dos membros da família da IL-12 pela IL-10 ocorre ao nível trascripcional.

Em resumo, esta tese mostra que a expressão da IL-10 é regulada de forma especifica para cada tipo de célula ou estimulo. Isto tem implicações na expressão nos membros da família da IL-12, afetando assim, a ativação celular e de células T.

TABLE OF CONTENTS

Figure Index

List of Abbreviations

ActD	Actinomycin D
AP-1	Activated protein 1
APC	Antigen-presenting cell
BCG	Bacillus Calmette-Guérin
BMDC	Bone marrow-derived dendritic cells
BMDM	Bone marrow-derived macrophages
c/EBPß	CCAAT/enhancer binding protein-ß
cDC	Classical dendritic cell
CARD	Caspase-associated recruitment domain
CLR	C-type lectin receptors
CRP	C-reactive protein
CXCL	CXC-chemokine ligand
DAMP	Damage-associated molecular pattern
DCs	Dendritic cells
DC-SIGN	Dendritic cell specific intracellular adhesion molecule-3-grabbing non-integrin
dsRNA	Double-stranded RNA
ERK	Extracellular signal-regulated kinase
FBS	Fetal bovine serum
GKS	Glycogen synthase kinase
HSS	DNasel hypersensitive site
iE-DAP	g-D-glutamyl-mesodiaminopimelic acid
IFN	Interferon
IKK	IκB kinase
IL	Interleukin
IRAK	IL-1receptor associated kinase
IRF	Interferon regulatory factors
ITAM	Immunoreceptor tyrosine-based activation motif
JAK	Janus kinase
JNK	JUN N-terminal kinases
LGP	Laboratory of genetics and physiology
LPS	Lipopolysaccharide
LRR	Lectin-rich repeats
MALT1	Mucosal-associated lymphoid tissue lymphoma translocation protein 1
MAPK	Mitogen activated protein kinases
MBL	Mannan-binding lectin
MDP	Muramyl dipeptide
mDC	Myeloid dendritic cell
MDA	Melanoma differentiation-associated gene
MHC	Major histocompatibility complex
miRNA	Micro RNA
mRNA	Messenger RNA
MyD88	Myeloid differentiation primary response 88
NF-κB	Nuclear factor - kappaB
NLR	NOD-like receptor

NOD	Nuclear-binding oligomerization
PAMP	Pathogen-associated molecular pattern
pDC	Plasmacytoid dendritic cell
PDCD4	Programmed cell death 4
PRR	Pattern-recognition receptor
RIG	Retinoic acid-inducible gene
RLR	RIG I-like receptor
SAP	Serum amyloid protein
SP	Specific protein
ssRNA	Single-stranded RNA
STAT	Signal transducer and activator of transcription
SYK	Spleen tyrosine kinase
TAK	TGFß-activated kinase
T_H	Helper T cell
TIR	Toll-interleukin 1 receptor
TIRAP	TIR domain containing adaptor
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TPL	Tumour progression locus
TRAF	TNF receptor associated factor
TRAM	TRIF related adapter molecule
T_{Reg}	Regulatory T cell
TRIF	TIR domain containing adaptor inducing interferon ß
TTP	Tristetrapolin

Introduction

The Innate Immune Response

Innate immunity is the first line of defence against foreign pathogens, providing efficient and immediate immune responses[1]. Activation of innate immune cells, like macrophages, neutrophils or dendritic cells is of major importance to control infection and maintain homeostasis. Moreover, it constitutes a pre-requisite for the later activation of acquired immune responses[2,3].

Macrophages, originally identified by Metchnikoff, are mononuclear phagocytes with a major role in the maintenance of tissue homeostasis, being the major mediators of the innate immune response[4]. Macrophages are highly heterogenic, reflection of their anatomical location and function specialization, namely osteoclasts (bone), alveolar macrophages (lung), histiocytes (interstitial connective tissue) and kupffer cells (liver)[5,6]. Moreover, several distinct macrophage populations are found either in the gut or in the secondary lymphoid organs[6]. Also macrophages sub-populations are found in immune-privileged sites, as the brain (microglia), eyes and testes[6]. Tissue resident macrophages maintain tissue homeostasis by operating as sentinels and control changes in physiology, having a role in development, metabolic processes and tissue repair, or in fighting pathogen invaders[4]. Lack of macrophages during tissue development can give rise to illness conditions, for example, lack of osteoclasts, caused by loss of bone-reabsorbing macrophages, leads to the development of osteoporosis[7]. Moreover, the role of macrophages in tissue development can impact ductal branching, neural networking and angiogenesis[7]. Tissue stress subsequent to injury or infection leads to the activation on tissue macrophages and to the recruitment of blood-circulating monocytes into the damaged tissue, which will differentiate into mononuclear phagocytes[8]. Activated macrophages will secrete several inflammatory mediators, including cytokines and nitric oxide, which in turn will activate microbicidal mechanisms, namely oxidative processes that contribute to the killing of foreign organisms[4]. For example, during *Mycobacterium tuberculosis* infection, alveolar macrophages will recognize and internalize the bacilli into phagosomes[9]. The phagosome maturation into phagolysosome will provide the release of hydrolytic enzymes that constitute one of the major microbicidal activities of macrophages[9]. Furthermore, macrophages activation also induces reactive oxygen species, and, in case of interferon-γ (IFNγ) and tumour necrosis factor-α (TNFα) activated macrophages, nitrogen reactive species. Macrophages also have a role in tumourigenesis, promoting tumour initiation, progress and metastasis[10], allergy and autoimmune disorders[6].

Dendritic cells (DCs), first described by Steinman and Cohn[11], sense the microenvironment and function as antigen-presenting cells (APCs) with the ability to mount adaptive immune responses. DCs have bone-marrow precursors that can give rise to either plasmacytoid DCs (pDC) and myeloid/classical DCs (mDC/cDC), commonly referred as DCs[12]. pDC are present in the blood and lymphoid tissues and are specialized in responding to viral infection, through foreign nucleic acid recognition, production type I IFN and acquisition of the capacity to present antigens, despite their low expression of major histocompatibility complex class II (MHC-II) and co-stimulatory molecules, compared to mDC[8,12].

Immature DCs are highly phagocytic cells that upon activation will secrete cytokines to promote their maturation and capacity of peptide digestion[8]. DCs maturation can be triggered upon PRR ligand binding[13]. Mature DCs are present at secondary lymphoid organs and express high levels of immunostimulatory molecules, such as peptide-MHC class I and II, as well as T cell co-stimulatory molecules as CD80 and CD86, that allow antigen presentation to naïve T cells and T cell priming[13,14]. DCs are cell specialized in tissue sensing, and can be subdivided into several subsets, namely: non-lymphoid tissues DCs, epidermal Langerhans cells, tissue-migratory DCs and lymphoid organ DCs[12]. Each DCs subset expresses a different set of PRRs that will induce a differential functional maturation, which will shape T cell response[13,14].

DCs and macrophages are specialized innate immune cells, with different functions, which combined can orchestrate innate immune responses against foreign pathogens.

In the next section specific subjects related to innate immune triggering and response development are reviewed.

Innate Immune Recognition

Innate immune recognition is mediated by germline-encoded PRRs that serve as first-line sentinels for pathogen recognition, having the ability to discriminate between different pathogen signature molecules, named Pathogen-associated molecular patterns (PAMPs)[15].

PAMPs can derive from virus, bacteria, fungi or parasites and are essential for their survival. These molecules include lipids, lipoproteins, proteins and nucleic acids[16].

Furthermore, in case of sterile inflammation, chronic infection or continuous injuries, immune cell recruitment is mediated through self-molecules production, named damage-associated molecular patterns (DAMPs)[17]. For instance, in case of cellular death by necrosis, the release of DAMPs, such

as extracellular ATP, mitochondrial formaldehyde peptides and mitochondrial DNA results in cellular stress or inflammation mediated by PRR activation[18,19].

PRRs can be expressed on the cell surface or in cell endosomes[16]. Moreover, PRRs can be secreted into the bloodstream and tissue fluids, like mannan-binding lectin (MBL), C-reactive protein (CRP) and serum amyloid protein (SAP) that are produced by the liver at early stages of infection[1]. Upon PRR activation, several intracellular signalling cascades will be triggered, leading to the production of inflammatory cytokines and type I IFN[16].

PRRs are broadly categorized into Toll-like Receptors (TLRs) and non-TLRs, which will be further addressed in the next sections.

TLR activation of innate immune responses

TLRs are type I integral transmembrane glycoproteins, composed by three domains: an extracellular (or cytoplasmatic in the case of endosomal TLRs) N-terminus domain that mediates PAMPs recognition through leucin-rich repeats (LRRs), a transmembrane domain and an intracellular C-terminus domain that triggers signalling pathways through a toll-interleukin (IL) 1 receptor (TIR)[15,16].

Ten different TLRs were identified in humans, TLR1 to TLR10, nine of them with homology in mice: TLR1 to TLR9[15]. In mice 3 additional TLRs were also identified: TLR11 to TLR13[15].

TLRs recognize a wide variety of PAMPs, including triacyl lipoproteins (TLR2/TLR1), diacyl lipoproteins (TLR2/TLR6), double-stranded RNA (dsRNA) (TLR3), lipopolysaccharides (LPS) and viral envelop lipoproteins (TLR4), flagelin (TLR5), single-stranded RNA (ssRNA) (TLR7 and TLR8), genomic rich DNA in unmethylated CpG DNA (TLR9), profin-like molecules (TLR11 and TLR12), uropathogenic bacteria (TLR11) and bacterial RNA (TLR13) [16,20,21]. The ligand for TLR10 is still unknown[16].

Depending on the type of family of recognized microbial molecules, TLRs assume different cellular localizations (Figure 1). In that sense, TLR1, TLR2, TLR4, TLR5, TLR6, TLR11 and TLR12 are expressed on the cell surface, whereas TLR3, TLR7, TLR8, TLR9 and TLR13 are expressed in endolysosomal compartments[16,20,21]. Interestingly, after triggering, TLRs that are expressed on the cell surface can be endocytosed and signal through endolysosomes[15,16,21,22].

After ligand recognition, TLRs can undergo heterodimerization or homodimerization acquiring a horseshoe-like structure that is essential for the initiation of the downstream signalling

cascade[3,15,16]. TIR domain activation leads to the recruitment of bridging adaptors: Myeloid Differentiation primary response 88 (MyD88), TIR domain containing adaptor protein (TIRAP), TIR domain containing adaptor inducing IFN-ß (TRIF) and TRIF related adapter molecule (TRAM)[23]. With exception of TLR3, all TLRs recruit MyD88 adaptor after ligand recognition[15,16]. TLR1, TLR2, TLR4 and TLR6 also use TIRAP as a bridging adaptor between TIR domain and MyD88[15,16]. TLR3 is the only TLR that signals exclusively via TRIF, whereas TLR4 is the only member of this family to signal via both MyD88 and TRIF. In the case of TLR4, TRAM is used as a bridging adaptor between TIR domain and TRIF (Figure 1). MyD88 dependent pathways recruit IL-1 associated kinase (IRAK) 1 protein kinases that activates TNF-associated factor (TRAF) 6. In turn, TRAF6 recruits TGF-ß-activated kinase (TAK) 1 that activates the inhibitor of kappaB kinase (IKK) complex and the phosphorylation of mitogen-activated protein kinases (MAPK), leading to the activation of nuclear factor –kappaB (NF-κB) subunits and activation of Activated protein 1 (AP-1) transcription factor for inflammatory cytokine transcription[16](Figure 1).

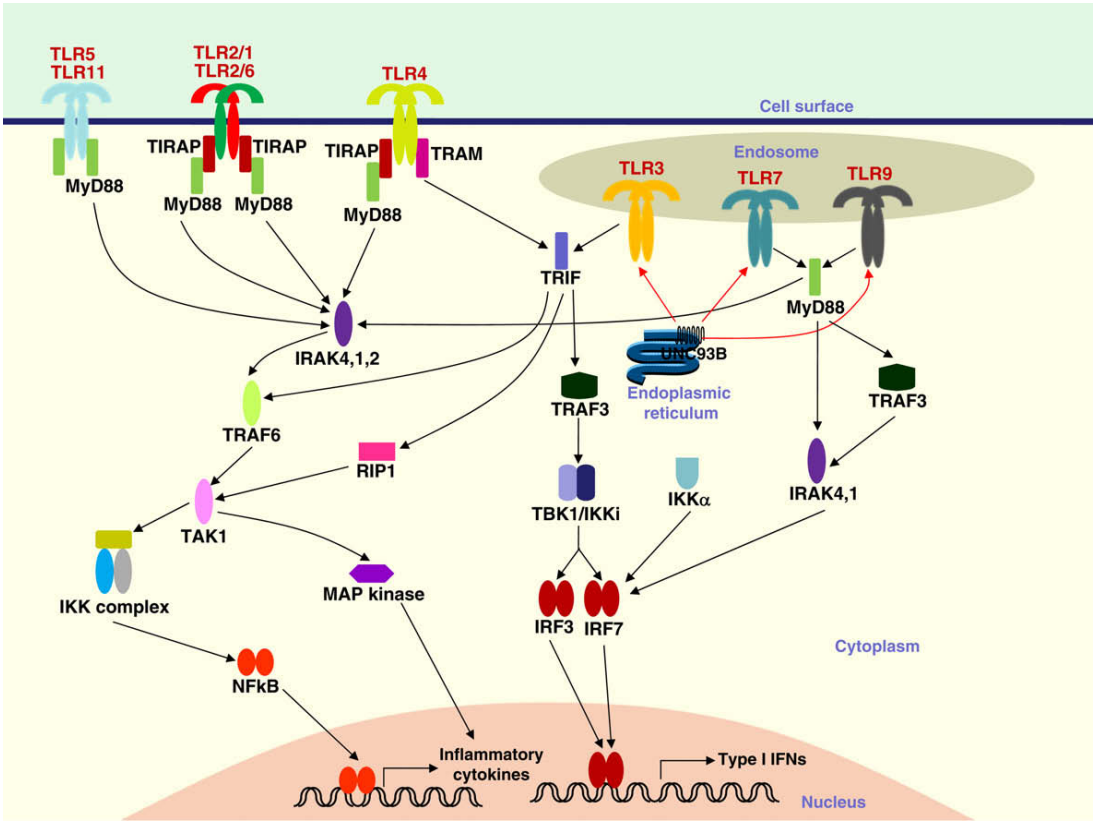

Figure 1: Toll-like receptor (TLR) signalling pathway. From Kumar, H. *et al*; Toll-like receptors and innate immunity. Biochem Biophys Res Commun, 2009.

MyD88- and TRIF-dependent pathways show convergent signalling cascades. TRIF downstream signalling also involves TRAF6 for NF-κB activation. Moreover, TRIF can recruit RIP1 that will activate TAK1[16]. In addition to NF-κB activation by TRAF6, TRIF can also activate TRAF3 that promotes TBK1/IKKi signalling complex that catalyses the phosphorylation of IFN regulatory factors (IRF) 3 and induces its nuclear translocation leading to the transcription of type I IFN[15](Figure 1).

