Current Topics in Microbiology and Immunology

8 Chapter 8

The regulation of IL-10 expression

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Abstract

Interleukin (IL)-10 is an important immunoregulatory cytokine and an understanding of how IL-10 expression is controlled is critical in the design of immune intervention strategies. IL-10 is produced by almost all cell types within the innate (including macrophages, monocytes, dendritic cells (DCs), mast cells, neutrophils, eosinophils and natural killer cells) and adaptive (including CD4$^+$ T cells, CD8$^+$ T cells and B cells) immune systems. The mechanisms of IL-10 regulation operate at several stages including chromatin remodelling at the *Il10* locus, transcriptional regulation of *Il10* expression, and post-transcriptional regulation of *Il10* mRNA. In addition, whereas some aspects of *Il10* gene regulation are conserved between different immune cell types, several are cell type- or stimulus-specific. Here we outline the complexity of IL-10 production by discussing what is known about its regulation in macrophages, monocytes, DCs and CD4$^+$ T helper cells.

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List of Abbreviations:

AHR	Aryl hydrocarbon receptor
AP-1	Activator protein 1
APC	Antigen presenting cell
ARE	AU rich element
ATF	Activating transcription factor 1
AUF-1	ARE/poly(U) binding degradation factor 1
BATF	basic leucine zipper transcription factor ATF-like transcription factor
BCL-6	B-cell lymphoma 6
BLIMP-1	PR domain zinc finger protein 1
BM	Bone marrow
C/EBP	CCAAT/enhancer-binding protein
cAMP	cyclic adenosine monophosphate
CBP	CREB-binding protein
CD40L	CD40 ligand
ChIP-Seq	Chromatin immunoprecipitation-sequencing
CNS	Conserved non-coding sequences
CREB	cAMP response element-binding protein
CRTC3	CREB-regulated transcription coactivator 3
DC	Dendritic cell
DLL	Delta-like Notch ligands
DRE	Distal regulatory element
DUSP1	Dual-specificity phosphatase-1
E4BP4	E4 promoter-binding protein 4
ERK	Extracellular signal-regulated kinase
E. coli	*Escherichia coli*
ETS-1	E26 transformation-specific 1
GATA3	GATA binding protein 3
GM-CSF	Granulocyte-monocyte colony stimulating factor
GSK3	Glycogen synthase kinase 3
HAT	Histone acetyl transferase
HDAC	Histone deacetylase
HMT	Histone methyl transferase
HSS	DNaseI hypersensitive sites
ICOS	Inducible T cell costimulator
IFN	Interferon
IL	Interleukin
IRF	Interferon regulatory factors
JDP	Jun dimerising protein
LPS	Lipopolysaccharide
MAP kinase	Mitogen-activated protein kinase
MARE	c-MAF responsive element
mDC	Myeloid dendritic cell
MHC	Major histocompatibility complex
miRNA	MicroRNA
MSK1/2	Mitogen- and stress-activated protein kinases ½
mTOR	mammalian target of rapamycin
M. tuberculosis	*Mycobacterium tuberculosis*
MyD88	Myeloid differentiation factor 88
NF-κB	Nuclear factor-κB
NFAT	Nuclear factor of activated T cells
PBX1	Pre-B cell leukaemia homeobox 1
pDC	Plasmocytoid dendritic cell
PDCD4	Programmed cell death 4
PGE2	Prostaglandin E2
PI(3)K	Phosphatidylinositol 3 kinase
PKA	Protein Kinase A
PKR	Protein-kinase R
PREP1	PBX-regulating protein 1
PRR	Pattern recognition receptor
RORγt	RAR-related orphan receptor gamma t
SIK2	Salt-inducible kinase 2

Sp1/3	Specific protein 1/3
STAT	Signal transducer and activator of transcription
SWI/SNF	Switching-defective-sucrose non-fermenting
SYK	Spleen tyrosine kinase
TBET	T-box transcription factor
TCR	T cell receptor
Tfh	T follicular helper cell
TGF	Transforming growth factor
Th	T helper cell
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TPL-2	Tumour progression locus 2
TRIF	TIR-domain-containing adapter-inducing interferon-β
TSS	Transcription start site
TTP	Tristetraprolin
UTR	Untranslated region

8.1 Introduction

The immune system has evolved to protect the host from a wide range of potentially pathogenic microorganisms. However, immune responses also have the potential to cause damage to the host if not adequately regulated. Interleukin-10 (IL-10) is an anti-inflammatory cytokine with a crucial role in preventing inflammatory and autoimmune pathologies by limiting the immune response to pathogens and microbial flora. However, the production of IL-10 can also inappropriately restrict protective immune responses and, in this context, IL-10 can contribute to chronic infection [1]. Thus, IL-10 is an important immunoregulatory cytokine and an understanding of how IL-10 expression is regulated is critical in the design of immune intervention strategies.

IL-10 is produced by cells within both the innate (including macrophages, monocytes, dendritic cells (DCs), mast cells, neutrophils, eosinophils and natural killer cells) and adaptive (including CD4$^+$ T cells, CD8$^+$ T cells and B cells) immune systems [2]. Cells within the innate immune system require stimulation by pathogen derived products via pattern recognition receptors (PRR) to induce IL-10 production [2]. T cells recognise microbial peptides presented in the context of major histocompatibility complexes (MHC) by antigen presenting cells (APC) including macrophages and DCs through the interaction with their T cell receptor (TCR). In contrast to the innate cells that can immediately produce IL-10 in response to environmental stimuli, naïve CD4$^+$ T cells first need to differentiate into the different T helper (Th) cell subsets, including Th1, Th2 and Th17 cells [3], in order to produce IL-10 [2].

The mechanisms of IL-10 regulation operate at several stages including chromatin remodelling at the *Il10* locus, transcriptional regulation of *Il10* expression, and post-transcriptional regulation of *Il10* mRNA. In addition, whereas some aspects of *Il10* gene regulation are conserved between different immune cell types, several may be cell type- or stimulus-specific. Here we outline the complexity of IL-10 regulation by discussing what is known about its regulation in macrophages, monocytes, DCs and CD4$^+$ Th cells.

8.2 Concepts of transcriptional gene regulation

Within chromosomes, DNA is wrapped around octamers of core histones to form nucleosomes, the basic units of eukaryotic chromatin. Multiple nucleosomes are arranged in a linear array along the DNA referred to as 'beads on a string' (accessible euchromatin) that can be further compacted into

30 nm fibres (inaccessible heterochromatin) upon the addition of linker histones [4] (Figure 1). Factors that influence the accessibility of chromatin include DNA methylation, nucleosome remodelling and covalent histone modifications such as acetylation or methylation [5] (Figure 1). Histone modifications, usually referred to as the histone code, have been reported to be associated with different functional elements such as promoters or distal regulatory elements (DRE), also known as enhancers or insulators, and are key to gene regulation [6] (Figure 1). Histone modifying enzymes such as histone acetyl transferases (HAT) and deacetylases (HDAC) or histone methyl transferases (HMT) alter histone 'tails' to form repressive or active marks, remodelling the chromatin from high density transcriptionally inactive heterochromatin to low density transcriptionally active euchromatin and *vice versa* thus governing gene expression [5]. Changes in the chromatin landscape can be monitored by DNaseI treatment that marks nucleosome-poor regions, indicative of 'open' chromatin, known as hypersensitive sites (HSS), where transcription factors can interact with the exposed regulatory DNA sequences (Figure 1).