Thus, adaptor recruitment initiates signalling cascades that culminate in the activation of transcription factors, namely NF-κB and IRFs, or in the phosphorylation of MAPK, such as extracellular signal-regulated kinases (ERK) 1/2, p38 and JUN N-terminal kinases (JNK)[16]. Transcription factors activation and nuclear translocation will promote a specific cellular transcriptomic program that culminates in expression of many immune mediators, such as cytokines[16,24] (Figure 1). In addition to the transcription of inflammatory cytokines, such as TNF or IL-6, and to type I IFN, the anti-inflammatory cytokine IL-10 is also induced upon TLR triggering, as discussed in more detail later.

The importance of TLRs is illustrated by the fact that several TLR polymorphisms have been associated with infection susceptibility and increased risk of cancer development[25]. For example, individuals that carry the D229G polymorphism in TLR4 are more susceptible to septic shock[26-28]. The TLR2 R753Q polymorphism is associated with *Borrelia burgdorferi, Treponema pallidum* and *Mycobacterium tuberculosis* susceptibility[26,29,30]. Additionally, the TLR2 R677W polymorphism has been associated with increased susceptibility to *M. tuberculosis* and *Mycobacterium leprae*[26,31,32].

Since part of the work developed in this thesis regards TLR2, this receptor is described in more detail in the next section.

TLR2

TLR2 is expressed on the cell surface and its triggering relies on the dimerization with either TLR1 or TLR6[33]. This differential dimerization enables TLR2 to discriminate molecular ligands present in the outer membrane of Gram-positive bacteria, as TLR2/TLR1 recognizes tri-acylated lipopeptide complexes, whereas TLR2/TLR6 recognizes diacylated lipopeptides[34-36].

Although TLR2 triggering depends on the dimerization with either TLR1 or TLR6, Farhat and colleges demonstrate that the downstream cascades activated upon triggering of TLR2/TLR1 or TLR2/TLR6 are the same[33]. Recently, Guan et al described the presence of TLR2/TLR10 pre-

formed dimers on the cell surface, but the biological function of such combination awaits to be determined[37]. Distinct TLR2 ligands can elicit distinct kinetic profiles of signalling and gene expression and consequently each heterodimer may prime a different physiological outcome[38]. Some authors described a possible TLR2 homodimerization, however further studies are needed to confirm the biological function of these homodimers[34,36]. TLR2 triggering can also be facilitated by co-receptors, as CD36, which binds to some TLR2 lipopeptides agonists, transferring them to CD14 that in turn presents them to the TLR2/TLR6 or TLR2/TLR1 complex[39,40].

More than 175 common TLR2 SNPs of low penetrance alleles have been reported in humans. Some of the SNPs promote a shift in the balance of pro and anti-inflammatory cytokines and have been associated with increased susceptibility for cancer development[25,41-43]. Some TLR2 polymorphisms modulate the host susceptibility or infection aggressiveness by bacteria, like *M. tuberculosis*[29,32], *Lepromatous leprosy*[44] and viral infections, namely cytomegalovirus[45] or hepatitis C virus[46]. TLR2 polymorphisms can also modulate the risk of development of Alzheimer's disease[47], Parkinson's disease[48], among others conditions.

The TLR2 Signalling Cascade

TLR2 triggering activates the MyD88-dependent pathway, which typically induces pro-inflammatory cytokines production[16,40]. After triggering, TLR2 recruits TIRAP, that functions as a bridging adaptor between the TIR domain and MyD88. MyD88 recruits IL-1 receptor associated kinase (IRAK) 4 that promotes the phosphorylation of IRAK1. Phosphorylated IRAK1 or IRAK2 promotes TRAF6 ubiquitination. Ubiquitinated TRAF6 activates the TAB2-TAK1-IKK complex that induces ubiquitination and phosphorylation of IκB. IκB degradation promotes NF-κB translocation to the nucleus for gene upregulation. TAB2-TAK1 complex can also activate MKK6 resulting in MAPK phosphorylation, which in turn will activate AP-1, triggering cytokine gene transcription[40]. (Figure 2)

In 1999, Underhill and colleagues described TLR2 recruitment into phagosomes[49]. In response to viral or bacterial ligands, internalized TLR2 activates, by an undetermined pathway, IRF7/3 leading to IFN-ß gene up-regulation[50] or activates IRF2/IRF1/Signal transducer and activator of transcription(STAT)1 for IFN-α gene up-regulation[51,52](Figure 2).

TLR2 activation also leads to the transcription of the anti-inflammatory cytokine IL-10 during several viral, fungal and bacterial infectious diseases[40]. The molecular mechanisms regulating IL-10 expression upon TLR signalling are detailed later in this thesis. For example, *Yersinia pestis*

hijacks TLR2/TLR6 inducing IL-10 production that leads to DC tolerance, as an evasion mechanism[53]. However, *Y. pestis* infection also triggers DCs via TLR2/TLR1 leading to the production of IL-12 and T cell priming[53]. The authors showed that TLR2$^{-/-}$ mice had a significant decrease in IL-10 production as well as an increase in the production of IFNγ, suggesting that TLR2 may have a role as immunomodulator. Furthermore, TLR6$^{-/-}$ mice showed residual levels of IL-10 as well as decreased bacterial burden[53].

Nevertheless, absence of TLR2 does not always leads to a decreased bacterial burden. For instance, TLR2$^{-/-}$ mice showed increased susceptibility to infection *Salmomella*[54].

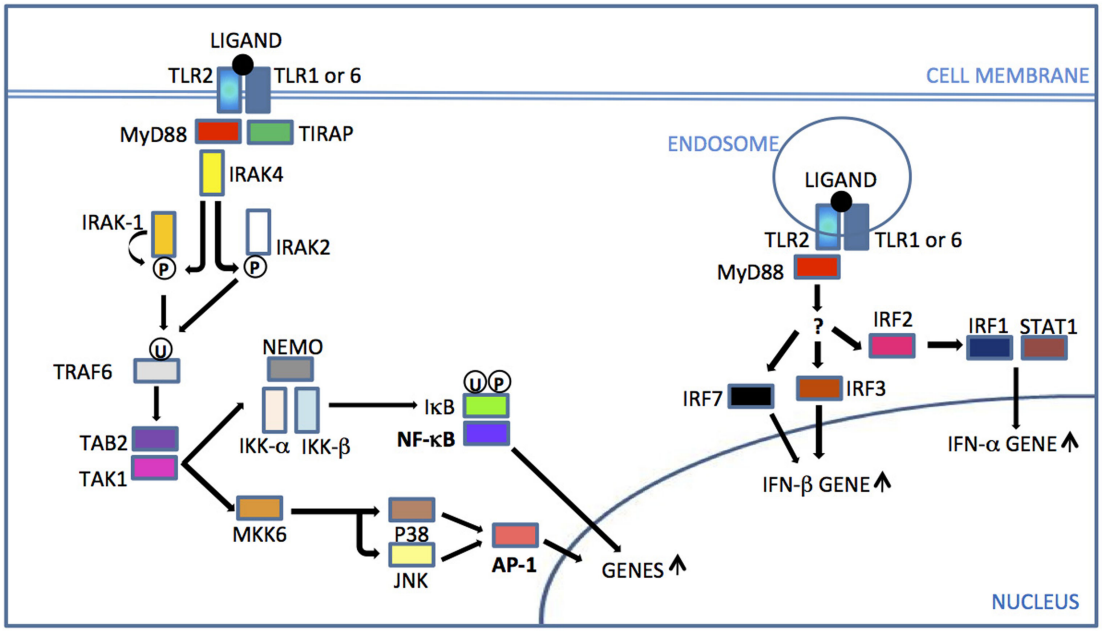

Figure 2: TLR2 signalling pathway. From Oliveira-Nascimento, L. et al; The role of TLR2 in infection and immunity; Frontiers in immunology; 2012.

Non-TLR activation of the innate immune responses

Several families of non-TLR PRRs have been described, including transmembrane proteins, as C-type lectin receptors (CLRs) and cytoplasmatic proteins, such as Retinoic acid-inducible gene (RIG)-I-like receptors (RLRs) and Nucleotide-binding oligomerization (NOD)-like receptors (NLRs)[55].

CLRs are transmembrane proteins that include Dectin-1, Dectin-2, manose receptor, C-type lectin receptor DC-SIGN and Mincle. CLRs are known to bind to carbohydrate structures present in fungal and bacterial cell walls, namely ß-glucan and mannan[56].

Three receptors have been identified in the RLR family, namely: RIG-I, melanoma differentiation-associated gene 5 (MDA5) and laboratory of genetics and physiology 2 (LGP2)[57]. RLRs recognize viral components in several cell types, triggering signalling cascades via Caspase-associated molecular pattern (CARD), resulting in the production of type I IFN and pro-inflammatory cytokines[57].

With 22 members, the NLR family best-characterized receptors are NOD1 and NOD2 that recognize structures of bacterial peptidoglycans, such as g-D-glutamyl-mesodiaminopimelic acid (iE-DAP) and muramyl dipeptide (MDP), respectively[58]. Upon ligand recognition, NOD1 and NOD2 signal through CARD leading to cytokine secretion[58].

Dectin-1

Dectin-1, a key receptor with critical biological functions upon infection, is a type II transmembrane NK-cell-receptor-like C-type lectins expressed in dendritic cells, macrophages, monocytes, neutrophils and a subset of T cells[59]. In contrast to other NK-cell-receptor-like C-type lectin, Dectin-1 receptor lacks cysteine residues in the stalk region, suggesting that the receptor can function as monomer[60]. Furthermore, Dectin-1 is self-possessed by an immunoreceptor tyrosine-based activation motif (ITAM) that mediates immune cellular activation[61].

Dectin-1 mediates the recognition of carbohydrates, specifically ß-1,3-linked and ß-1,6-linked glucans, as well as undefined ligands on T cells[60,62,63]. Engagement of Dectin-1 by ß–glucans activates internal signalling cascades that lead to cytokine and chemokine production, including TNF, CXC-chemokine ligand 2 (CXCL2, also known as MIP-2), IL-2, IL-6, IL-12 and IL-10[61,64-67].

ITAM-like motive phosphorylation mediates the triggering of the signalling cascade downstream of Dectin-1 through the recruitment of Raf-1 and tandem SH2-domain- containing protein spleen tyrosine kinase (SYK) [65]. Activation of Raf-1 kinase promotes gene transcription though NF-B recruitment[56]. SYK requirements differ among cell-types[60]. In macrophages SYK is required for respiratory burst induction, but not for phagocytosis[68]. DCs require SYK for Dectin-1 mediated IL-10 and IL-12 production, playing also a role in the dectin-1 mediated phagocytosis[65,69](Figure 3).

Recruitment of SYK activates downstream signalling pathways through CARMA-1-related adaptor protein CARD9, which binds to mucosal-associated lymphoid tissue lymphoma translocation protein (MALT)1 and Bcl-10 to promote IKK kinase complex activation that culminates in the activation of the transcription factor NF-κB[65,68,70]. Dectin-1/SYK signalling depends on CARD9 for

NF-κB activation[70]. CARD9$^{-/-}$ DCs are impaired to produce TNFα upon zymosan stimulation, whereas TLR stimulation is not affected. Moreover, IL-6 and IL-2 production is considerably decreased in CARD9$^{-/-}$ DCs upon infection with *C. albicans* [70] Consequently, CARD9$^{-/-}$ mice are more susceptible to infection, with higher *C. albicans* burden[70].

Several authors report the involvement of MAP kinases phosphorylation, such as ERK[67,71] in the Dectin-1 downstream signalling cascade.

There are some evidences that suggest that Dectin-1 can induce intracellular signalling through a non-SYK signalling pathway, either in macrophages and DCs, however it requires the involvement of collaborative signalling from TLRs[65,72], which is further detailed in the next section.

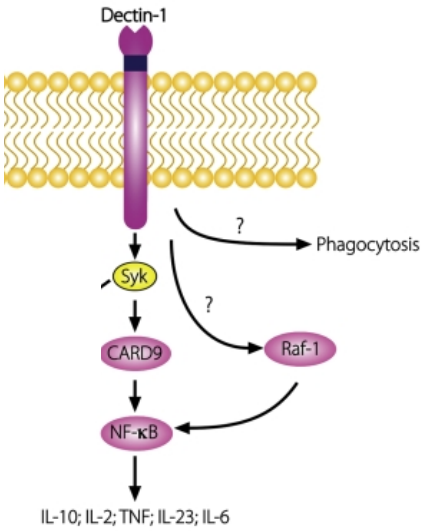

Figure 3: Dectin-1 signalling Pathway. Adapted from Reid, D. et al; Pattern-recognition receptors: recent insights on Dectin-1; Current Opinion in Immunology, 2009

Cooperation between Dectin-1 and TLR 2

Complex microorganisms present an extensive diversity of PAMPs that may be recognized by different PRRs, thus orchestrating the innate immune response.

The molecular basis underlying the cooperation between Dectin-1 and TLR2 for the induction of several cytokines had been subject of investigation. It is known that both receptors collaborate to induce optimal cytokine responses in macrophages and dendritic cells. Moreover, SYK and CARD9 induced signalling by Dectin-1 is required for this collaboration with TLR2[73,74]. Underhill and colleges showed that in macrophages and dendritic cells dectin-1 and TLR2 triggering

synergistically led to the production of IL-12 and TNF[64]. This observation was further corroborated for macrophages infected with *Mycobacterium smegmatis*, Bacillus Calmette-Guérin (BCG), *Mycobacterium avium* and *M. tuberculosis*[75]. Moreover, the synergistic effect between Dectin-1 and TLR2 can lead to higher induction of IL-23, IL-6 and IL-10 production in dendritic cells, when compared to TLR2 activation alone[76].

Cytokines

In response to PRR activation, innate immune cells produce several cytokines. These cytokines are important mediators of innate responses, as well as key molecules for the induction of the acquired immune response. Also, IL-10, an anti-inflammatory cytokine, has a prominent function in regulating the immune response, preventing inflammatory and autoimmune pathologies[24].
In this thesis special attention was given to IL-10 and IL-12 family cytokines. For this reason, these molecules are introduced in more detail below.

Interleukin-10

Interleukin-10 (IL-10) is an anti-inflammatory cytokine that exerts a suppressive action on immune cell activation, controlling the pro-inflammatory cytokine production with repercussions on the activation and intensity of the acquired immunity[77]. IL-10 belongs the IL-10 cytokine family, which is located in chromosome one, and also encompasses IL-19, IL-20 and IL-22[78,79].
IL-10 is expressed by innate and acquired immune cells, as dendritic cells, macrophages, mast cells, NK cells, eosinophils and neutrophils and T cells subsets as T_H1, T_H2, T_H17, T_{Reg}, $CD8^+$ T cells and B cells[24]. Excess of IL-10 can limit or subvert an immune response, however normal levels of IL-10 proved to be extremely important in preventing autoimmune reactions and protecting against diseases that result from exaggerated inflammatory responses[80]. Moreover, IL-10 abrogation, in the context of an immune response, can result in an increased extent of immune pathology, mainly related with tissue damage[81,82].
IL-10 activity is mediated through IL-10 receptor (IL-10R) binding, a transmembranar protein formed by 2 subunits, IL-10R1 and IL-10R2, being IL-10R2 an accessory molecule for signalling[83].

Upon IL-10 binding, signal transduction is initiated through Janus kinase (JAK)/ STAT[83,84]. The IL-10/IL-10R binding leads to the phosphorylation of JAK1, expressed by IL-10R1, and Tyk2, expressed by IL-10R-2. Tyrosine phosphorylation will thereafter activate STAT3 and STAT1 for cytokine and chemokine production[83].

Regulation of IL-10 production by innate immune cells

IL-10 production needs to be finely regulated, as excessive IL-10 expression can limit the immune response, whereas absence of IL-10 can lead to immunopathology[24].

Among the several layers for IL-10 regulation, one of them involves chromatin remodelling that enhances or silences *Il10* gene expression[24]. The mouse *Il10* locus in macrophages, DCs and T cells is composed by several DNaseI hypersensitive sites (HSSs)[85-87]. Saraiva et al identified, in macrophages and DCs, a decondensed and hyperacetylated HSS-4.5 that suggested to be a transcriptionally active element[85]. Besides chromatin remodelling, epigenetic imprinting of the *Il10* locus is associated with high levels of IL-10 production seen either in T_H2 cells and macrophages[85,88]. GATA binding protein 3 (GATA3) was suggested to be the initiator of chromatin remodelling and histone acetylation in mice *Il10* locus in T_H2 cells[85]. Since macrophages, DCs and other IL-10 producing T_H cells do not express higher levels of GATA3, it is possible that chromatin remodelling is induced by specific mechanisms depending on the cell type[85].

Macrophages and DCs differ in their expression of PRRs, which translates into a different awareness to products derived from pathogens and into differential activation of signalling cascades. As a result, the expression of cytokines, including of IL-10, varies between these two cell types. As for the signalling cascades induced upon PRR triggering in these cells and regulating IL-10 expression, a major attention has been dedicated to MAPK. There are three main MAPKs involved: ERK, p38 and JNK[24]. Indeed, Boonstra et al showed that IL-10 production differs in macrophages and DCs upon TLR9 ligand CpG activation, a difference that could be related to distinct activation of signalling cascades[89]. Kaiser et al defined a direct relationship between the strength of ERK activation and the production of IL-10[90]. Macrophages, due to a higher activation of ERK, showed a greater production of IL-10. The same reasoning applies to DCs, which show lower ERK activation upon TLR triggering, and which express intermediate levels of IL-10 and to pDC where no ERK activation, nor IL-10, were detected after TLR stimulation[90]. These findings are corroborated with studies in tumour progression locus 2 (TLP2) and NF-κB (precursors of ERK

activation) deficient cells [90,91]. ERK-independent mechanisms can also prime IL-10 production. Activation of p38 upon TLR recognition leads to increased expression of IL-10. p38 activation contributes for IL-10 expression upon CpG stimulation, an agonist of TLR9, and is present in signalling pathways of macrophages and DCs[90]. p38 is activated concomitantly with ERK but their signalling cascades do not relate to each other. It is likely that the two signalling pathways induced by ERK and p38 cooperate for maximal IL-10 production[92,93]. As mentioned before, the downstream activation of NF-κB and ERK in DCs triggering through Dectin-1 is required for IL-10 production in these cells.

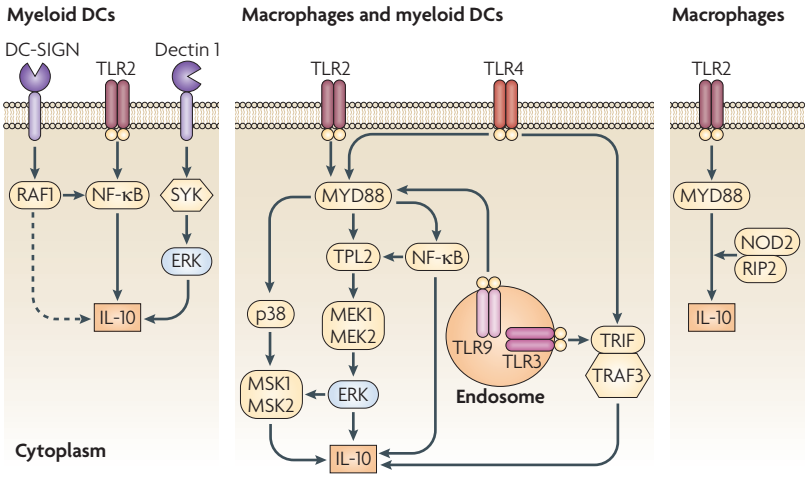

Figure 4: IL-10 regulation by innate immune cells. From Saraiva, M. and O'Garra, A.; The regulation of IL-10 production by immune cells; Nature Reviews Immunology; 2010.

The regulation of IL-10 expression also implies inhibitory processes. IFNγ promotes a negative regulation of IL-10 by acting directly through inhibition of ERK and p38, or indirectly by inducing compounds such as glycogen synthase kinase 3 (GKS3) which subsequently inhibit the binding of AP1 to the IL-10 promoter, resulting in IL-10 transcription inhibition[94,95].

Another layer of regulation of IL-10 gene expression relies on the activation of specific transcription factors[24]. Humans and mice present a high level of homology in the IL-10 promoter, namely in certain putative binding sites for transcription factors[24]. Specific protein 1 (SP1)[96], SP3[97], CCAAT/enhancer binding protein-ß (C/EBPß)[98], IRF1[99] and STAT3 have been suggested to bind and transactivate IL-10 in macrophages and T cells, either in human and mouse cell lines. Moreover, it was showed that NF-κB p50 subunit binds to the IL-10 promoter in a human T cell lymphoma cell line[100].

Finally, post-transcriptional control of IL-10 can be induced by p38 activation, which associated with inactivation of Tristetrapolin (TTP), an RNA-destabilizing factor that enables degradation of IL-10 mRNA by triggering the gathering of RNA decay machinery[101-104]. We have recently shown that differential TLR stimulation can also lead to a differential rate in the *Il10* mRNA degradation. Compared to TLR2-stimulated macrophages, TLR4 stimulated cells show an increased *Il10* mRNA stability, which was dependent on TRIF-mediated activation of the p38 signalling pathway (Teixeira-Coelho, M.; Guedes, J. et al, submitted).

IL-10 mRNA can also be a target of miR-106, which negatively regulates the IL-10 expression, miR-466l that up-regulates IL-10 expression by antagonizing the TTP-mediated IL-10 degradation and miR-21 that positive regulates IL-10 expression by repressing the mRNA encoding programed cell death 4 (PDCD4)[105-107]. Thus, IL-10 expression is also regulated at the post-transcriptional level by miRNA.

The Interleukin-12 family of cytokines

The IL-12 family encompasses heterodimeric cytokines, namely IL-12, IL-23, IL-27 and IL-35[108]. The cytokines of the IL-12 family are heterodimeric proteins that comprise a α-chain (p19, p28 or p35) and a ß-chain (p40 or ebi3)[109]. The p40 chain can heterodimerize either with p35, to form IL-12, or with p19, to form IL-23. Ebi3 can heterodimerize with p28 or p35 to form IL-27 or IL-35, respectively[108].

Due to the different biological functions of the bioactive molecules, IL-12 family cytokines are provided with positive and negative feedback control. In this sense, IL-12 and IL-23 have pro-inflammatory characteristics that impact T_H1 and T_H17 development, respectively[110-112]. On the other hand, IL-27 is an immunoregulatory cytokine and IL-35 is anti-inflammatory molecule that inhibits the development of T_{Reg}[113]. Therefore, positive regulation is achieved with IL-12 and IL-23 and negative feedback is regulated by IL-27 and IL-35[108,111].

The biological activity of IL-12, IL-23, IL-27 and IL-35 is induced upon interaction of each cytokine with its receptor through JAK/STAT phosphorylation[108]. The receptor chains are also shared among the IL-12 bioactive molecules. IL-12 signals upon binding to the IL-12 receptor (IL-12R), with p40 interacting with IL-12Rß1, essential for high-affinity binding, and p35 interacting with IL-12Rß2 that is required for signal transduction[108]. IL-23 signals via IL-12Rß1 and IL-23R, being IL-12Rß1 required for high-affinity binding and IL-23R for the signal transduction[108]. IL-27 signals via gp130

and IL-27R, also known as WSX-1, for functional signal transduction[114]. IL-35 signal via IL-12Rß2-gp130 or can signal through homodimerization of gp130 or IL-12Rß2[115]. Receptor chain sharing and competition for binding could help to maintain IL-12 family immune balance.

The expression of the IL-12 family monomers is tightly controlled at the transcriptional level. p40 transcriptional regulation has been the subject of extensive studies. It is known that p40 is tightly regulated by multiple factors, including NF-κB[116], IRF-1[117,118], IRF5[119], IRF-8[117], PU.1, C/EBP[120], AP-1[121] and Rel proteins[120]. IRF1[118,122], IRF3[123], IRF5[119] represent key factors in the regulation of p35 expression. p19 subunit was found to be negative regulated by RelA[124,125] and c-Rel[125,126] and positively regulated by p52[125], all members of the NF-κB family. Additionally, p19 is also regulated by IRF5[119], ATF-2[127], SMAD-3[127] and AP-1[128]. A lot less is known about p28 and ebi3 transcriptional regulation. Expression on p28 can be induced via IRF1 and IRF3[129]. Upon TLR stimulation, NF-κB and PU.1 transcription factors promote the transactivation of ebi3 subunit[130].

As referred before, IL-12 is a pro-inflammatory cytokine produced by macrophages, dendritic cells and B cells[131]. IL-12 promotes IFNγ production by activated T cells, constituting a positive feedback loop for IL-12 production that facilitates T_H1 differentiation[132]. As IL-12, IL-23 is a pro-inflammatory cytokine predominantly expressed by dendritic cells and phagocytic cells[112]. IL-23 expression can be augmented through T cell CD40 interactions, establishing a positive feedback response that enhances IL-23 expression[112]. IL-23 stabilizes IL-17 expression and T_H17 differentiation, however, IL-23 is not the differentiation factor for the T_H17 phenotype[133]. IL-27 is an immunomodulatory cytokine produced by antigen-presenting cells. IL-27 induces T_{Reg} cell population development (Tr1 and iTr35) that limits T_H1, T_H2 and T_H17 responses through IL-10 production[134,135]. In combination with IL-2 or IL-12, IL-27 can exhibit stimulatory effects by inducing IFNγ producing T cells[108]. Moreover, IL-27 can activate STAT1 and that transcription factor T-bet that sensitizes T cells to respond to the signal that facilitates T_H1 responses[136].

IL-35 is specifically produced by T_{Reg} cells and mediates the suppression of T cell proliferation[113]. IL-35 can generate iTr35 cells and mediates their suppression exclusively via IL-35[137].

It is well established that IL-10 reciprocally regulates the expression of IL-12 and IL-23 members[138,139]. The negative regulation that IL-10 exerts on IL-12 production impacts T_H1 cell differentiation[140]. Moreover IL-10 can inhibit T_H17 differentiation through negative regulation of the IL-23 expression[141]. In fact, IL-$10^{-/-}$ mice, present high levels of IL-12 and IL-23 expression that promote chronic inflammation, which leads to the development of enterocolitis[142].

Overall, the immune balance is thus established via the differential production of cytokines over time and in different anatomical locations and also via the function of IL-10. Understanding how these various events are regulated will contribute to uncover targets for immune modulation, with an impact in infectious and auto-immune diseases.

Aims

The equilibrium between pro- and anti-inflammatory cytokines is important to maintain a balanced immune response, preventing immunopathology. IL-10 is an anti-inflammatory cytokine, produced among several cell types, that regulates immune cellular activation and expression of pro-inflammatory cytokines. Understanding the molecular mechanisms underlying the regulation of IL-10 expression in macrophages and dendritic cells is of major importance, considering that IL-10 exerts a negative control over cell activation and cytokine production. Upon infection, triggering of PRRs by PAMPs is a major contributor to the production of IL-10 by innate immune cells. However, the molecular mechanisms that are involved in the tight regulation of IL-10 expression by these cells need to be better understood. Thus, in the first results chapter of this thesis we aimed at:

- Understanding how differential TLR versus non-TLR activation, impacts IL-10 production in macrophages and dendritic cells;
- How differential IL-10 expression by PRR-activated macrophages and DCs can be modulated by the distinct activation of MAPKs;
- Investigating the mechanisms underlying multiple PRR activation in the regulation of IL-10 by dendritic cells.

Reciprocal regulation of IL-12 and IL-23 by IL-10, either in macrophages or in dendritic cells, is also of major importance to the development of the immune response, since this family of cytokines impacts the differentiation of T helper cells and thus the acquired immune response. Thus, in the second part of the results presented in this thesis, we aimed at:

- Understanding the differential regulation of the expression of all IL-12 family members in TLR-stimulated macrophages versus DCs based on their distinct IL-10 production;
- Investigating how IL-10 impacts the transcription of all monomers of the IL-12 family upon TLR stimulation of macrophages and DCs.

MATERIALS AND METHODS

Mice

C57BL/6 WT and IL-10 deficient mice were purchased from Charles River (Barcelona, Spain), TLR2 deficient mice derived from a C57BL/6 background (kindly provided by Anne O'Garra, NIMR) were maintained in a closed breeding colony at the ICVS facilities. Bone marrow was extracted from age (8 – 12 week old) and sex matched mice. Minimums of 2 animals per strain were used for each experiment to overcome cell variability. Animals were euthanized by CO_2 poisoning. All protocols were performed according to the European Union Directive 86/609/EEC, and were previously approved by the national authority *Direcção Geral de Veterinária*.

Reagents

All reagents were prepared as indicated by each company and used at the following concentration, unless otherwise is stated. Pam_2CSK_4 (Invivogen) and Curdlan from *Alcaligenes faecalis* (Invivogen) were used at 100ng/mL. Pam_3CSK_4 (Invivogen) was used at 2μg/μL. Zymosan (Invivogen) was used at 100μg/mL. CpG (Invivogen) was used at 2μM and Poly I:C (Invivogen) was used at 20μg/mL. LPS *Salmonella Minnesota* (Sigma) was used at 25ng/mL. ERK (SB0203580) and p38 (PD0325901) inhibitors were a kind gift from Anne O'Garra, NIMR and were used at 2,5μM and 0,1μM, respectively.

Bone marrow derived macrophages (BMDM)

Bone marrow cells were flushed from mice femurs and tibiae with complete RPMI (cRPMI) medium [containing 10% of Fetal Bovine Serum (FBS; Gibco), 1% Sodium pyruvate 100nM (Gibco), 1% HEPES 1M (Gibco), 1% L-glutamine (Gibco) and 0,05mM 2-mercaptoethanol (2-ME; Sigma)]. Bone marrow cells were cultured in cRPMI supplemented with 20% L929-cell-conditioned medium (LCCM) in 8-cm plastic petri dishes (Sterilin) in 8 mL at a density of 0.5 x 10^6 cells/mL. On the day 4 of differentiation, 10 mL of supplemented medium was added to the cultures and at day 7 adherent cells were harvested, counted and stimulated as appropriately.

Bone marrow derived dendritic cells (BMDC)

Bone marrow cells were flushed from mice femurs and tibiae and cultured in cRPMI medium supplemented with 20% granulocyte-macrophage colony-stimulating factor (GM-CSF) in T75 cm^2 flasks in 25 mL at a density of 2 x 10^5 cells/mL. At day 3 and day 6, 25 mL of supplemented medium was added or replaced, respectively. At day 8 cells were harvested, counted and stimulated as appropriately.