8.3   Structure of the IL-10 gene locus

The genes encoding both human and murine IL-10 are located within the *Il10* gene family cluster on Chromosome 1 of the respective genomes [7]. The *Il10* gene is flanked upstream by other members of the IL-10 family of cytokines *Il19*, *Il20* and *Il24*, and downstream by the *Mapkapk2* (MAP kinase-activated protein kinase 2) gene (Figure 2a). The *Il10* gene itself is comprised of 5 exons and 4 introns followed by a 3' untranslated region (UTR) spanning around 5 kb in total on the forward strand (Figure 2b and c). The *Il10* transcription start site (TSS) is relatively well defined, with proximal downstream TATA and CCA[A/G in mice]T elements within the core promoter (Figure 2d) characteristic of a 'sharp' or 'focused' promoter that is thought to be associated with tissue-specific transcription often in an environmentally responsive manner [6]. There is a high degree of homology between the human and mouse *Il10* gene loci with a number of conserved non-coding sequences (CNS, named according to their distance in kb from the *Il10* TSS) and shown to be present using VISTA-Point software [8] (Figure 2b and c). This is indicative of an evolutionary constraint most likely resulting from functional relevance of these regions, some of which have been shown to be functionally characterized as enhancers of *Il10* gene expression [9-13].

Changes in chromatin structure at the *Il10* gene locus are fundamental to the regulation of its expression. Several studies in a variety of cells including both macrophages and Th cells have

investigated the pattern of DNaseI HSSs at the *Il10* gene locus. In bone marrow (BM)-derived macrophages, four HSS sites have been identified at positions -4.5, -2, -0.12 kb upstream and +1.65 and +2.98 kb downstream of the *Il10* TSS following their stimulation with pattern recognition receptors (PRR) ligands including lipopolysaccharide (LPS), CpG and zymosan A [14] (Figure 2c). The HSS -4.5 kb was also present in BM derived DCs when stimulated with the same ligands [14]. Unstimulated macrophages also showed some degree of sensitivity to DNaseI digestion at these sites suggestive of poised chromatin conformation in the resting state. This may be in line with the immediate expression of the *Il10* gene in these cells upon exposure to appropriate stimuli. In contrast, the *Il10* gene locus in naïve T cells is in a closed, transcriptionally inactive conformation with only one HSS -8.8 kb upstream of the *Il10* TSS [12] (Figure 2b). Substantial remodelling of the *Il10* gene locus occurs upon differentiation into Th1 and Th2 cells, as demonstrated by the increased number of HSS sites detected, most of which were found to be present in both Th1 and Th2 cells [12-15] (Figure 2b and c, black arrows). A few HSS sites were more prominent in Th2 cells including HSS -30.4, -29.8, -21, -17.5 and -0.12 kb downstream and +6.45 kb upstream of the *Il10* gene TSS [12] (Figure 2b and c, green arrows). These differences may account for the enhanced production of IL-10 in Th2 cells when compared to in Th1 cells, although this is yet to be investigated. In contrast to Th1 and Th2 cells, little is known about the chromatin conformation at the *Il10* gene locus in Th17 cells. Interestingly, many of the HSS studied were common between macrophages, DCs and Th cells, apart from HSS -4.5 kb which was macrophage and DC specific [14]. Moreover, in most instances, the DNaseI HSS detected correspond with the computationally obtained CNS sites (Figure 2b and c), further supporting a regulatory role for these CNS.

Additional evidence informing on the accessibility of the *Il10* gene locus comes from studies on histone modifications as markers of permissive *versus* repressive chromatin. In IL-10-producing macrophages, the presence of hyperactylation of histone H4 (active mark) was detected at the -4.5 and -1.2 kb HSS regions [14]. In Th1 and Th2 cells, the presence of H3K4me3 (active mark) and absence of H3K27me3 (repressive mark) was shown across the *Il10* gene locus by chromatin immunoprecipitation-sequencing (ChIP-Seq) [16]. These histone marks indicate a transcriptionally competent state of the *Il10* gene locus, although their presence does not always correlate with active gene expression. The possible candidates initiating chromatin remodelling at the *Il10* gene locus will be discussed in some detail later.

8.4 Molecular Signals and Transcriptional Regulation of IL-10 Expression

8.4.1 The Regulation of IL-10 in Macrophages and Dendritic Cells

Almost all cells within the innate immune system, with the exception of plasmacytoid DCs (pDC) [17-19], have been shown to produce IL-10. These include macrophages, monocytes and myeloid (or conventional) DCs (mDC), which largely require stimulation via PRRs to induce IL-10 production [2]. Additionally, co-stimulation of DCs with a PRR ligand and CD40 ligand (CD40L) or immune-complexes can enhance the production of IL-10 [20, 21]. Importantly, other signals, such as those received from autocrine or paracrine cytokines, can also modulate IL-10 production [22-25]. Thus, the ultimate level of IL-10 produced by macrophages or mDCs is determined by the integration of several molecular signals (Figure 3a).

8.4.1.1 The Regulation of IL-10 Downstream of Pattern Recognition Receptors

Amongst the PRRs, the Toll-like receptor (TLR) family is the best studied. TLRs can be expressed at the plasma membrane or endosomally and recognise a range of ligands including bacterial lipopeptides (TLR 2), viral RNAs (TLRs 3 and 7), LPS found in the cell wall of Gram-negative bacteria (TLR 4), flagellin (TLR 5), and CpG DNA (TLR 9) [26]. It has been shown that TLR expression may vary amongst macrophage, monocyte and DC subsets [27, 28]. For example in humans, TLR 7 and TLR 9 are more highly expressed on pDCs than monocytes or other DC subsets [27, 28]. In contrast, human monocytes have been shown to more highly express TLR 2 and TLR 4 [27]. Of note, these differences may not be conserved in the murine system where it has been shown that the majority of TLR mRNAs (with the exception of TLRs 3, 5 and 7) are similarly expressed in DC subsets [29]. The ligation of several TLRs including 2, 4, 5, 7 and 9 has been shown to induce the production of IL-10 in human and murine macrophages and mDCs [17, 18, 30-32], although, macrophages and mDCs may not have the same capacity to produce IL-10. For example, TLR 3 activation has been shown to induce IL-10 production in macrophages but not mDC [17], and TLR 9 ligation induces IL-10 production more readily in murine macrophages than mDC, and not at all in pDC, despite all of these cell types expressing TLR 9 [17, 18]. Importantly however, in mDCs, the co-ligation of TLR 2 and Dectin-1, a C-type Lectin receptor which recognises fungal β-glucans [33], has been shown to enhance the level of IL-10 production relative to TLR2 or Dectin-1 stimulation alone [34]. This demonstrates that PRRs are able to cooperate in their induction of IL-10, a concept which may have relevance in the context of infection when several PRR ligands are likely to be present at the same time.

Downstream of TLR ligation, adaptor proteins are essential for signal transduction. The adaptor myeloid differentiation factor 88 (MyD88) is required for signalling downstream of all TLRs with the exception of TLR 3 which utilises the adaptor TIR-domain-containing adapter-inducing interferon-β (TRIF). TLR 4 is the only TLR that mediates signal transduction through both MyD88 and TRIF [26] and indeed both adaptors are required for optimal IL-10 production downstream of this receptor [17]. TLR signalling through these adaptor proteins leads to the activation of several signalling pathways including mitogen-activated protein kinase (MAP kinase) pathways, the phosphatidylinositol 3 kinase (PI(3)K)-AKT pathway, the nuclear factor (NF)-κB pathway and the activation of interferon regulatory factors (IRF). These downstream pathways collectively regulate the production of TLR-induced cytokines, including IL-10 [26] (Figure 3a).

Several studies have shown that the activation of the MAP kinases extracellular signal-regulated kinase (ERK) 1 and ERK 2 (here referred to collectively as ERK) is critical for the production of IL-10 in macrophages and mDCs downstream of TLRs 2, 4 and 9 [18, 35-37], and in *Mycobacterium tuberculosis (M. tuberculosis)* infected macrophages and monocytes [38]. In keeping with this, tumour progression locus 2 (TPL-2), the MAP 3-kinase upstream of TLR-induced ERK activation [39], which is itself regulated by the NF-κB family member p105 [40], is also required for IL-10 production in TLR stimulated macrophages and mDCs, and in *M. tuberculosis* infected macrophages and monocytes [18, 35, 38] (Figure 3a and b). In these cell types, the TPL-2/ERK pathway also negatively regulates type I IFN [18, 38] which as discussed in more detail later, is a positive regulator of IL-10 production [22, 38, 41-43] (Figure 3b). However, despite the relatively higher level of type I IFN produced in $Tpl2^{-/-}$ macrophages, mDCs and monocytes, IL-10 production remains reduced compared to wild type cells [18, 38]. Together, this demonstrates a central role for the TPL-2/ERK pathway in the regulation of IL-10 that cannot be compensated for by elevated autocrine type I IFN production. Further supporting an important role for ERK in the regulation of IL-10, differential ERK activation has been correlated with the different levels of IL-10 produced by macrophages, mDCs and pDCs [18] and ERK activation promotes IL-10 production in mDC downstream of the non-TLR PRR Dectin-1 [44, 45].

Downstream of MAP kinase signalling transcription factors including activator protein 1 (AP-1), a heterodimeric transcription factor composed of dimers of the Fos, Jun, Jun dimerising protein (JDP) and activating transcription factor (ATF) family members are activated [46]. In TLR stimulated macrophages and mDCs, the AP-1 member c-FOS has been associated with the ERK-dependent positive regulation of IL-10 [18, 23, 30, 36]. In contrast to TLR-induced IL-10 production however, Dectin-1-dependent IL-10 induction requires the upstream kinase spleen tyrosine kinase (SYK) and

is not dependent on c-FOS [44, 45, 47] (Figure 3a). In addition to the activation of AP-1, in macrophages co-stimulated with a TLR 4 ligand and immune-complexes, ERK activation has been shown to promote the phosphorylation of histone H3 at sites across the *Il10* promoter [48, 49]. This histone modification enhanced the accessibility of the *Il10* promoter, providing a permissive chromatin structure for the binding of transcription factors such as the constitutively expressed specific protein 1 (Sp1) [48, 49], shown to bind within the proximal *Il10* promoter at a site critical for transactivation of the *Il10* gene [50] (Figure 2d). Of note, in addition to Sp1, Sp3 has been reported to bind at the *Il10* promoter and transactivate the *Il10* gene in the Drosophila SL2 cell line [51]. The concept of chromatin remodelling at the *Il10* locus prior to *Il10* gene expression is in keeping with the dependence of *Il10* gene expression on the activity of SWI/SNF (switching-defective-sucrose non-fermenting, also known as BRG1 associated factors in mammals) nucleosome remodelling complex which enhances accessibility of certain genetic loci in response to TLR stimulation [52].

Similarly to ERK, the MAP kinase p38 has been shown to positively regulate IL-10 production in TLR stimulated macrophages, monocytes and mDCs [23, 32, 37, 53-55] (Figure 3a). In a TLR 4 stimulated human monocytic cell line, p38 was proposed to induce Sp1 binding to an alternative upstream Sp1 binding site which was important for the transactivation of the *Il10* gene in this system [56]. Also downstream of p38, pre-B cell leukaemia homeobox 1 (PBX1) and PBX-regulating protein 1 (PREP1) have been shown to promote IL-10 production in response to apoptotic cells [57].

ERK and p38 may also cooperate in their regulation of IL-10 production, demonstrated by the finding that both ERK and p38 contribute to the activation of mitogen- and stress-activated protein kinases MSK1 and MSK2 which in turn promote IL-10 production in TLR 4 stimulated macrophages [58]. Downstream of MSK1 and MSK2, the transcription factors cyclic adenosine monophosphate (cAMP) response element-binding protein (CREB) and ATF1 were found to bind the *Il10* promoter (Figure 2d) [58]. CREB and ATF1 binding at the *Il10* gene locus and transactivation of the *Il10* promoter has also been demonstrated in a cAMP treated human monocytic cell line [59]. CREB has further been shown to induce IL-10 production in response to Zymosan, a yeast derived combined TLR 2/Dectin-1 stimulus [60].

The third MAP kinase, JNK, has also been proposed to positively regulate IL-10 production in TLR stimulated macrophages and monocytes [23, 32, 61]. However, the downstream mechanisms of this

are unclear and detailed studies of the role of JNK in the regulation of IL-10 have until recently been complicated by the lack of specific inhibitors for this kinase.

TLR-induced signalling through the PI(3)K-AKT pathway also contributes to IL-10 production in macrophages, monocytes and mDCs [62-64] (Figure 3a). In TLR 4 stimulated mDCs and *Leishmania* infected macrophages, this has been shown to be mediated at least in part through the inhibition of glycogen synthase kinase 3 (GSK3)-β [63, 65], a constitutively active kinase which inhibits the production of IL-10 by antagonising the DNA binding activity of CREB [66]. It has also been proposed that the PI(3)K-AKT pathway activates ERK [62] and this may promote IL-10. The PI(3)K-AKT pathway additionally leads to the downstream activation of mammalian target of rapamycin (mTOR) and this also promotes the production of IL-10 [63, 64] (Figure 3a). Recently it has been shown that the MAP kinase p38 can also activate mTOR, providing an additional mechanism whereby p38 activation promotes the production of IL-10 production [67]. mTOR may further regulate IL-10 production through the activation of signal transducers and activators of transcription (STAT) 3 [64], reported to bind at a STAT consensus site within the proximal *Il10* promoter, important for transactivation of the *Il10* gene [68] (Figure 2d). Of note however, STAT3 activation also occurs downstream of the IL-10 receptor [69] and in human monocyte-derived macrophages STAT3 has been implicated in the feed-forward loop whereby IL-10 positively regulates its own production [24]. Thus, whether STAT3 regulates IL-10 downstream of PRR stimulation via mTOR, or downstream of autocrine IL-10 signalling, or both, is unclear.

NF-κB is also activated in response to TLR ligation (Figure 3a) and the finding that IKK2 deficient macrophages, which have impaired NF-κB activation, show reduced production of IL-10 [70] implicated NF-κB in the regulation of IL-10. Although the interpretation of this result is difficult as IKK2 is also upstream of TPL-2 activation [40], later studies have continued to support a role for NF-κB in the regulation of IL-10. For example, an NF-κB binding site is present at HSS -4.5 kb upstream of the *Il10* TSS and recruits the NF-κB subunit p65 in TLR 4 stimulated macrophages [14] (Figure 2c). Further, p105 (encoded by *Nfkb1*) has been shown to regulate IL-10 production through both NF-κB-dependent and TPL-2/ERK-dependent pathways [35]. NF-κB p50 homodimer recruitment to an NF-κB site proximal to the *Il10* TSS has also been shown to promote IL-10 production in TLR 4 stimulated macrophages (Figure 2d) and the complexing of these p50 homodimers with the co-activator CREB-binding protein (CBP) further enhances *Il10* promoter activity [71]. Finally, p65 binding at an additional site in the *Il10* promoter (Figure 2d) has been reported to upregulate IL-10 in dsRNA-stimulated macrophages and this was dependent on protein-kinase R (PKR) [61].

Several other transcription factors have been implicated in the regulation of *Il10* gene expression in PRR stimulated macrophages and/or mDCs. The transcription factor c-MAF has been reported to bind at the *Il10* promoter (Figure 2d) and enhance IL-10 production in LPS stimulated macrophages, although this mechanism of IL-10 regulation may be most relevant in the context of IL-4 which increases the recruitment of c-MAF to the *Il10* promoter [25]. Several CCAAT/enhancer-binding protein (C/EBP) sites have been identified within the *Il10* promoter [72]. In a human monocyte cell line, binding of C/EBPα and β to a C/EBP site proximal to the TATA box was important for basal *Il10* gene expression and transactivation of the *Il10* gene in response to cAMP [72]. Roles for C/EBPβ and C/EBPδ in cooperation with Sp1, have also been identified in the expression of *Il10* in a TLR 4 stimulated murine macrophage cell line [73]. C/EBPβ has further been shown to mediate *Il10* expression downstream of the A_{2A} adenosine receptor in *Escherichia coli* (*E. coli*) infected macrophages [74]. Of note, the synergistic induction of IL-10 in adenosine/*E. coli* infected macrophages was dependent on p38 activation [74] suggesting that p38 may be upstream of C/EBPβ activity. The ligand-activated transcription factor aryl hydrocarbon receptor (AHR) is additionally reported to be required for maximal production of IL-10 in TLR 4 but not TLR 9 stimulated macrophages, suggesting a stimulus specific requirement for AHR in the regulation of IL-10 [75].