Cell stimulation for cytokine production

BMDM and BMDC were harvested, re-suspended in 5% FBS cRPMI and the number of cells was adjusted to 0.5 x 10^6 cells/well in a 24-well plate. Cells were stimulated and cytokine release was measured after 6 and/or 24 hours post incubation at 37 ºC and 5% CO_2 by specific commercial ELISA kit for IL-10, IL-6 and TNF (all from eBioscience), according to the manufacturer's instructions.

mRNA stability assay

BMDMs and BMDCs were plated in 24 well plates at 1x10^6cells/mL in a final volume of 500µL and stimulated with TLR agonists as previously described. The mRNA stability was determined with the addition to the cell culture medium of Actinomycin D (ActD) (Gibco, Invitrogen), at a final concentration of 10 µg/ml. ActD is an antibiotic that binds to DNA, immobilizing the associated transcription complex, by interfering with the elongation of RNA. Thus, upon addition of ActD the transcription process is stopped and by measuring the amount of RNA present in the culture post-ActD addiction we can access the mRNA degradation rates. The cells for total RNA extraction were recovered in TRIzol® reagent immediately before ActD treatment and 30, 60 and 90 minutes after treatment. The mRNA stability data analysis was performed by calculating the percentage of mRNA transcripts present at each time point after ActD addition, considering 100% the level of mRNA expression at 0 minutes before the addition of ActD.

Quantitative Real-time PCR analysis

BMDM and BMDC were cultured in 24-well plates at a density of 0,5 x 10^6 cells/well. After stimulation, RNA was extracted using TRIzol® Reagent (Sigma), according to the manufacturer's instructions. Total RNA was reversed transcribed using Reverter AidTH H Minus Strand cDNA Synthesis kit (Fermentas). The resultant cDNA template was used for quantification of target genes by real-time PCR analysis with SYBR green detection system. Ubiquitin or HPRT were used as housekeeping genes. Each primer was used at a final concentration of 0.4μM. SYBR Green based reactions were performed according to the following parameters: 95ºC for 15 minutes, 39 cycles of 95ºC for 15 seconds, 58ºC for 20 seconds, and 70ºC for 15 seconds, followed by 65ºC for 5 seconds and 95ºC for 5 seconds. TaqMan based reactions were performed according to the following parameters: 50ºC for 2 minutes, 95ºC for 10 minutes, 40 cycles of 95ºC for 15 seconds and 60ºC for 1 minute, followed by 60ºC for 1 minute.

The following primers were used:

SYBR Green primers	
Ubiquitin sense	5'-TGGCTATTAATTATTCGGTCTGCAT-3'
Ubiquitin antisense	5'-GCAAGTGGCTAGAGTGCAGAGTAA-3'
IL-10 sense	5'-TTTGAATTCCCTGGGTGAGAA-3'
IL-10 antisense	5'-GCTCCACTGCCTTGC-TCTTATT-3'
TNF sense	5'-GCCACCACGCTCTTCTGTCT-3'
TNF antisense	5'-TGAGGGTCTGGGCCATAGAAC-3'
IL-6 sense	5'-ACACATGTTCTCTGGGAAATCGT6-3'
IL-6 antisense	5'-AAGTGCATCATCGTTGTTCATACA-3'

TaqMan primers	
HPRT	Uni gene ID: Mm.299281
IL12a	Uni gene ID: Mm.103783
IL23a	Uni gene ID: Mm.125482
IL-27	Uni gene ID: Mm.222632
Ebi3	Uni gene ID: Mm.256798

Table 1: Primers

Biological triplicates for each condition were run. Different genes were analysed in the same run to avoid inter-run variations. Relative gene expression was determined using the ΔCt calculation method, were the amount of target, normalized to the housekeeping gene Ubiquitin, is given by $1{,}8^{\Delta Ct}x100000$, where Ct is the cycle number of detection threshold.

Cell-lysate preparation and Western blot analysis

BMDM and BMDC were cultured in a 24-well plate at a density of 1 x 10^6 cell/mL/well in 1%FBS cRPMI and rested during 5 hours before stimulation. At each time point, post stimulation, cells were washed in ice-cold apyrogenic PBS and resuspended in lysis buffer (containing 100mM Tris-HCL pH8, 10% glycerol, 1mM EDTA pH8, 5mM $MgCl_2$, 50mM NaCl, 1%NP-40, dH_2O) and a proteinase inhibitor cocktail (Sigma). Cell lysates were boiled at 95ºC during 5 minutes. Protein samples were electrophoresed in a 12% SDS polyacrylamide gel and transferred onto Trans-Blot® Turbo™ Transfer System (Bio-Rad). Membranes were blocked in 5% low fat milk PBS-Tween 0.1% washed in PBS-Tween 0.1% and incubated for overnight at 4ºC with primary antibody, washed and incubated for 1 hour at room temperature with HPR-labelled anti-rabbit secondary antibody (Cell Signalling). Anti-p-p38 and anti-p38 total were purchased from Cell Signalling Technology; anti-p-ERK and anti-ERK total were purchased from Invitrogen. Quantity One 1-Danalysis Software version 4.6.9 for Mackintosh (Bio-Rad) was used for image quantification.

Statistical analysis

Quantitative data were represented in terms of mean ± SEM. Statistical analysis was performed using GraphPad Prism version 5.00 for Mackintosh (GraphPad Software, www.graphpad.com). Data from independent experiments was analysed by comparison to a defined control value using two-tailed Student's T-tests for two-group analysis and Two-way ANOVA with multiple comparisons for grouped analysis. p values <0.05 were considered significant ($^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$).

RESULTS – PART I

Differential IL-10 expression by BMDM and BMDC upon TLR2 activation

Previous data from our laboratory provided initial evidence for a differential regulation of IL-10 induction in macrophages versus dendritic cells responding to TLR2 stimulation. In particular, the profile of IL-10 secretion by bone-marrow derived macrophages (BMDM) and bone-marrow derived dendritic cells (BMDC) upon infection with *M.* tuberculosis was different. To further investigate the molecular bases underlying these observations, BMDM and BMDC were stimulated with Pam_3CSK_4 (a TLR2/TLR1 pure ligand). Induction levels of *Il10* mRNA were assessed by RT-PCR at 30 minutes, 1, 3, 6 and 24 hours post Pam_3CSK_4-stimulation (Figure 1A). At 24 hours post stimulation supernatants were collected and IL-10 production was measured by ELISA (Figure 1B). Our data showed that BMDM and BMDC display a distinct kinetics of *Il10* mRNA induction. 30 minutes post stimulation we observed a 566-fold increase of *Il10* mRNA in BMDM compared to BMDC, and those high levels of expression were maintained until 1 hour post-stimulation. At 3 hours we observed a decay in the *Il10* mRNA in BMDM and that was maintained throughout the time (Figure 1A). BMDC showed a continuous induction of *Il10* mRNA until 6 hours post Pam_3CSK_4-stimulation, when the peak of expression was achieved, albeit at levels that were much lower than those observed in BMDM (Figure 1A). The distinct *Il10* mRNA kinetics between BMDM and BMDC had an impact on the IL-10 protein production, with higher quantities being produced by BMDM and consistently poor to bellow detection limit in TLR2/TLR1-stimulated BMDC (Figure 1B). An initial interpretation of the data was that BMDC were not responding to TLR2/TLR1 triggering. To further investigate this hypothesis, we measured pro-inflammatory cytokine expression and production, namely IL-6 and TNF, upon stimulation of BMDM and BMDC with Pam_3CSK_4. In contrast to *Il10*, BMDC showed a quicker induction of *Il6* mRNA after Pam_3CSK_4-stimulation than BMDM, however both cell types achieved the peak of expression at 6 hours post stimulation (Figure 1C). Both BMDC and BMDM produced IL-6, although BMDC produced significantly higher quantities of this cytokine than macrophages (Figure 1D). Moreover, compared to BMDM, BMDC achieved a quicker but lower peak of *Tnf* mRNA expression at 1 hour post Pam_3CSK_4 stimulation (Figure 1E) that likely contributed to an significantly higher TNF production (Figure 1F).

Based on these observations we concluded that TLR2/TLR1 triggering in BMDC induces a pro-inflammatory cytokine profile that favors IL-6 and TNF over IL-10 production. In contrast, TLR2/TLR1 activation in BMDM favored IL-10 induction.

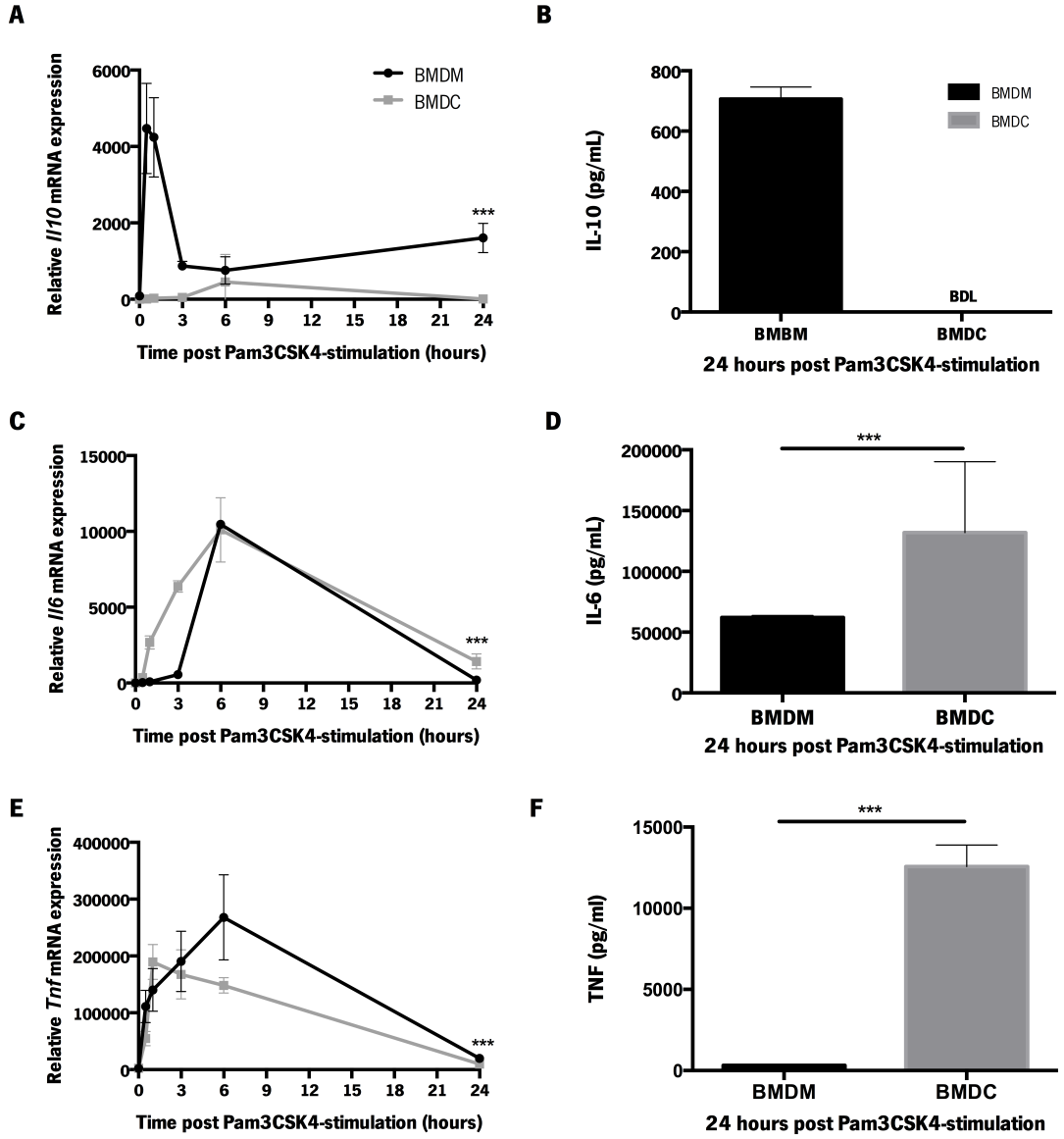

Figure 1: Differential IL-10 expression upon TLR2/TLR1 stimulation in BMDM and BMDC. WT BMDM (black) and BMDC (grey) were stimulated with Pam_3CSK_4 – TLR2/TLR1 ligand – and the relative mRNA induction for *Il10* (A), *IL6* (C) and *Tnf* (E) was assessed by RT-PCR at the indicated time points. All samples were normalized to ubiquitin. Cell culture supernatants were collected at 24 hours post stimulation and cytokine production was measured by ELISA for IL-10 (B), IL-6 (D) and TNF (F). Data represents mean ± SD of triplicates; *** $p \leq 0.001$. Data is representative of two independent experiments. BDL=bellow detection limit.

Lack *Il10* mRNA induction by TLR2 in BMDC is not dependent on the ligand dose, nor on the heterodimerization of TLR2 with TLR1 or TLR6

Although in the previous section we demonstrated that lack of IL-10 induction in BMDC stimulated via TLR2/TLR1 was not due to a general poor triggering of this TLR, we still considered that the strength of the TLR activation may not have been enough for IL-10 expression. We next compared

the response of WT BMDM and BMDC stimulated with increasing doses of Pam_3CKS_4, ranging from 0.5 to 10 μg/mL. Cell supernatants were collected at 24 hours and cytokine production was measured by ELISA. Our data showed that BMDM produced considerable amounts of IL-10 from the lower to the highest concentrations of Pam_3CSK_4 used, in opposite to what happened to BMDC, in which residual amounts of IL-10 were detected only at the higher concentrations of ligand (Figure 2A). As before, BMDC responded to all doses of ligand with IL-6 and TNF production, as did BMDM (Figure 2B and 2C). Taken together these results suggest that upon TLR2/TLR1 stimulation, BMDC are not able to produce considerable levels of IL-10.

It is known that TLR2 can dimerize either with TLR1 or TLR6[33,36]. We investigated if the lack of IL-10 production by BMDC was dependent on the type of TLR2 dimerization. For that, WT BMDC were stimulated with Pam_3CSK_4 (TLR2/TLR1 ligand) or Pam_2CSK_4 (TLR2/TLR6 ligand). IL-10 protein production was measured by ELISA, 24 hours post stimulation (Figure 2D). Our data showed that the amounts of IL-10 produced by BMDC upon TLR2 stimulation are consistently low, independently of the heterodimerization of TLR2 with TLR1 or TLR6. In all, our data suggest that BMDC are poor producers of IL-10 in response to TLR2.