8.4.1.2 *Negative Regulators of IL-10 Production*

Negative mechanisms of IL-10 regulation in PRR stimulated macrophages and monocytes have also been identified, in keeping with the important biological functions of IL-10 in various scenarios, and the need for tight control of its expression. For example, in a TLR 4 stimulated macrophage cell line, the chromatin modifying activity of HDAC11 has been shown to inhibit IL-10 production, possibly by impairing the transition of repressed to active chromatin at the *Il10* locus and restricting the binding of Sp1, STAT3 and PolII to the proximal *Il10* promoter [76]. Recently, IRF5, the expression of which is induced downstream of TLR ligation, has been identified as a negative regulator of IL-10 in human GM-CSF differentiated monocytes [77]. This study also showed recruitment of IRF5 to the IL-10 promoter, suggesting a direct mechanism of transcriptional inhibition [77]. IL-10 is additionally negatively regulated at the post-transcriptional level, discussed in detail later.

8.4.1.3 The Modulation of IL-10 Production by Autocrine and Paracrine Factors

Signalling induced by environmental cytokines can modulate the level of IL-10 produced by PRR stimulated macrophages or mDCs (Figure 3a). A key example of this is the effect of IL-10 on itself. In addition to the already mentioned activation of STAT3 which can enhance IL-10 production [24], IL-10 signalling has been shown to upregulate the expression of *Tpl2* [78] (Figure 3b) which also positively regulates IL-10 [18, 35, 38]. In contrast, IL-10 can induce the expression of dual-specificity phosphatase-1 (DUSP1) which negatively regulates p38 activation, a positive regulator of IL-10 production [79].

Type I interferon (IFN) has been shown to enhance the production of IL-10 from TLR 4 stimulated murine macrophages and human monocytes [22, 41, 42]. Type I IFN also promotes IL-10 production in *M. tuberculosis* infected macrophages [38, 43]. Reported mechanisms of type I IFN enhancement of IL-10 production in PRR stimulated cells include the recruitment of IRF1 and STAT3 to the *Il10* promoter in a human B cell line [80], the activation of STAT1 in murine macrophages [81] and the activation of the PI(3)K-AKT pathway followed by the subsequent inactivation of GSK3-β in human DCs [82]. It has also been reported that IL-27 is required for the maximal induction of IL-10 by type I IFN in TLR 4 stimulated murine macrophages [83], however another study reported that resting murine macrophages are unresponsive to IL-27 and that IL-27 had an inhibitory effect on IL-10 production in human monocytes via STAT1 [84]. Thus, the role of IL-27 and STAT1 in the regulation of IL-10 remains incompletely understood. In contrast to type I IFN, in TLR 2 activated human macrophages, IFN-γ has been shown to reduce the production of IL-10 through the inhibition of MAP kinases and enhancement of GSK3-β activity [23]. This led to the suppression of the transcription factors AP-1 and CREB, which both positively regulate IL-10 [23].

The enhancement of IL-10 by the lipid-derived molecule prostaglandin E2 (PGE2) in TLR 4 activated macrophages has also been reported [85], illustrating that factors other than cytokines can modulate the production of PRR induced IL-10 production. A model for the mechanism of PGE2 mediated enhancement of IL-10 has been proposed in which PGE2 treatment activates protein kinase A (PKA) to phosphorylate salt-inducible kinase 2 (SIK2) [86, 87]. This inhibits the ability of SIK2 to phosphorylate CREB-regulated transcription coactivator 3 (CRTC3), permitting CRTC3 to translocate to the nucleus and act as a coactivator of CREB, ultimately enhancing IL-10 production [86, 87].

Thus, PRR activation induces IL-10 production in macrophages and mDCs with further modulation of IL-10 levels by other environmental cues. However, there are still many incompletely understood aspects of IL-10 regulation in macrophages, monocytes and DCs. For example, how signalling pathways from multiple PRRs, or PRRs in combination with signals from environmental factors such as cytokines are integrated to either positively or negatively regulate *Il10* expression is not clear. Further, it is not fully understood if the ability of certain PRRs (e.g. TLR 3 and TLR 9) to more efficiently induce the production of IL-10 in macrophages compared DCs [17, 18] is a consequence of cell type-specific signal transduction pathways, or if it is also contributed to by a differing chromatin state of the *Il10* locus in the distinct immune cell types. In addition, most molecular studies investigating the regulation of *Il10* have been conducted in TLR 4 stimulated cells. Thus, the continuation of studies delineating the common and distinct factors required for *Il10* expression downstream of other TLR and non-TLR PRRs will enhance our understanding of IL-10 regulation in various immune scenarios.

8.4.2   Transcriptional Regulation of IL-10 in CD4$^+$ T Helper Cells

Within the adaptive immune system, regulation of the *Il10* gene expression has been most extensively characterised in CD4$^+$ Th cells. In contrast to macrophages and DCs that can instantly produce IL-10 in response to microbial products, naïve CD4$^+$ T cells first need to differentiate into the different effector Th cell subsets in order to produce IL-10 [2]. The different Th cells subsets include Th1, Th2 and Th17 cells, each of which expresses a unique combination of cytokines that promote their specific effector functions [3]. The distinct Th cell fates are determined by the STAT family of transcription factors signalling downstream of cytokines present in the environment during T cell activation and are ultimately controlled by the subsequent induction of lineage specific master regulators. Th1 differentiation is driven predominantly by IL-12 signalling through STAT4 [88] the expression of the master regulator T-box transcription factor (TBET) that induces the production of the Th1 hallmark cytokine IFN-γ [89]. Th2 differentiation requires IL-4 signalling through STAT6 and the expression of the master regulator GATA binding protein 3 (GATA3) [90] that induces the production of Th2 hallmark cytokines IL-4, IL-5 and IL-13 [91]. Th17 differentiation is dependent on transforming growth factor (TGF)-β and IL-6, the latter of which signals through STAT3, and the expression of the master regulator RAR-related orphan receptor gamma t (RORγt) that induces the production of the Th17 hallmark cytokine IL-17 [92, 93]. Interestingly, despite the differences in their developmental requirements outlined above, IL-10 has been shown to be produced by all of the different Th cell subsets [2]. In fact, the production of IL-

10 by Th cells is tightly interlinked with each of their differentiation programmes. For example, strong TCR signalling, together with IL-12 and STAT4 are required for IL-10 production in Th1 cells [94], while IL-4 activation of STAT6 signalling and GATA3 are essential for IL-10 production in Th2 cells [16, 94-97], and TFG-β, IL-6 and STAT3 have been shown to drive the production of IL-10 in Th17 cells [98, 99]. Additionally, type I IFN has been shown to promote IL-10 production by human CD4$^+$ T cells [41, 100, 101], although the downstream mediators of type I IFN and its effects on the different Th cell subsets are not defined (Figure 3c). Thus, the discrete signals required for IL-10 induction are tightly linked with those involved in directing the differentiation of each unique Th cell subset.

The current model of how Th cell identity is established involves a layered architecture of transcription factors induced downstream of the TCR and cytokine signalling pathways. The TCR signals are thought to induce pioneer factors able to bind closed chromatin that are, together with specific STATs, involved in the establishment of permissive epigenetic patterns across a broad range of Th cell genes in differentiating CD4$^+$ T cells [102]. In contrast, targets of the master regulator transcription factors are thought to be more limited to a set of key genes crucial for lineage specificity, be it those involved in lineage reinforcement or in the limitation of alternative fate [102]. Since the production of IL-10 is so closely intertwined with T helper lineage specification, it is likely that this model would also apply to the regulation of IL-10, whereby layers of transcription factors, induced downstream of TCR and cytokine signalling or other environmental cues, act together to promote *Il10* gene expression (Figure 3c). However, the specific signalling cascades and transcription factor complexes that determine IL-10 production by the different Th cell subsets are still unclear.