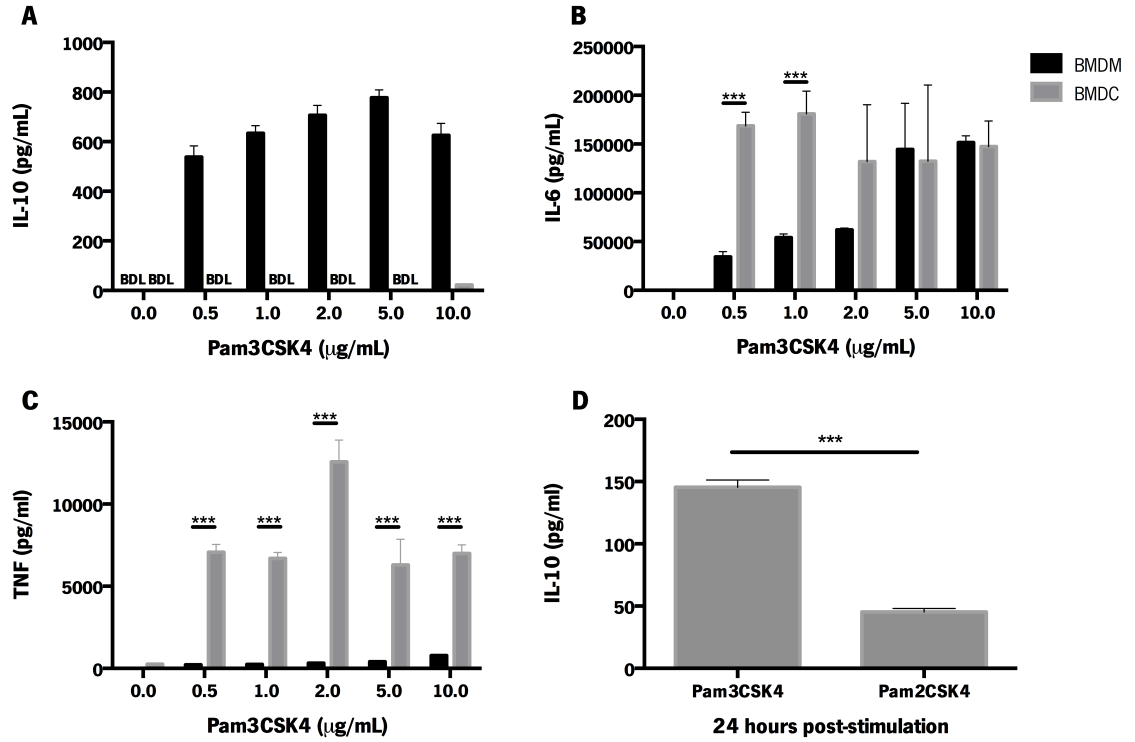

Figure 2: Cytokine production by BMDC and BMDM upon stimulation with Pam_3CSK_4 increasing doses. WT BMDM (black) and BMDC (grey) were stimulated with the indicated doses of Pam_3CSK_4 (TLR2/TLR1 ligand) and at 24 hours post-stimulation cytokine production was determined by ELISA for IL-10 (A), IL-6 (B) and TNF (C). WT BMDC (Grey) were stimulated with Pam_3CSK_4 (TLR2/TLR1 ligand) or Pam_2CSK_4 (TLR2/TLR6 ligand). Supernatants were collected at 24 hours post-stimulation and IL-10 production was measured by ELISA (D). Data represents mean ± SD of triplicates; *** $p \leq 0.001$. Data is representative of two independent experiments. BDL=bellow detection limit.

TLR activation of BMDM and BMDC leads to distinct *Il10* kinetics profiles

Next, we tested whether other TLR-ligands induced IL-10 production in BMDC. We stimulated BMDM and BMDC with Pam_3CSK_4 as a control or with LPS (TLR4 ligand) or CpG (TLR9 ligand). A kinetic assay was performed and induction levels for *Il10* mRNA were assessed by RT-PCR at 0, 1, 3 and 6 hours post-stimulation (Figure 3A and 3B). Supernatants were collected at 24 hours post-stimulation and IL-10 protein was measured by ELISA (Figure 3C and 3D). Our data showed that independently of the stimuli, BMDM showed a peak of *Il10* mRNA induction at 1 hour post stimulation that is followed by a rapid decrease (Figure 3A). As for BMDC, only a modest induction of IL-10 expression was observed independently of the TLR stimulation, which was accompanied by low IL-10 secretion (Figure 3B and 3D).

Our data suggest that IL-10 is differently regulated according to the cell type, and that within the same cell type, different TLR stimuli leads to differential IL-10 responses.

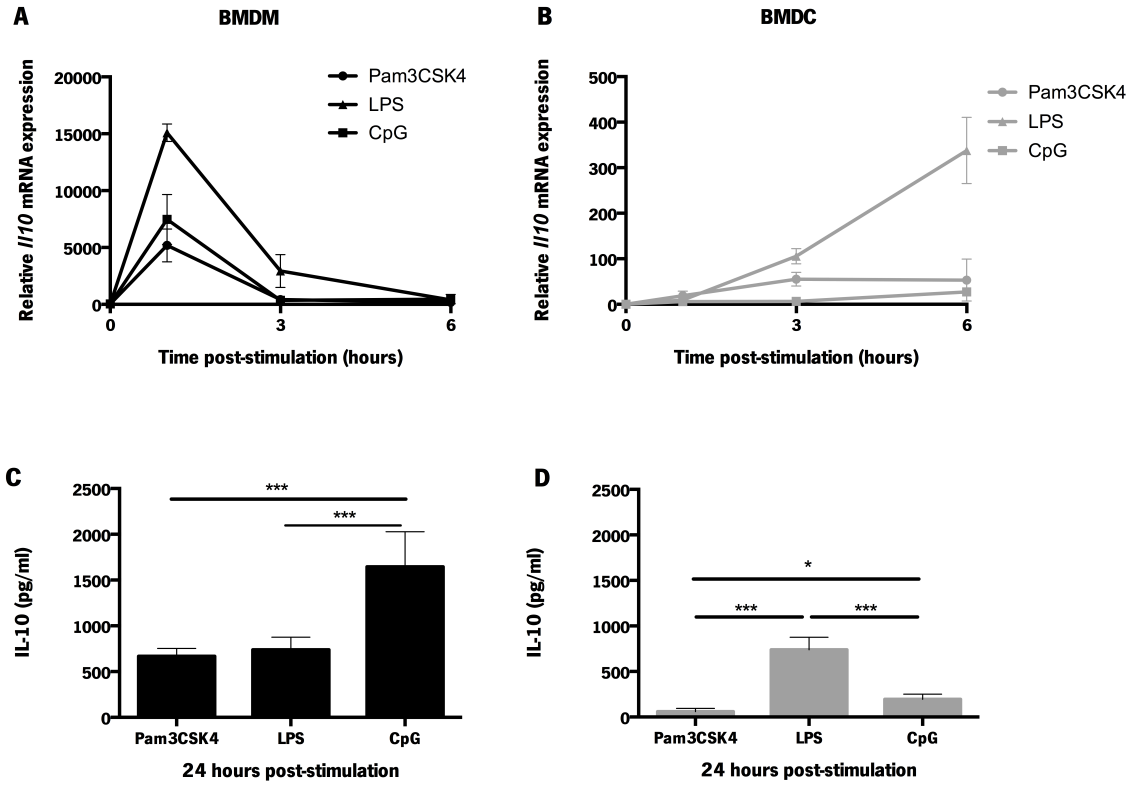

Figure 3: BMDC are poor IL-10 producers in response to TLR2, TLR4 or TLR9 signalling. WT BMDM (black) and BMDC (grey) were stimulated with Pam_3CSK_4, LPS or CpG for the referred time points. *Il10* gene expression was determined by RT-PCR (A,B). All samples were normalized to ubiquitin. For the same conditions, supernatants were collected at 24 hours post stimulation, for analysis of cytokine production be ELISA for BMDC (C) and BMDM (D). Data represents mean ± SD of triplicates; *** $p \leq 0.001$. Data is representative of one independent experiment.

Dectin-1 triggering induces IL-10 expression/secretion by BMDC

IL-10 induction in innate immune cells may also be triggered by non-TLR stimulation. According to the literature, Dectin-1 – ß-glucan receptor – is highly expressed in dendritic cells when compared to macrophages. Taking this into consideration, we compared the IL-10 production in BMDM and BMDC upon Dectin-1 triggering. For that, we stimulated BMDM or BMDC with different concentrations of Curdlan (Dectin-1 ligand) ranging from 2 to 500 μg/mL. IL-10 production was measured by ELISA, 24 hours post-stimulation. BMDC were more sensitive to Dectin-1 stimulation than BMDM, producing significantly higher amounts of IL-10 than BMDM (Figure 4). The amounts of IL-10 produced were dependent on the dose of Curdlan used to stimulate BMDM or BMDC. Our data thus indicate that BMDC are not generally impaired to produce IL-10, doing so in response to certain stimuli, such as Dectin-1 triggering.

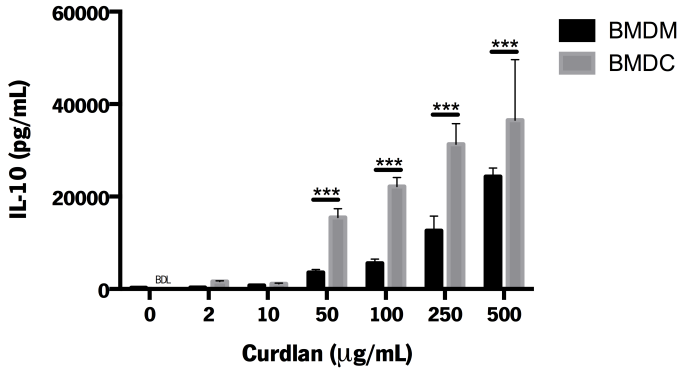

Figure 4: BMDC are more responsive to Dectin-1 signalling. WT BMDM (black) and BMDC (Grey) were stimulated with increasing doses of Curdlan and supernatants were collected 24 hours post cell stimulation. Cytokine production was tested by ELISA. Data represents mean ± SD of triplicates; *** $p \leq 0.001$. Data is representative of two independent experiments. BDL=bellow detection limit.

Triggering by Dectin-1 induces higher ERK activation in BMDC than TRL2 stimulation

Upon Dectin-1 and TLR2 triggering, several downstream signalling cascades are activated, culminating in MAPK activation[40]. Moreover, IL-10 production can be modulated by the activation of ERK and p38[24,90,143]. For these reasons, we investigated the activation of ERK and p38 in either BMDM or BMDC upon Dectin-1 or TLR2 stimulation. For that, WT BMDC and BMDM were

stimulated with Pam_3CSK_4 (TLR2/TLR6 ligand) or Curdlan (Dectin-1 ligand) during 0, 30, 60, 90, 120, 180 minutes. Cell lysates were recovered at each time point and protein analysis was accessed by western-blot. Our data showed that the activation of p38 occurred in both BMDM and BMDC upon TLR2 stimulation, although at an initially higher level for BMDM (Figure 5A). in contrast, ERK activation was only transiently detected in BMDM upon TLR2-stiomualtion, but at low levels (Figure 5B and 5C). Although the ERK activation triggered by this stimulus appeared faster in BMDM, in contrast to what was observed for TLR2 stimulation of BMDC, with Dectin-1 stimulation we were able to detect ERK activation for all time points tested (Figure 5E and 5F).

Considering the data on p38 and ERK activation, we hypothesized that ERK activation was key for IL-10 induction in BMDC. To test this hypothesis, WT BMDC were stimulated with Curdlan alone or in the presence or ERK or p38 inhibitors. Supernatants were recovered 24 hours post-stimulation and IL-10 production was measured by ELISA. Our data showed that IL-10 production by BMDC was abrogated in the absence of ERK (Figure 5E). Inhibition of p38 had no effect on the amount of protein produced by Dectin-1 stimulated BMDC (Figure 5E). thus, ERK activation is essential for IL-10 production in BMDC.

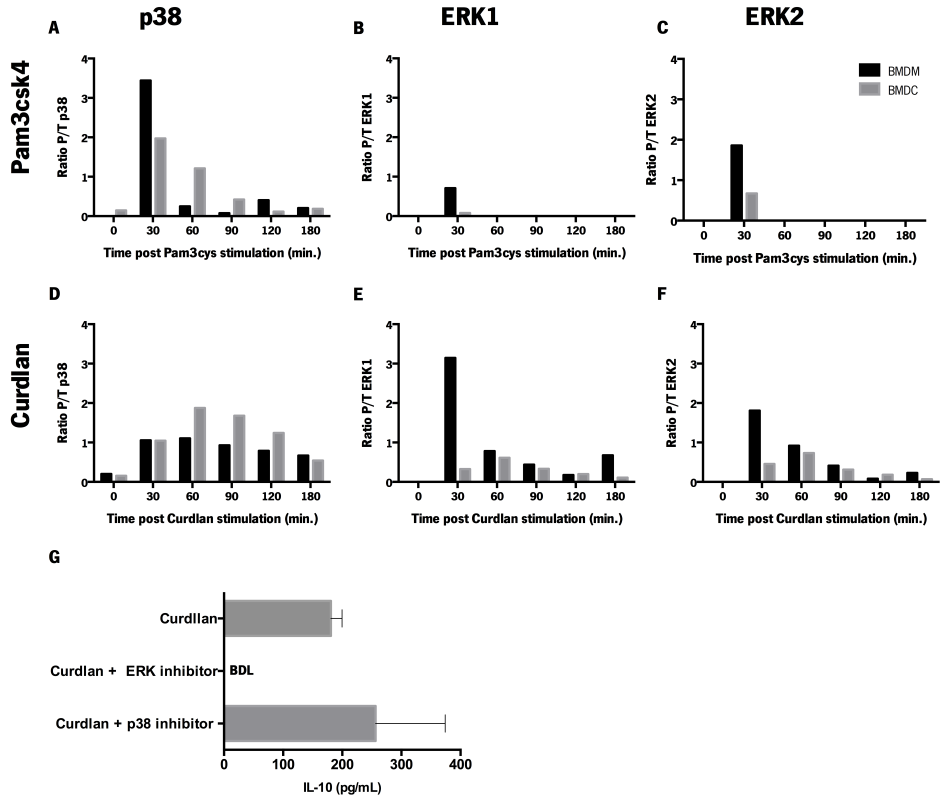

Figure 5: ERK activation is required for IL-10 production in BMDC. WT BMDM (black) and BMDC (grey) were stimulated with Pam_3CSK_4 (A-C) or Curdlan (D-E) and cell lysates were recovered at 0, 30, 60, 90, 120 and 180 minutes. ERK and p38 activation was accessed by western-blot. WT BMDC were stimulated with Curdlan in the presence or absence of ERK or p38 inhibitors (G). Cell supernatants were recovered 24 hours post-stimulation and IL-10 protein was measured by ELISA. Data is representative of two independent experiments.

Synergy between Dectin-1 and TLR2 promotes an inhibitory effect in the IL-10 production in BMDC

Previous studies suggest that Dectin-1 and TLR2 synergise for maximal IL-10 production in macrophages/dendritic cells[73]. Our data showing that high ERK activation was achieved in BMDC stimulated with Curdlan, whereas high p38 activation was observed in TLR2 stimulated BMDC may shed light into molecular mechanisms underlying such studies. Indeed ERK activation is known to be important for transcriptional induction of IL-10[90,144], whereas p38 activation contribute to the stabilization of *Il10* mRNA[103]. We therefore questioned if the stimulation of BMDC through Dectin-1 could rescue IL-10 production in BMDC stimulated via TLR2. For that, we stimulated BMDC with Curdlan (Dectin-1 ligand) and Zymosan (Dectin-1+TLR2/TLR6 ligand). Supernatants were collected 24 hours post stimulation and cytokine production was evaluated by ELISA. In line with the literature we observed that BMDC produced higher levels of IL-10 upon TLR2/Dectin-1 stimulation, as compared to Dectin-1 stimulation alone (Figure 6A). To further understand the impact of TLR2 in the collaborative response, we stimulated BMDC with a fixed dose of Pam_3CSK_4 in combination with increasing doses of Curdlan ranging from 0.25 to 100 μg/mL. In parallel WT BMDC were stimulated with the same doses of Curdlan alone. Protein production was determined by ELISA, 24 hours post stimulation. Our data showed that BMDC produced IL-10 protein in a dose dependent manner, either when stimulated with Curdlan alone or when stimulated with Curdlan in combination with Pam_3CSK_4 (Figure 6B). Interestingly, when TLR2/TLR1—Dectin-1 signal was triggered we saw a significantly reduction in the levels of IL-10 produced by BMDC, suggesting that TLR2/TLR1—Dectin-1 signalling has an inhibitory effect in the IL-10 production (Figure 6B). To better understand the molecular mechanisms underlying this differential regulation, we decided to investigate the transcriptional kinetics of *Il10* mRNA upon BMDC stimulation with Dectin-1, TLR2 or Dectin-1/TLR2. For that, BMDC were stimulated with Pam_3CSK_4 (TLR2/TLR1 ligand), Curdlan (Dectin-1 ligand) or both ligands combined. *Il-10* mRNA induction was measured by RT-PCR at 0, 1, 3, 6 and 24 hours post stimulation. Our data showed similar kinetics between BMDC stimulated with Curdlan alone and Curdlan combined with Pam_3CSK_4, (Figure 6C). As before, stimulation of BMDC with Pam_3CSK_4 alone led to poor induction of IL-10 (Figure 6C) and poor IL-10 production (Figure 6B).