8.4.2.1   The Regulation of IL-10 by T Cell Receptor Signalling

Following TCR activation, numerous signalling pathways are initiated leading to the induction, nuclear translocation and/or activation of downstream transcription factors including AP-1, nuclear factor of activated T cells (NFAT) and NF-κB [103] (Figure 3c).

Similarly to macrophages and DCs, the activation of the MAP kinase ERK has been shown to be a common positive regulator of IL-10 in the different Th cell subsets [94]. Of note, in contrast to TPL-2 driven ERK activation downstream of TLRs in macrophages and DCs [39], in T cells ERK is activated via Ras downstream of the TCR [104]. As already mentioned, the most prominent

family of transcription factors induced downstream of ERK is AP-1. Binding of AP-1, comprised of JunB and to a lesser extent c-Jun [13], at the HSS +6.45 kb in the *Il10* gene locus in Th2, but not Th1 cells has been demonstrated to enhance IL-10 expression using luciferase reporter assays [12, 13] (Figure 2c). Furthermore, retroviral expression of c-Jun and JunB in activated naïve CD4$^+$ T cells showed a five-fold increase in IL-10 and to a lesser extent IFN-γ production, while expression of a dominant negative c-Jun protein decreased the amount of IL-10 produced, thus confirming the role of Jun family proteins in IL-10 regulation [13]. More recently, the basic leucine zipper ATF-like transcription factor (BATF), another AP-1 family member, and the transcription factor IRF4 have been shown to cooperatively bind at CNS-9, in Th2, Th9 and Th17 cells as well as CD4$^+$ T cells stimulated in the presence of IL-21 [9]. BATF has further been demonstrated to positively regulate IL-10 expression in Th2 cells, although its effect is only revealed in the absence of BATF3 [105]. Of note, the production of IL-4 in the Th2 driven *Batf$^{-/-}$Batf3$^{-/-}$* CD4$^+$ T cells was also significantly affected, indicative of a more general defect in the Th2 differentiation pathway as opposed to just IL-10 production [106]. In keeping, *Irf4$^{-/-}$* CD4$^+$ T cells stimulated in the presence of IL-21 failed to express IL-10 mRNA when compared to the wild type T cells, although the effect on other cytokines was not assessed [9]. Mutating either the IRF or AP-1 motif within CNS-9 resulted in a diminished luciferase reporter activity in IL-21 stimulated CD4$^+$ T cells consistent with functional cooperation between these factors in *Il10* gene regulation [9] (Figure 2c). Furthermore in Th2 cells, IRF4 alone has been shown to positively regulate IL-10 by binding to the *Il10* promoter and at CNS+6.45 [11] (Figure 2c). Of note, similarly to the effect of BATF on Th2 differentiation, IRF4 also increased the production of IL-4 in these cells by binding and transactivating the *Il4* promoter, showing that IL-4 and IL-10 can be co-regulated in Th2 cells, suggesting a more general effect on Th2 cell differentiation, accompanied by IL-10 production [11].

Upon TCR activation, NFAT1 translocates from the cytoplasm into the nucleus where it is known to interact with AP-1 and other transcriptional partners to promote cytokine gene transcription [107]. NFAT1 has been shown to bind regions in the *Il10* promoter in Th2 and in intron 4 of the *Il10* gene locus in Th1 cell lines [15]. In a later study, NFAT1 binding further upstream at CNS-9 together with IRF4 has been shown to synergistically enhance IL-10 expression in Th2 cells using luciferase reporter assays [10] (Figure 2c). Furthermore, blocking of NFAT signalling with a cyclosporine A inhibitor also decreased IL-10 mRNA expression in differentiated Th2 as well as Th1 cells, although it is not known what effect it had on the hallmark cytokine production and therefore could represent a generalised effect on Th cell differentiation [10].

NF-κB activation downstream of the TCR has been reported to play a role in the production of Th1 [108], Th2 [109] and Th17 hallmark cytokines [110]. Although NF-κB h□□□ □□□□□□□□□□□□□□□□□□□□□□□□□□ □□□□□□ □□ known about its role in IL-10 regulation in Th cells other than the reported binding of NF-κB□complexes at the□*Il10* gene locus in a human T lymphoma line [111].

Thus, even though CD4^{+} T cell activation through the TCR is crucial for Th cell differentiation, the roles of factors downstream of the TCR in the regulation of IL-10 are only partially characterised and warrant further investigation.

8.4.2.2   The Regulation IL-10 by Cytokine Signalling

In contrast to what is known about TCR signals promoting IL-10 production, there is a large body of evidence supporting a role for STAT and SMAD proteins downstream of cytokine receptors in the regulation of IL-10 expression in Th cells, although again this may be tightly linked to the differentiation of each Th cell subset. STAT1 downstream of IL-27 [98, 112, 113], STAT3 downstream of IL-27, IL-21 or IL-6 [98, 99, 114], STAT4 downstream of IL-12 [94] and STAT6 downstream of IL-4 [94] have all been linked to IL-10 production in Th cells (Figure 3c). Furthermore, IL-10 could potentially also enhance its own production in T cells through the activation of STAT3, as previously shown in macrophages [24]. However, it is important to note these functional studies use STAT deficient T cells that are not responsive to the cytokines and thus unable to differentiate into each discrete Th cell subset. Direct binding of the various STATs to the *Il10* locus has recently been demonstrated for STAT4 in intron 4 in Th1 cells and STAT6 in the promoter region in Th2 cells (Figure 2c), where it is thought to facilitate the acquisition of the activating H3K4me3 and loss of the repressive H3K27me3 histone marks, thus increasing the accessibility of the *Il10* gene locus to transcription factors [16]. In Th17 cells, STAT3 has been shown to bind in an intron 4 in the *Il10* gene locus [9] (Figure 2c), although its role in the remodelling of the *Il10* gene locus is yet to be determined.

SMADs downstream of TGF-β signalling have been shown to regulate IL-10 production in Th cells, albeit at the expense of inhibiting Th1 and Th2, but not Th17 differentiation [94]. In particular, SMAD4 has been shown bind and transactivate the *Il10* gene promoter in Th1 but not in

Th2 cells [115] (Figure 2d), while SMAD3 together with GATA3 has been shown to positively regulate IL-10 production in response to TGF-β in a Th2 cell line [116].

8.4.2.3 The Role of T Helper Cell Master Regulators in IL-10 Regulation

Given the role of master regulators in the differentiation of Th cell subsets, to which the production of IL-10 is tightly coupled, it is possible that IL-10 production in Th cells is further regulated by the lineage specific master regulator transcription factors induced mainly downstream of the STAT signalling but also of TCR. Recent genome wide studies of Th cells following the binding of p300, a marker of active enhancers, in wild type *versus* STAT deficient Th cells transfected with lineage specific master regulators, have identified a small subset of enhancers that are regulated directly by GATA3 [117] and to a lesser extent also by TBET [117] and RORγt [118]. Namely, overexpression of GATA3 in STAT6 deficient Th2 cells resulted in the recovery of half of the Th2-specific STAT6 dependent p300 binding sites including many in the *Il4* gene locus [117]. Similarly, overexpression of Tbet in STAT4 deficient Th1 cells resulted in the recovery of only about a quarter of the Th1-specific STAT4 dependent p300 binding sites including many in the *Ifng* gene locus [117]. The fact that GATA3 binding can precede enhancer activation in developing thymocytes [119] does suggest that it may function as a pioneer-like factor at certain genetic loci. For example in Th2 cells, GATA3 has been shown to drive the expression of IL-4 by both mediating the remodelling of the *Il4* gene locus [120, 121] and transactivation of its promoter [90]. GATA3 also binds and facilitates remodelling of the *Il10* gene locus, but it does not transactivate the *Il10* promoter [95, 96] (Figure 2d). It remains to be determined whether GATA3 acts as a true pioneer-like transcription factor or whether its action is aided by other factors in order for it to mediate the remodelling of the *Il10* gene locus. Furthermore, the fact that GATA3 alone is not able to transactivate the *Il10* promoter and is not required for IL-10 production in differentiated Th2 cells [97] illustrates the need for additional factors that promote *Il10* gene expression in differentiated Th2 cells. Importantly, GATA3 is only expressed to a high level in Th2 cells, implying that other mechanisms are likely to regulate IL-10 production in the different Th cell subsets that express no or low levels of GATA3. Since both TBET and RORγt can, similarly to GATA3, regulate a subset of genes independently of the STAT proteins it would be interesting to know whether they can also alter the accessibility of the *Il10* gene locus in addition to the hallmark cytokines in Th1 and Th17 cells.