Taken together, these results suggest that the differential IL-10 regulation of single Dectin-1 or combined Dectin-1/TLR2 stimulation is post-transcriptionally regulated. Since these data contrasts what has been shown by other authors[73], future experiments are required to explain the observed discrepancies. This is discussed in detail in the discussion section of this thesis.

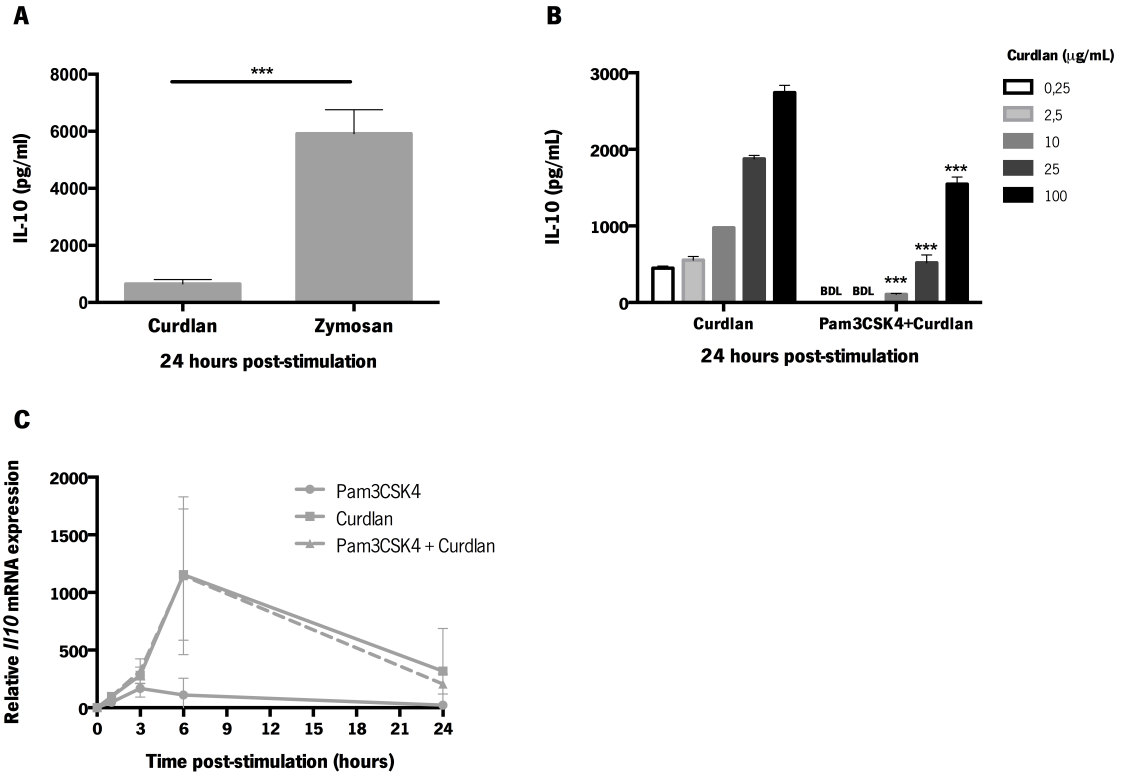

Figure 6: Inhibitory synergistic effect induced by TLR2 and Dectin-1 signalling. WT BMDC were stimulated with Curdlan or Zymozan (A). WT BMDC were stimulated with increasing doses of Pam_3CSK_4 or Curdlan (B). IL-10 production was measured by ELISA at 24 hours post cell stimulation. BMDC were stimulated with Pam_3CSK_4 alone, Curdlan alone or Curdlan in association with Pam_3CSK_4. *Il10* gene expression was measured at the referred time points by RT-PCR. (C) All samples were normalized to ubiquitin. Data represents mean ± SEM of triplicates; *** $p \leq 0.001$. Data is representative of two independent experiment. BDL = bellow detection limit

Results – Part II

BMDM secrete earlier and increased IL-10 than BMDC in response to TLR stimulation

As mentioned in detail before, IL-10 is an anti-inflammatory cytokine known to regulate the expression of several pro-inflammatory cytokines upon infection, avoiding immunopathology[24]. Among them, certain cytokines of the IL-12 family have been described to be directly regulated by the expression of IL-10.

Data described in the previous chapter showed that IL-10 is differently expressed in TLR-stimulated BMDM and BMDC. We now started by investigating how different TLR stimuli influence the kinetics IL-10 production by BMDM and BMDC. WT BMDM and BMDC were stimulated with Pam_3CSK_4 (TLR2 ligand), Poly I:C (TLR3 ligand), LPS (TLR4 ligand) or CpG (TLR9 ligand). At 1, 3, 6 and 24 hours post stimulation supernatants of the stimulated cultures were recovered and IL-10 production was measured by ELISA. In line with our previous observations and with the literature, TLR-stimulated BMDM displayed significantly higher levels of IL-10 than BMDC (Figure1). Moreover, for all TLRs tested BMDM showed an earlier production of IL-10 than BMDC (Figure1).

In summary, our data showed that upon TLR stimulation, BMDM produced earlier and enhanced levels of IL-10 than BMDC.

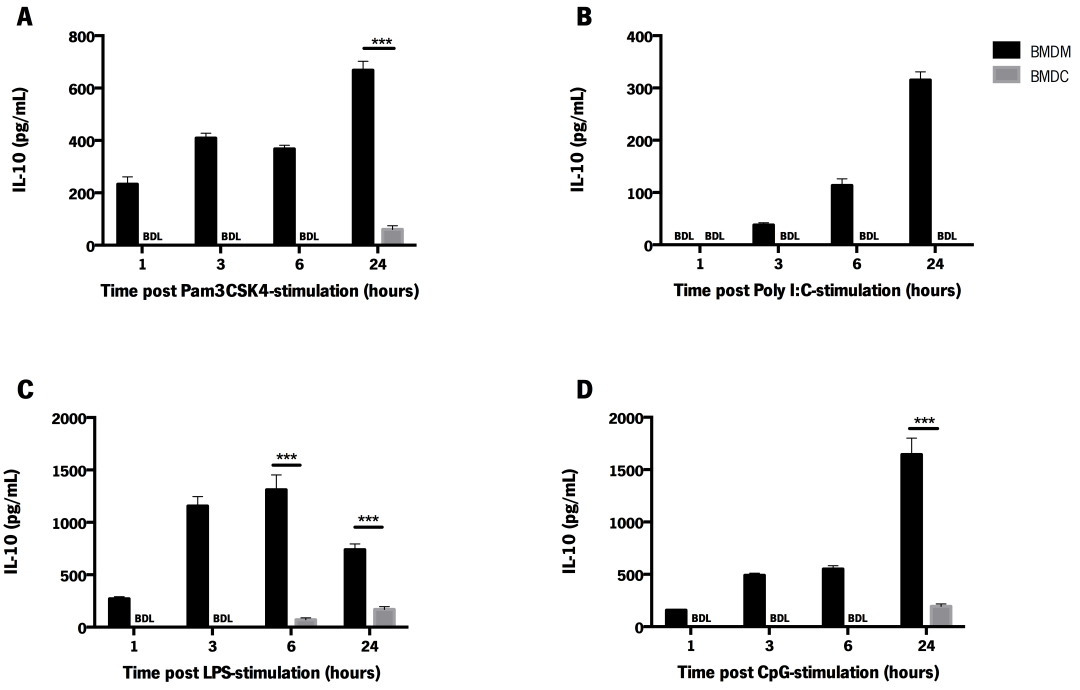

Figure 1: Earlier and higher IL-10 production in TLR-stimulated BMDM than BMDC. WT BMDM (black) and WT BMDC (grey) were stimulated with Pam_3CSK_4 (TLR2 ligand) (A), Poly I:C (TLR3 ligand) (B), LPS (TLR4 ligand) (C) or CpG (TLR9 ligand) (D). Supernatants were collected at 0, 1,3, 6 and 24 hours post stimulation and IL-10 production was measured by ELISA. Data represents mean ± SD of triplicates; *** $p \leq 0.001$. Data is representative of two independent experiments. BDL=bellow detection limit.

Differential regulation of IL-12p40, IL-12p70, IL-23 and IL-27p28 production by BMDM and BMDC upon TLR2, TLR3, TLR4 and TLR9 triggering

Taking into consideration the results from Figure 1, we next questioned whether the IL-12 family bioactive molecules were being differentially regulated in BMDM versus BMDC and weather that would correlate with the differences observed inIL-10 production. To test our hypothesis, WT BMDM and BMDC were stimulated with Pam_3CSK_4 (TLR2 ligand), Poly I:C (TLR3 ligand), LPS (TLR4 ligand) or CpG (TLR9 ligand). At 24 hours post stimulation supernatants were recovered and IL-12p40, IL12p70, IL-23 and IL-27p28 cytokine production was measured by ELISA. The production of IL-35, the fourth member of the IL-12 family, was not measured due to the unavailability of immunoassays to this molecule. BMDC produced all IL-12 family cytokines tested upon TLR stimulation, with a marked IL-12p40, IL-12p70 and IL-23 production (Figure 2). Furthermore, we found that TLR stimulation of BMDM led to poor to no secretion of IL-12p40, IL-12p70 or IL-23, but did induce the secretion of IL-27, which in case of TLR2 or TL3 stimulation was higher than that observed in BMDC (Figure 2). Our data suggest that cytokines of the IL-12 family are differently regulated in TLR-stimulated BMDM and BMDC. Moreover, the differences observed suggest a reciprocal regulation of these cytokines by IL-10.

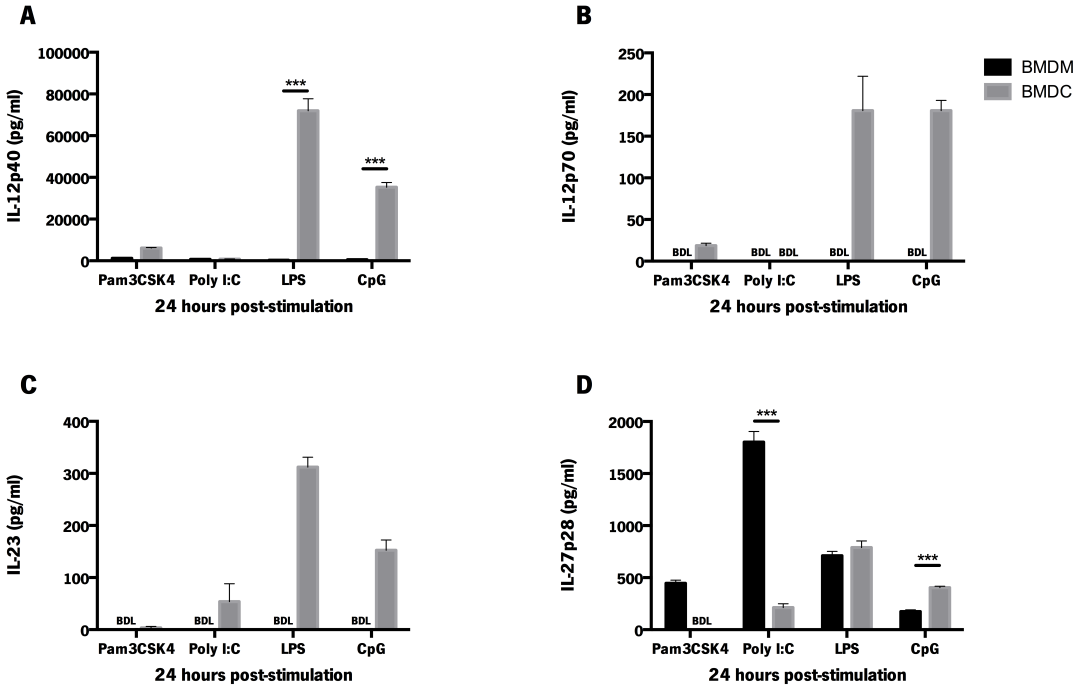

Figure 2: Differential Regulation of IL-12 family bioactive molecules by BMDM and BMDC. WT BMDM (black) and WT BMDC (Grey) were stimulated with Pam_3CSK_4 (TLR2 ligand), Poly I:C (TLR3 ligand), LPS (TLR4 ligand) and CpG (TLR9 ligand). Supernatants were collected 24 hours post stimulation. IL-12p40 (A), IL-12p70 (B), IL-23 (C) and IL-27p28 (D) production was measured by ELISA. Data represents mean ± SD of triplicates. Data is representative of two independent experiments. ** $p \leq 0.01$, *** $p \leq 0.001$. Data is representative of two independent experiments. BDL=bellow detection limit

Distinct transcriptional regulation *Il12a* (p35), *Il12b* (p40), *Il23a* (p19), *Il27a* (p28) and *Il27b* (ebi3) in BMDM versus BMDC

The IL-12 family cytokines are heterodimers[108]. Each cytokine is formed by two monomers. IL-12 is constituted by p35 and p40, IL-23 by p19 and p40, IL-27 by p28 and ebi3 and IL-35 by p35 and ebi3[108]. Thus an increase in the bioactive molecules as shown in Figure 2 can be due to an increase in each or both monomers, which are transcriptionally regulated. Taking into consideration the differences observed in the secretion of IL-12 family members upon TLR stimulation of BMDM versus BMDC (Figure 2), we next investigated if the differences observed at the protein level were related to differential transcription of the various monomers. For that, we evaluated the kinetic profile of all IL-12 monomers upon stimulation with TLR ligands. WT BMDM and BMDC were stimulated with Pam_3CSK_4 (TLR2 ligand), Poly I:C (TLR3 ligand), LPS (TLR4 ligand) or CpG (TLR9 ligand) as before and at 1, 3, 6 and 24 hours post-stimulation the amount of *Il12a*, *Il12b*, *Il23a*, *Il27a* and *Il27b* mRNA was accessed by RT-PCR and normalized to HPRT. BMDC displayed a higher *Il12a*, *Il12b* and *Il23a* mRNA induction, compared to BMDM (Figures 3A-3L). Nevertheless, BMDM exhibited a higher *Il27a* and *Il27b* mRNA induction, compared to BMDC (Figure 3M-3T). These results are in line with the protein data (Figure 2), suggesting that the regulation of the IL-12 family bioactive molecules in BMDM and BMDC is transcriptional.

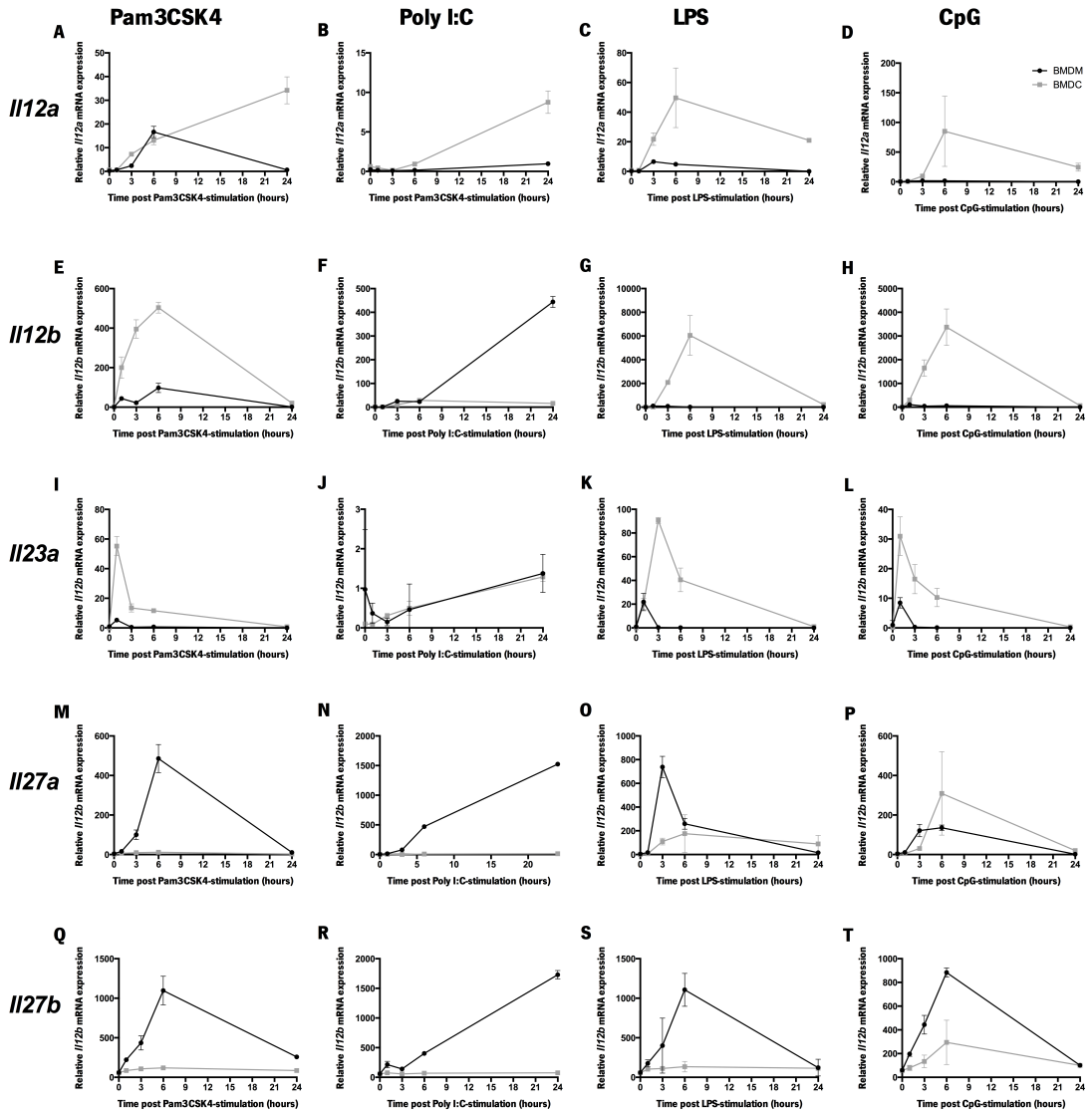

Figure 3: Differential transcriptional regulation by TLR stimulated BMDM and BMDC. BMDM (black) and BMDC (grey) were stimulated with Pam_3CSK_4 (TLR2 ligand), Poly I:C (TLR3 ligand), LPS (TLR4 ligand) and CpG (TLR9 ligand). Relative mRNA induction for *Il12a* (A-D), *Il12b* (E-H), *Il23a* (I-L), *Il27a* (M-P) and *Il27b* (Q-T) was assessed by RT-PCR at the indicated time points. All samples were normalized to HPRT. Data represents mean ± SD of triplicates. Data is representative of two independent experiments.

IL-10 limits the production of IL-12 family cytokines by TLR-stimulated BMDM

To access the role of IL-10 in the regulation of IL-12 family bioactive molecules, we next investigated if the lack of IL-10 impacted the ability of BMDM or BMDC to produce IL-12 family cytokines. For that, WT and IL-$10^{-/-}$ BMDM or BMDC were stimulated with the TLR ligands Pam_3CSK_4 (TLR2 ligand), Poly I:C (TLR3 ligand), LPS (TLR4 ligand) and CpG (TLR9 ligand). At 24 hours post-stimulation supernatants were recovered and IL-27p28 and IL-12p70 cytokine production was measured by ELISA. Both IL-27p28 and IL-12p70 were produced in higher

amounts by BMDM in the absence of IL-10, except for TLR3 stimulated BMDM (Figures 4A and 4C). This result may be explained by the observation that TLR3-stimulated BMDM produced only low amounts of IL-10 (Figure 1B). Regarding BMDC, the absence of IL-10 had no effect on the p28 and p70 production (Figure 4B and 4D), probably due to the low levels of IL-10 that these cells produce upon TLR stimulation (Figure 1).

Our data demonstrated that the presence of IL-10 exerts a negative regulation of IL-12 family bioactive molecules impairing or limiting the ability of BMDM to response to TLR stimulation with the secretion of these molecules. Of note, whereas the findings obtained for IL-12p70 confirmed the studies by other authors[89,138], the observation that IL-27p28 secretion by BMDM in novel. Also, the effect of IL-10 on IL-23 is currently under investigation, but preliminary results point to a role of IL-10 in limiting also IL-23 in TLR-stimulated BMDM.

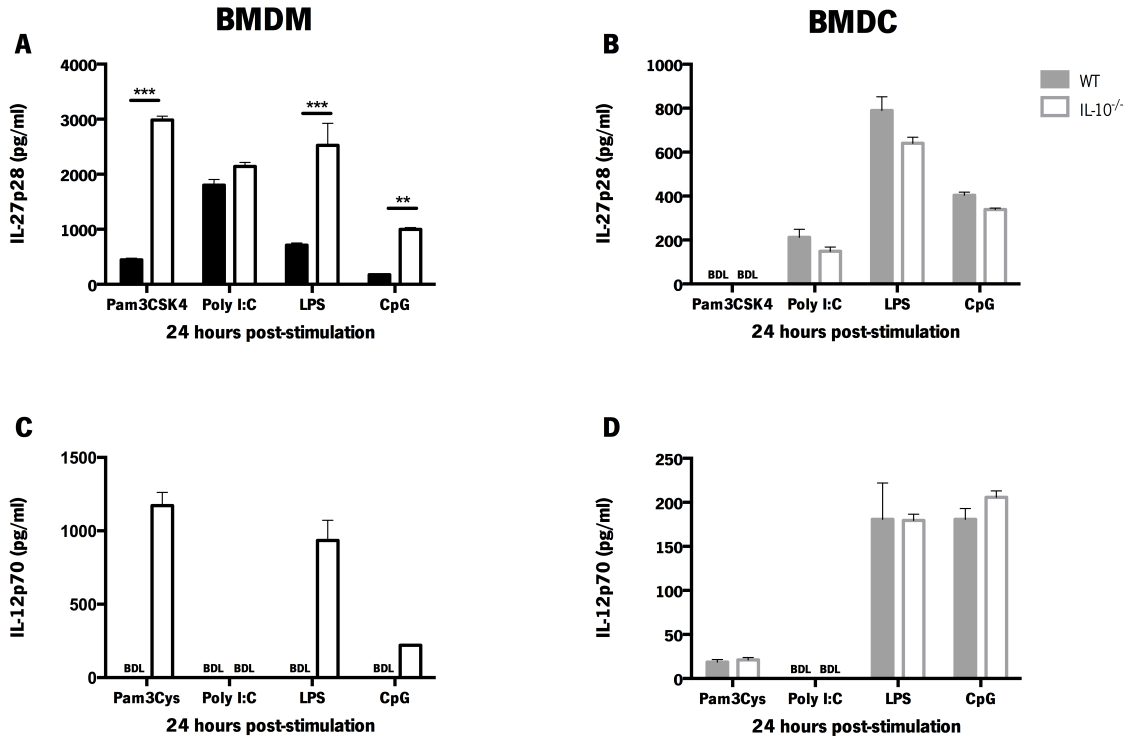

Figure 4: IL-10 deficiency led to higher IL-27p28 and IL-12p17 production by BMDM with no impact in BMDC. WT or IL-10$^{-/-}$ BMDM (left) and BMDC (right) were stimulated with Pam_3CSK_4 (TLR2 ligand), Poly I:C (TLR3 ligand), LPS (TLR4 ligand) and CpG (TLR9 ligand). Supernatants were collected 24 hours post stimulation. IL-27p28 (A, B) and IL-12p70 (C, D) production was measured by ELISA. Data represents mean ± SD of triplicates;** $p \leq 0.01$, *** $p \leq 0.001$. Data is representative of two independent experiments. BDL=bellow detection limit

IL-10 regulates the production of IL-12 family bioactive molecules through the transcriptional regulation of the monomers

As showed before, IL-12 bioactive molecules are composed by monomers that are transcriptionally regulated upon TLR stimulation of BMDM or BMDC (Figure 3). Thus, we questioned how the monomers transcriptional profile was regulated by BMDM and BMDC in the presence or absence of IL-10. WT and IL-10 $^{-/-}$ BMDM and BMDC were stimulated with LPS (TLR4 ligand) and *Il12a* (p35), *Il12b* (p40), *Il23a* (p19), *IL27a* (p28) and *Il27b* (ebi3) mRNA induction was accessed by RT-PCR at 0, 1, 3, 6 and 24 hours post-stimulation. We chose LPS as a stimulus because we observed that both BMDM and BMDC were able to produce IL-10 upon TLR4 stimulation (Figure 1).

When compared to WT, IL-10$^{-/-}$ BMDM showed a consistent and marked increase in the transcription of IL-12 monomers, which was more pronounced in the cases of *Il12a* (p35) (Figure 5A), *Il12b* (p40) (Figure 5C) and *Il27a* (p28) (Figure 5G) subunits. IL-10$^{-/-}$ BMDC showed less pronounced differences, which were mainly seen in the case of the *Il23a* (p19) monomer (Figure 5B).

Our data thus suggest that IL-10 regulates the production of IL-12 family bioactive molecules in TLR stimulated BMDM through the transcriptional regulation of the IL-12 monomers.

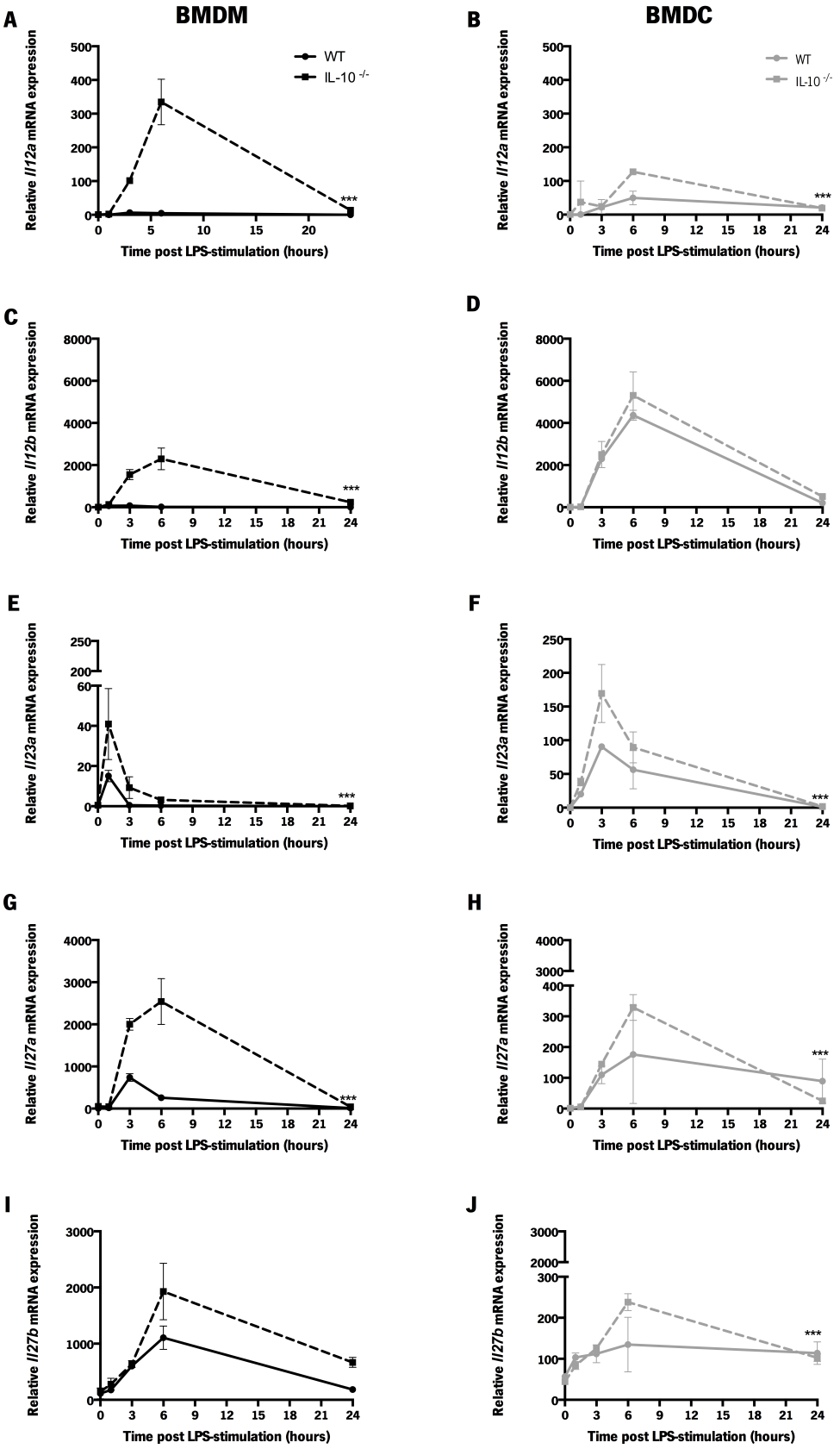

Figure 5: Il-10 deficiency impacts the transcription of IL-12 monomers in TLR-stimulated BMDM. BMDM (black) and BMDC (grey) were stimulated with LPS (TLR4 ligand). Relative mRNA induction for *Il12a* (A; B), *Il12b* (C, D), *Il23a* (E, F), *Il27a* (G, H) and *Il27b* (I, J) was assessed by RT-PCR at the indicated time points. All samples were normalized to HPRT. Data represents mean ± SD of triplicates. *** $p \leq 0.001$; Data is representative of two independent experiments.