8.4.2.4 The Role of Other Transcription factors in IL-10 Regulation

Although originally described as a Th2 specific transcription factor [122], c-MAF expression is detectable in all the different Th cells subsets, where its expression has been shown to correlate with the levels of IL-10 [94]. In Th2 cells, c-MAF is thought to direct the expression of IL-4 by binding to a c-MAF responsive element (MARE) in the proximal promoter of the *Il4* gene [122], the action of which has further been shown to be augmented by IRF4/NFAT1 [123] and AP-1 (JunB) [124]. However, c-MAF alone is thought not to be able to transactivate the *Il10* gene promoter [125]. It is yet to be determined whether c-MAF in combination with other factors such as NFAT or AP-1, similarly to their role in the regulation of IL-4, can also regulate IL-10 production, both of which have been shown to bind the *Il10* gene locus as discussed above. The expression of c-MAF in Th1 cells is driven by IL-12, however, the role of c-MAF in regulating IL-10 is only correlative whereby the expression of c-MAF decreases together with IL-10 upon treatment with a MEK inhibitor that abrogates ERK activation [94]. Furthermore, it is unclear whether ERK activation is induced downstream of the TCR via Ras, or of IL-12R via TPL-2 [94, 126], or both. In the so called 'Tr1' cells, CD4$^+$ T cells that co-secrete IFN-γ and IL-10 differentiated *in vitro* in the presence of IL-27 or IL-27 and TGF-β [127], c-MAF together with AHR have been implicated to play a role in the positive regulation of IL-10 production [128]. As such, knocking down c-MAF or AHR in 'Tr1' cells has been shown to correlate with a decrease in *Il10* mRNA expression and both have been demonstrated to bind and synergistically transactivate the *Il10* gene promoter [128] (Figure 2d). The induction of c-MAF in the 'Tr1' setting is thought to be further regulated by IL-21 and possibly by inducible T cell costimulator (ICOS) since both IL-21 and ICOS deficient T cells show reduced IL-10 production and c-MAF expression [129]. Of note, both IL-27 [130] and IL-21 [131] are thought to also activate ERK. Downstream of TGF-β signalling c-MAF has been shown to play a role in the differentiation programme of Th17 cells [132], where it is primarily thought to act as a repressor of proinflammatory gene expression e.g. of *Rora*, *Runx1*, *Il1r1*, *Ccr6*, and *Tnf* but where it can also act as an inducer of genes involved in the attenuation of inflammation, including of *Il10* [118]. c-MAF has been further shown to positively regulate IL-10 (and possibly IL-17) in Th17 cells, but to have negative effect on IL-22 production, as demonstrated using retroviral transduction and siRNA knock down assays [132]. In Th17 cells, c-MAF has additionally been shown to bind and transactivate the *Il10* gene promoter in a luciferase reporter assay [99] (Figure 2d). Taken together, these findings indicate that c-MAF could act as a common transcriptional regulator of IL-10 in different immune cell types, as suggested by both the Th cell and macrophage studies described above. However, at least in Th2 cells, it is unclear whether c-maf is involved in differentiation rather than IL-10 production *per se*.

The transcriptional repressor E4 promoter-binding protein 4 (E4BP4) encoded by the *Nfil3* gene has recently been shown to play a role in positively regulating the production of IL-10 in Th1 and Th2 cells independently of their hallmark cytokines IFN-γ and IL-4, respectively [133] (Figure 2c). E4BP4 has been shown to bind to intron 4 and to 3' UTR at the *Il10* gene locus in Th2 cells and, owing to the decrease in histone acetylation in Th2 cells from *Nfil3*^{-/-} mice, E4BP4 is thought to regulate the permissive status of the *Il10* gene locus [133]. Specifically, E4BP4 has been shown to increase *Il10* gene expression in a GATA3-independent manner, as demonstrated by retroviral transduction studies in *Nfil3*^{-/-} and *Gata3* conditional knockout cells [133]. Thus, E4BP4 could be a dominant factor responsible for epigenetic regulation controlling accessibility of the *Il10* gene locus in Th1 cells, akin to GATA3 in Th2 cells. Although it is not yet known which signalling pathways lead to the induction of *Nfil3* expression in T cells, in macrophages *Nfil3* is an IL-10 responsive gene [134]. Of note, overexpression of E4BP4 does not allow Th17 differentiation [118], implying that other mechanisms must operate to allow IL-10 production in Th17 cells. Moreover, *Nfil3*^{-/-} mice develop mild diarrhoea reminiscent of colitis observed in *Il10*^{-/-} animals [133]. It is not clear, however, whether this is due to the possible lack of IL-10 in T cells or myeloid cells, a Th cell imbalance, or all of these.

PR domain zinc finger protein 1 (BLIMP-1) encoded by the *Prdm1* gene is a transcriptional repressor induced downstream of the TCR that has also been shown to positively regulate IL-10 production in CD4^{+} T cells, as demonstrated by decreased IL-10 production in *Prdm1*^{fl/fl} *Lck Cre*^{-} CD4^{+} T cells, which *in vivo* is accompanied by pathology and increased inflammation [135]. However, in addition to the observed decrease in IL-10 production, *Prdm1*^{fl/fl} *Lck Cre*^{-} CD4^{+} T cells produce increased amounts of IL-2 and IFN-γ□□□□□□□□□heir IL-4 production is not affected and the effect on IL-17 production is unknown [135]. BLIMP-1 downstream of IL-27 has further been demonstrated to mediate IL-10 induction in CD4^{+} [136] and CD8^{+} T cells [137]. IL-10 production by CD4^{+} Treg cells is also regulated by BLIMP-1, primarily by its synergistic effect with IRF4 on histone acetylation at the *Il10* gene promoter [138]. However, the molecular mechanism of how BLIMP-1 regulates IL-10 production in Th cells is not known. Hence the severe colitis that is observed in *Prdm1*^{fl/fl} *Lck Cre* mice may be due to the collective effect of defective thymic output, decreased Treg cell IL-10 production and/or excessive effector T cell function [135].

Of note, BLIMP-1 expression is regulated by B-cell lymphoma 6 (BCL-6), itself a transcriptional repressor typically involved in the establishment of T follicular helper (Tfh) cell identity [139-141]. Similarly to what is known about BCL-6 regulation in B cells, the action of BCL-6 in T cells is in turn suppressed by BLIMP-1 [140]. BCL-6 expression is driven by IL-6 and IL-21 and it functions

mainly by inhibiting the expression and/or function of TBET, GATA3 and RORγt master regulator transcription factors thus suppressing the Th1, Th2 and Th17 differentiation programmes, respectively, while concurrently inducing the expression Tfh cell related genes [142, 143]. In view of the reciprocal regulation of BLIMP-1 and BCL-6, it is possible that the role of BLIMP-1 in IL-10 regulation is indirect via the repression of BCL-6 and subsequent upregulation of GATA3 [144].

8.4.2.5   The regulation of IL-10 by Notch Signalling

In addition to the TCR and cytokine signals, signalling via Notch following interaction with ligands induced on macrophages and DCs by pathogen-derived signals have been shown to have an impact on the differentiation of CD4$^+$ Th cells as well as their production of IL-10 (Figure 3c). In particular, the engagement of Delta-like Notch ligands (DLL) has been shown to favour the development of Th1 cells, while ligation of Jagged preferentially leads to Th2 cell differentiation [145]. DLL-1 and DLL-4 Notch ligands have further been shown to induce IL-10 but have little effect on IFN-γ production in Th1 cells [146]. Of interest, Jagged1, typically associated with inducing IL-4 production via Notch, has paradoxically been identified as the ligand of CD46 [147] driving the production of IL-10 in human T cells that co-secrete IFN-γ [148]. However, the exact molecular mechanisms of IL-10 regulation by Notch signalling are not yet known and warrant further investigation.

8.4.2.6   Negative Regulators of IL-10 Production

Similarly to macrophages and DCs, negative mechanisms of IL-10 regulation have also been identified in Th cells.

E26 transformation-specific 1 (ETS-1) is a member of the ETS family of transcription factors, the expression of which is high in resting T cells but decreases following their activation [149]. Of note, ETS-1 can be further regulated at a post-transcriptional level by ERK [150], although it is not known whether this is the case in T cells. ETS-1 has been shown to negatively regulate IL-10 production in Th1 and Th2 cells, as demonstrated by enhanced IL-10 in *Ets1*$^{-/-}$ CD4$^+$ T cells stimulated under Th1 and Th2 skewing conditions, though the effect of ETS-1 deficiency on the hallmark cytokines has not been investigated [151]. Of note, ETS-1 has previously been shown to positively regulate IFN-γ together with TBET [152]. ETS-1 has been to shown to downregulate the expression of IL-10 in Th1 cells via its interaction and recruitment of HDAC1 to HSS -0.12, +1.65

and +2.98 kb at the *Il10* gene locus, thus maintaining its relatively closed conformation [151] (Figure 2c).

PU.1, another member of the ETS family, is expressed specifically in Th2 cells where it inhibits the production of Th2 hallmark cytokines including IL-10 by interfering with DNA binding of the master regulator GATA3, although a direct effect PU.1 on the *Il10* gene locus has not been reported [153]. In addition to GATA3, PU.1 also complexes with IRF4, a transcription factor correlating with high levels of IL-10 expression in Th2 cells, thus preventing the ability of IRF4 to bind and to transactivate the *Il10* gene promoter [11].

In summary, the above studies indicate that the activity of transcription factors downstream of the TCR, cytokine signalling and other environmental cues regulate the production of IL-10 at multiple levels ranging from increasing the accessibility of the *Il10* gene locus to cooperative binding of transcription factors. This allows the assembly of transcription factor complexes at discrete sites along the *Il10* gene locus which likely act as platforms to integrate TCR and cytokine driven signals allowing the transcription of the *Il10* gene in a context specific manner. However, the components of the transcription factor complexes and their temporal recruitment to the *Il10* gene locus during the differentiation of the various Th cell subsets remain unclear and require further investigation. However, the tight relationship between Th cell differentiation versus IL-10 production poses a challenge in the future studies in this field. Furthermore, it will be important to determine what are the common or cell type specific requirements for IL-10 production in the Th cell subsets. Ultimately, these studies will need to be recapitulated in human cells, as most of the data to date has been generated using mouse as a model organism.

8.5 Postranscriptional regulation of IL-10

Post-transcriptional control of cytokine production is a key step for the regulation of the amount of cytokines produced by cells of the immune system [154, 155] (Figure 1). This layer of regulation is critical to ensure that the rapid transcription of cytokines in response to an initial stimulus, such as microbial infection, is also rapidly turned off to avoid excessive pro-inflammatory cytokine production likely resulting in tissue damage, or the development of chronic infection in the case of an anti-inflammatory cytokine such as IL-10. One of the first pieces of evidence of post-transcriptional regulation of *Il10* expression came from studies of the 3'UTR of the *Il10* mRNA, which showed the existence of AU rich elements (AREs), capable of mediating mRNA decay in a

lymphocyte cell line [156]. Another study, however, concluded that the same destabilizing effect of the 3'UTR of *Il10* mRNA was not observed in RAW 264.7 macrophages [157]. AREs are found in many cytokine 3'UTRs and mark short-lived mRNA, as they recruit various ARE-binding proteins that increase or decrease mRNA stability [154]. The ARE-binding protein that was identified in a genome wide analysis to target *Il10* mRNA is tristetraprolin (TTP) [158]. TTP also promotes the rapid decay of other cytokine mRNAs, including that of tumour necrosis factor (TNF) [159], IL-6 [160] and granulocyte-monocyte colony stimulating factor (GM-CSF) [161], by initiating the assembly of RNA decay machinery thus causing the elimination of bound mRNAs [162, 163]. In line with a role for TTP in regulating *Il10* mRNA stability, the half-life of *Il10* mRNA in TLR 4 stimulated macrophages was increased in the absence of this molecule [158, 164]. Consequently, in the absence of TTP the amount of IL-10 produced by TLR 4 stimulated macrophages was found to be higher [164]. More importantly, higher IL-10 levels were also described *in vivo* in the serum of LPS-treated mice where TTP was ablated from myeloid cells [165]. However, such differences in IL-10 production were not found in TLR 4 stimulated TTP deficient DCs [166], potentially due to increased p38 activity in these cells [167]. Indeed, TTP is negatively regulated by the MAP kinase p38 [168-171], which although needed for TTP expression, also restrains the mRNA destabilizing activity of TTP [172]. Although the impact of p38 control of TTP-mediated mRNA destabilization varies with target mRNAs, in the case of IL-10, the inhibition of p38 activation allows for elevated TTP activity and thus enhanced TTP-mediated mRNA decay [171]. In a recent study, the activity of p38 during the inflammatory response was shown to qualitatively and temporally control TTP activity, thus leading to a timely induction of cytokine mRNA decay [165]. This temporal control of TTP by p38 is influenced by different mechanisms including by TRIF signalling, as shown by comparing the activity of p38 activity and the IL-10 mRNA decay in macrophages stimulated through TLR 2 or TLR 4 (Teixeira-Coelho et al, in press). Although less studied than TTP, the RNA-binding protein ARE/poly(U) binding degradation factor 1 (AUF1) is also known to bind to the 3'UTR of the IL-10 mRNA. Diminished levels of AUF1 correlated with increased half-life of the IL-10 mRNA in a melanoma cell line [173]. More recently, the translocation of AUF1 from the nucleus to the cytosol of TLR 4 stimulated macrophages was found to be regulated by DUSP1 [174]. Consequently, in the absence of DUSP1, both *Il10* mRNA stability and IL-10 secretion were increased [175].

IL-10 has been shown itself to regulate the stability of cytokine mRNAs, including its own, in TLR 4 stimulated blood monocytes [176]. More recently, this effect of IL-10 was found to be dependent on TTP. In addition to being a target of TTP, IL-10 is itself an activator of TTP, by increasing TTP expression and reducing late p38 activity [164, 177]. Thus, in situations when IL-10 is being

produced, TTP is activated and a post-transcriptional program for cytokine gene regulation is initiated, mediated by IL-10 induction of mRNA decay. It is possible that this mechanism results in limiting IL-10 translation, thus helping in guaranteeing the appropriate balance of the immune response.

Additionally to the 3'UTRs that control the mRNA stability and decay rates, 5'UTR regions are also linked to post-transcriptional gene regulation by mediating the binding rate of mRNA to the ribosomal 40S subunit, thus controlling the mRNA translation [178]. Two alternative variants of the 5'UTR sequences were found in *Il10* mRNA synthesized after TLR 4 stimulation of blood cells, suggesting that modulation of IL-10 protein translation may also be implicated in the network of post-transcriptional mechanisms regulating IL-10 [179].