IL-10 does not influences p19 mRNA stability

Interestingly, the expression of *Il23a* (p19) mRNA in WT versus IL-10$^{-/-}$ TLR-stimulated BMDM and BMDC showed a statistical different kinetic, with higher *Il23a* (p19) induction in IL-10$^{-/-}$ than WT cells. These finding is in agreement with the increased secretion of IL-23 in IL-10$^{-/-}$ BMDM or BMDC upon stimulation with LPS (data not shown). We next questioned if besides the transcriptional regulation, IL-10 was also regulating at the post-transcriptional level the monomer p19. For this, WT and IL-10$^{-/-}$BMDM or BMDC were stimulated with LPS (TLR4 ligand) and at 1 or 3 hours post stimulation, Actinomicyn D (ActD) was added to BMDM and BMDC cultures, respectively. The time of ActD treatment corresponded to the peak of *IL23a* mRNA expression observed for BMDM and BMDC, respectively. Total mRNA was collected at 30, 60 and 90 minutes post ActD treatment. The remaining *Il23a* (p19) mRNA levels were measured by RT-PCR and normalized to HPRT expression and further normalized to the results collected at 0 minutes of ActD treatment for each type of cell. Our data showed no differences in the *Il23a* mRNA stability between WT and IL-10$^{-/-}$, either for BMDM and BMDC (Figures 6A and 6B).

In summary, our results suggest that the presence of IL-10 regulates IL-23 through monomer transcriptional regulation.

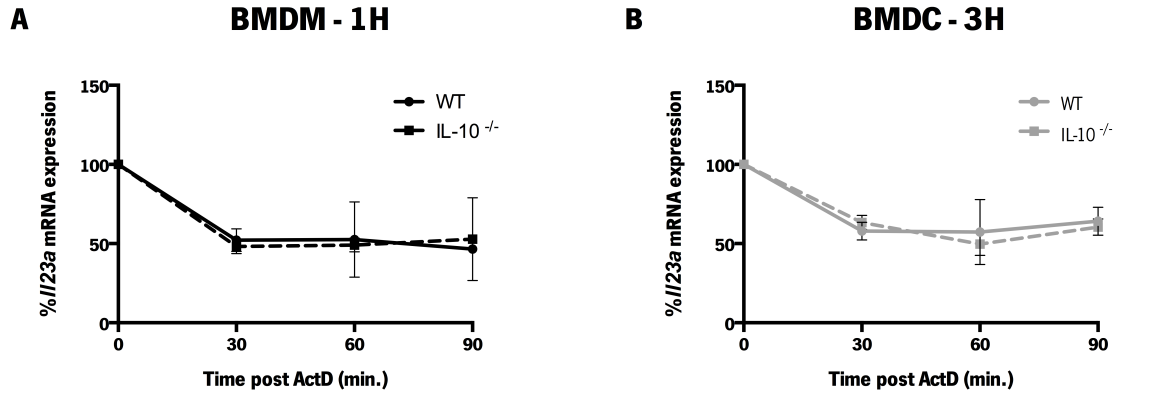

Figure 6: *Il23a* mRNA decay in BMDM and BMDC. BMBM (A, black) and BMDC (B, grey) were stimulated with LPS (TLR4 ligand) for 1 and 3 hours, respectively, followed by addition of ActD to stop transcription. After 30, 60 and 90 minutes *Il23a* mRNA was quantified by RT-PCR. All samples were normalized to HPRT. Data represents mean ± SD of triplicates. Data is representative of two independent experiments.

Discussion

Macrophages and DCs have distinct physiologic functions in the innate immune response, thus we were interested in understanding how these cells are differently triggered upon PAMPs recognition. In particular, we wanted to investigate the molecular events triggered in macrophages or DCs activation upon TLR and non-TLR stimulus. Moreover, we wanted to understand how these events impacted the production of IL-10 and IL-12 family members.

Data generated in our lab showed that BMDM and BMDC differently express IL-10 upon *M. tuberculosis* and that TLR2 is in this case a major contributor for the production of IL-10 (Teixeira-Coelho, M.; unpublished data). Furthermore, several studies show that BMDM are stronger producers of IL-10 than BMDC upon TLR2 stimulation[89]. We thus aimed at firstly understanding how IL-10 expression is regulated in TLR2 stimulated BMDM versus BMDC. Our results showed a differential IL-10 gene transcription, with BMDM exhibiting higher and earlier *Il10* gene expression than BMDC. Consistent with these data, BMDM produced higher amounts of IL-10 than BMDC. Because BMDC showed residual levels of *Il10* gene induction, we questioned if these cells were being activated by the TLR2/TLR1 stimulus. To access that, we measured *Il-6* and *Tnf* mRNA induction and cytokine production in BMDM and BMDC. We observed that BMDC were producing higher amounts of IL-6 and TNF compared to BMDM. Thus, BMDC were not impaired in their response to the TLR2/TLR1 ligand, but instead this stimulus was favouring a pro-inflammatory response in the near absence of IL-10. Moreover, our results showed that the lack of IL-10 production by TLR2/TLR1 activated BMDC was not dependent on the ligand concentration, as even at higher doses BMDC only produced residual amounts of IL-10. In contrast, IL-6 and TNF achieved the maximal levels even at low ligand concentrations.

Lipopeptides recognition by APCs triggers TLR2 signalling in a TLR1- or TLR6-dependent manner[36]. Seen that the poor IL-10 production in BMDC was not related to a poor response of these cells to TLR2/TLR1 ligand, we investigated whether the type TLR2 heterodimerization impacted the induction of IL-10. We observed that BMDC were producing modest amounts of IL-10 after being stimulated either with TLR2/TLR1 or TLR2/TLR6. This result is in accordance with the work of Farhat and colleagues, that suggest that the TLR2 heterodimerization with TLR1 and TLR6 expands the ligand spectrum, allowing the recognition of different lipopeptides, but does not lead to differential signalling[33].

Because TLR2 heterodimerization and concentration did not impact the amount of IL-10 produced by TLR2 stimulated BMDC, we questioned if upon other TLR stimulation IL-10 was produced by BMDC. We observed that BMDM expressed consistently higher amounts of IL-10 protein upon TLR

stimulation than BMDC, and that *Il10* mRNA induction closely paralleled the pattern of protein expression. Taking the previous published studies and our systematic approach in measuring IL-10 mRNA and protein in BMDC versus BMDM stimulated via different TLRs, it is clear that DCs are somehow limited to produce IL-10 in response to TLRs.

It as been previously shown that Dectin-1 triggering leads to IL-10 production[71]. For instance, in response to zymosan, a Dectin-1/TLR2 ligand, BMDC produce high levels of IL-10[145], which is mediated by the activation of the SYK pathway[65]. As we show in this thesis, in contrast to what was observed with TLRs, in response to Dectin-1 BMDC produced higher amounts of IL-10 than BMDM. Furthermore, IL-10 production by Dectin-1 stimulated BMDC was observed even at low doses of ligand. Therefore, in spite of the limited production of IL-10 upon TLR stimulation, BMDC are not impaired in their capacity of secreting IL-10. We therefore now have a model in which different cell types stimulated with different PRR ligands show distinct IL-10 production. Thus, unravelling the molecular pathways that discriminate between TLR and Dectin-1 responses in these cells will shed light into potential targets for IL-10 modulation. To start addressing this question, we compared the kinetics and strength of ERK and p38 activation in BMDM versus BMDC stimulated with TLR2 or Dectin-1. Activation of these MAPKs mediates IL-10 production in response to TLR signalling[90,101,103]. We therefore examined the possibility that differential ERK and p38 phosphorylation could be happening in TLR2 versus Dectin-1-stimulated BMDM and BMDC. We observed that ERK activation in BMDC was higher upon Dectin-1 stimulation than upon TLR2 and that, consistent with this, inhibition of ERK activation led to abrogation of IL-10 production in Dectin-1 stimulated BMDC. Furthermore, we also observed that ERK activation is nearly undetected in BMDC upon TLR2 stimulation. This fact may explain the lack of IL-10 production by these cells under TLR2-stimulation. I will now be interesting to understand if other TLR stimulation also fail to induce ERK activation in BMDC, as well as the molecular determination for this. The low Il-10 production under conditions that lack ERK activation is in line with previously published studies[90,92,144]. This is likely due to the requirement of ERK for the induction of cFOS, shown to regulate IL-10 expression[90,146]. Also, ERK is known to be activated downstream of Dectin-1, with a major role in the Dectin-1 dependent IL-10 production in BMDM[71]. IL-10 expression can also be regulated by p38 phosphorylation either in TLR4 or TLR9 activated macrophages and dendritic cells. However, as we show here, p38 activation is not enough to promote IL-10 induction. Also, in the case of BMDC stimulation via Dectin-1 we now show that p38 signalling is not involved in IL-10 production, thus discriminating the MAPK requirements for maximal IL-10 production upon TLR

versus non-TLR signalling. It is possible that the main role of p38 in regulating IL-10 is at the post-transcriptional level[101,103]. More recently was showed that, prolonged p38 activation upon TRIF activation leads to enhanced IL-10 transcription (Teixeira-Coelho, M.; Guedes,J. et al; submitted), thus providing further evidence for the role of p38 as a post-transcriptional regulator of IL-10.

Dectin-1 and TLR2 can induce collaborative induction of inflammatory responses in macrophages and dendritic cells, acting synergistically to mediate cytokine production, namely IL-10, IL-12 or TNF[64,147]. However, the molecular mechanisms underlying the synergistic effect of Dectin-1 and TLR2 are not understood. Surprisingly we observed that despite the similar *Il10* mRNA induction between Dectin-1 and Dectin-1/TLR2 stimulated BMDC, the combination of the two ligands led to a reduction in the IL-10 production when compared to single Dectin-1 stimulation. These results may suggest that the IL-10 production upon co-stimulation of Dectin-1/TLR2 is being post-transcriptionally regulated. However, our data opposes that of Dennehy et al suggested, since our results suggest an inhibitory effect on the IL-10 production induced by cooperation between Dectin-1/TLR2[76]. One possible explanation relies on the differences between the ligands that were used: we used a commercially available ß-glucan derived from *Alcaligenes faecalis* whereas Dennehy et al used a highly purified ß-glucan isolated from *Saccharomyces cerevisiae*. The different ß-glycan ligand could lead to a differential BMDC activation, and consequently to a distinct IL-10 cytokine production. Also, in our study we increased the doses of ß-glucan and maintained that for TLR2 agonists, whereas Dennehy did the opposite. It is possible that depending on the TLR2/Dectin-1 balance the signalling cascades differ, thus leading to different IL-10 production. Further experiments are needed and will be performed to clarify the observed discrepancies.

Despite our observations that combined stimulation of BMDC with Dectin-1 and TLR2 may be, at least in certain scenarios, inhibitory for IL-10 production, we did observe that Zymosan stimulation of these cells led to maximal IL-10 production. This finding was expected, but the underlying molecular mechanism is not known. We hypothesized that Dectin-1 may provide the ERK activation needed for IL-10 induction. Then TLR2 signalling may bring high p38 activation which could stabilize the IL-10 mRNA (Teixeira-Coelho, M. Guedes, J. et al; submitted)[101,103]. Thus the activation of these two MAPK would allow maximal IL-10 production by BMDC. Further studies are needed to confirm this hypothesis and also if similar observations are found with Dectin-1 and TLR2. In summary, in future experiments we wish to further understand why TLR-stimulated BMDC are poor inducers of ERK activation and explore the mechanisms underlying the cooperation between Dectin-1 and TLR2. Moreover, it would be interesting to investigate if Dectin-1 can synergise with

other TLRs. Furthermore, because BMDC are maturated from bone-marrow precursors in vitro in the presence of GM-CSF, it would be important to confirm our results in mice splenic DCs, which are already differentiated, and mostly importantly in monocyte-derived DCs from human samples.

In addition to the differences at the level of IL-10 production observed in TLR-stimulated BMDM and BMDC, these cells also differ with respect to other cytokines. Gerosa el al suggested that selective engagement of distinct combinations of PRRs leads to a differential regulation of IL-12 and IL-23 production by human monocyte derived DC, that consistently express higher levels of IL-12 and IL-23 than human monocyte derived macrophages[147]. According to Boonstra and colleagues, IL-10 secreted by macrophages and DCs upon TLR4 and TLR9 stimulation, supressed their IL-12p70 production[89]. Recently, Roses et al reported that the differential TLR triggering of DCs impacts the regulation of IL-12 and IL-23 production[148]. Specifically, single TLR agonists tend to induce IL-23 production, whereas combined TLR stimulation is required for commitment to IL-12 production[148]. In the same work, the authors show that LPS triggering leads to IL-23 production, but no IL-12 in monocyte-derived DCs[148]. Our work shows that, opposite to monocyte-derived DCs, BMDC are activated by TLR4 triggering, and are able to produce considerable amounts of IIL-12p70, as well as IL-12p40, IL-23 and IL-27p28. In line with our results, Siegemund et al showed that upon TLR4 triggering BMDC produce consistently higher amounts of IL-12p40, IL-12p70, IL-23 and IL-27p28 than macrophages[149]. The data presented here is in line with previous results showing that DCs are better producers of IL-12 and IL-23 than macrophages upon TLR triggering. However, we now show that this does not apply to all members of the IL-12 family, as production of IL-27p28 and expression of p28 and ebi3 were higher in TLR-stimulated BMDM than BMDC. Of note, Dennehy et al showed that following TLR2 triggering BMDM and BMDC have similar amounts of IL-12p70 production[76], whereas we show that TLR2 stimulation led to undetectable levels of IL-12p70 in BMDM.

The differential expression of IL-12 and IL-23 in macrophages versus DCs has been previously addressed at the molecular level. In particular, IRF5 was shown to be an important player[119]. Taking into consideration the differences in the kinetics of IL-10 production by TLR-stimulated BMDM and BMDC we now explored whereas IL-10 could also explain the different production of IL-12 family cytokines in these cells. To characterize the effect of IL-10 in the regulation of IL-12 bioactive molecules, we compared WT and IL-10 deficient BMDM and BMDC. Our results showed that the absence of IL-10 in BMDM leads to an increased production of IL-12p70 and IL-27p28 for all TLRs tested, with exception of TLR3. This result may be explained as TLR3 stimulated BMDM

produce low amounts of IL-10. No significant differences were seen between TLR stimulated WT and IL-10 deficient BMDC. This result may be related to the fact that TLR stimulated WT BMDC produce residual amounts of IL-10, that consequently have no effect on the IL-12p70 and IL-27p28 production.

Taken into consideration the results obtained from LPS stimulated WT and IL-10$^{-/-}$ BMDM and BMDC, we next showed that IL-10 reciprocally regulates IL-12 family cytokines, through transcriptional regulation of IL-12 monomers. Moreover, for p19 and p40 we show no contribution of IL-10 for post-transcriptional regulation.

In summary, in this thesis we demonstrate that BMDM are more potent inducers of IL-10 than BMDC, and consequently, BMDC are major producers of pro-inflammatory cytokines, as IL-12. The differential triggering of BMDM and BMDC upon the same PRR stimuli is likely related to their physiological functions. Macrophages are expected to remain in situ, controlling infection through the activation of microbicidal mechanisms and recruitment of inflammatory cells to the lung, therefore immunoregulation is essential to prevent tissue damage. As for DCs, PRR triggering will induce their maturation and provide the co-stimulation to promote their migration to secondary lymphoid organs. The production of stimulatory cytokines is essential for T cell priming and control of the magnitude of T cell differentiation, and could be compromised by IL-10.

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