MicroRNAs (miRNA) have also been shown to contribute to the post-transcriptional mechanisms regulating TLR signalling and subsequent cytokine production, including that of IL-10 [180]. For example, hsa-miRNA-106a binds to the 3'UTR of the IL-10 mRNA and negatively regulates its expression in different cell lines [181]. Also, miRNA-466l was shown to upregulate TLR-induced IL-10 expression in macrophages by antagonizing TTP-mediated *Il10* mRNA degradation and extending the half-life of *Il10* mRNA [182]. MiRNA-21 induced upon TLR 4 stimulation targeted the effector molecule programmed cell death 4 (PDCD4) and promoted IL-10 production [183].

Thus, a growing body of evidence clearly demonstrates that IL-10 production by immune cells is controlled at the post-transcriptional level along with many other cytokines. Since the majority of the studies have been performed in TLR-activated macrophages, it will be interesting to study these potential mechanisms in other immune cells. For example, in CD4$^+$ T cells, p38 was found to be involved in regulating the stability of IL-4 and IL-5 mRNAs [184], but such studies are lacking in relation to IL-10.

8.6   Concluding remarks

IL-10 mediates the fine balance between immunosuppression and immunopathology in a number of settings. Therefore, a thorough understanding of the molecular mechanisms that regulate the expression of this cytokine is crucial for the design of immune intervention strategies. The subject of IL-10 regulation is complicated by the fact that IL-10 can be expressed by almost all cells within both the innate and adaptive immune systems, despite the fact that each cell type responds to

different stimuli. This complexity is further compounded by the numerous positive and negative regulatory networks which integrate to determine the final level of IL-10 production.

In macrophages and DCs, although several mechanisms have been identified which regulate the production of IL-10, most studies focus on single PRR stimuli, whereas pathogens likely activate multiple PRRs. As such, how the signals from different PRRs co-operate to modulate IL-10 production versus the production of different proinflammatory cytokines remain only partially understood. Additionally, the production of IL-10 in response to damage associated molecular patterns (DAMP) has to date not been investigated. These studies would provide a better understanding of the regulation of IL-10 in the context of infection and sterile inflammation. In addition, the mechanisms controlling the distinct levels of IL-10 produced by the different myeloid populations, for example the relatively higher level of IL-10 production by macrophages compared to DCs, are poorly defined. Fully investigating whether the signalling pathways induced by the same TLRs are fundamentally different in macrophages, DCs and myeloid populations from distinct anatomical locations, or if the chromatin state of the *Il10* locus is different between these cell types would greatly enhance our understanding of how IL-10 is regulated.

In T cells, several signalling pathways and downstream transcription factors regulating IL-10 have been identified in the different Th cell subsets. However, the ultimate challenge lays in distinguishing whether these factors are truly a requirement for IL-10 expression as opposed to a requirement for Th cell differentiation, or both. Furthermore, many of the studies to date concentrate on a single Th cell subset. A concurrent analysis of the all the subsets would allow us to determine whether there are common or Th cell type specific factors for IL-10 production in the different Th cell subsets. Lastly, owing to the heterogeneity within each of the Th cell populations with respect to hallmark cytokine as well as IL-10 production, it would be important to investigate these separately in order to obtain more meaningful results.

Therefore, for all cell types, including those currently less studied but known to produce IL-10, such as mast cells, B cells or regulatory T cells, the future challenge is to decipher the complex IL-10 regulatory networks including the temporal recruitment of transcription factors and their hierarchies of binding to the *Il10* gene locus. The use of genome-wide, high-throughput, high resolution and bioinformatic approaches will complement traditional molecular and biochemical methods and be key to gaining a better understanding of IL-10 regulation in the different immune cell types. Finally, it will be important to identify the immune scenarios in which these various

factors regulate IL-10 production *in vivo* in order to use this knowledge in the fine-tuning of immune intervention strategies.

Figure Legends:

Figure 1: Concepts of gene regulation.

Mechanisms governing gene expression operate at several levels including chromatin accessibility, active regulation of transcription as well as post-transcriptional regulation. Within chromosomes, DNA is wrapped around octamers of core histones forming nucleosomes, the basic units of eukaryotic chromatin. Multiple nucleosomes are then arranged along the DNA as 'beads on a string' (accessible euchromatin). The compaction of chromatin into 30 nm fibres is achieved upon the addition of linker histones to nucleosomes giving rise to inaccessible, transcriptionally inactive heterochromatin. Depicted are features governing the accessibility of chromatin including DNA methylation, histone modifications or DNase hypersensitive sites (HSS). Open chromatin allows for transcription factor binding to gene regulatory elements such as distal regulatory elements (DRE) or promoters and initiation of gene expression by RNA polymerase. Once transcribed, mRNA can further be subject to post-transcriptional regulation at the 3'UTR by AU rich (ARE)-binding proteins that increase or decrease mRNA stability by preventing or aiding its degradation by RNA decay machinery, respectively.

Figure 2: A diagram of mouse chromosome 1 showing the *Il10* gene locus.

(a) *Il10* gene and its neighbours, horizontal arrows (blue) depict orientation of gene expression. (b) Detail of DNA surrounding the *Il10* gene locus, vertical arrows denote HSS sites present equally in Th1 and Th2 cells (black) or more prominently in Th2 cells (green) aligned against Vista plot of the murine sequence *versus* human homology, pink peaks denote CNS. (c) *Il10* gene locus and its regulatory elements again aligned against Vista plot of the murine sequence *versus* human homology, pink peaks denote CNS, purple peaks denote exons and turquoise peak denotes 3' UTR. Arrows denote HSS sites with their relative position to the *Il10* TSS indicated, and the cell type in which they have been detected depicted (macrophage, DC, Th1 and Th2), note that HSS -0.12 and +6.45 kb have been shown to be more prominent in Th2 over Th1 cells. The binding sites of transcription factors that have been shown to regulate *Il10* gene expression in the different cell types (indicated by different colour borders) are also shown. (d) *Il10* gene proximal promoter, depicts further binding sites of transcription factors regulating *Il10* gene expression.

Figure 3: Signals that induce IL-10 expression in representative cells of the innate (macrophages and DCs) and adaptive (Th cells) immune system.

(a) The expression of *Il10* is induced by signals downstream of PRRs, such as TLRs, in macrophages and mDCs. Downstream of TLR ligation the recruitment of the adaptor molecules

MYD88 and TRIF are critical for the activation of downstream signalling pathways. Of these, ERK and p38 MAP kinases, the PI(3)K-AKT pathway and the NF-κB pathway have been shown to have roles in the induction of *Il10* gene expression. Importantly, several of these pathways also mediate the expression of pro-inflammatory cytokines. Non-TLR PRRs can also induce IL-10 production. An example of this is Dectin-1, which is expressed mainly on DCs and induces IL-10 production via a SYK and ERK dependent pathway. *Il10* expression is further modulated by signals induced by surrounding cytokines such as type I IFN and IL-10 itself which promote IL-10 production, and IFN-γ which inhibits IL-10 production through the inhibition of MAP kinase and PI(3)K-AKT pathways. (b) The TPL-2/ERK pathway induces IL-10 production which positively feeds back to upregulate TPL-2. In contrast, the TPL-2/ERK pathway negatively regulates IFN-β production, itself a positive regulator of IL-10. The negative regulation of IFN-β by this pathway is both dependent and independent of IL-10 production. This complex molecular network also includes the regulation of pro-inflammatory cytokines e.g. TNF and IL-12, which are positively and negatively regulated by the TPL-2/ERK pathway, respectively. (c) The expression of IL-10 in CD4$^+$ T cells is initiated by signalling downstream of the TCR and costimulatory molecules such as CD28 and involves the MAP kinase ERK, the calcineurin-NFAT and the NF-κB pathways, amongst others. It is further modulated by signals downstream of cytokine receptors via STAT or SMAD proteins that are also critical for the differentiation of naïve CD4$^+$ cells into the different Th cell subsets. At least in Th2 cells, IL-10 production is further regulated by the lineage specific master regulator transcription factor GATA3 induced mainly downstream of the STAT6 signalling but also of TCR. In addition to the TCR and cytokine signals, signalling through Notch ligands induced on macrophages and DCs by pathogen-derived signals has an impact on the differentiation of CD4$^+$ Th cells as well as their IL-10 production.

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