ANALYSIS OF NFAT5 EXPRESSION AND ACTIVITY IN RESPONSE TO TOLL-LIKE RECEPTORS

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A Simo

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THESIS ABSTRACT

Stimulation of Toll-like receptors (TLRs) in cells of the innate immune system activates the expression of a proinflammatory and antimicrobial gene program controlled by a network of transcriptional regulators. We show that NFAT5, which belongs to the Rel family of transcription factors and was previously characterized as an osmostress responsive factor, is required for the expression of a group of TLR-responsive genes in macrophages, such as *Nos2*, *Il6* and *Tnf*. NFAT5 recruitment to its target genes is dependent on IKKβ activity, *de novo* protein synthesis and is sensitive to histone deacetylases. Interestingly, NFAT5 is essential in the response to low doses of TLR ligands, regulating specific gene subsets depending on the stimulus strength. We also show that macrophages use NFAT5 to facilitate chromatin accessibility, allowing the recruitment of transcriptional regulators such as p65/NF-κB, c-Fos and p300 to its target regions. We use *Nos2* as a gene whose induction is NFAT5-dependent especially at low doses of LPS to demonstrate that NFAT5 controls the recruitment of p65 by facilitating the activity of H3K27 demethylases, without influencing the binding of Polycomb repressive complex 2 or JMJD3. Altogether, this thesis characterizes NFAT5 as a novel regulator of the immune response to low pathogen load involved in the control of local chromatin accessibility.

RESUMEN DE LA TESIS

En las células del sistema inmunitario innato, la estimulación de los receptores de tipo Toll (TLR) activa la expresión de un programa génico pro-inflamatorio y antimicrobiano que está controlado por una red de reguladores transcripcionales. Hemos demostrado que el NFAT5, perteneciente a la familia de factores de transcripción Rel y previamente caracterizado como un factor de respuesta a estrés osmótico, es importante para la expresión de un grupo de genes de respuesta a TLRs, entre ellos *Nos2*, *Il6* y *Tnf*. El reclutamiento del NFAT5 a sus genes diana requiere la actividad de IKKβ, la síntesis *de novo* de proteínas y es sensible a la acción de las deacetilasas de histonas. Resulta interesante el hecho de que el NFAT5 es esencial para responder a bajas dosis de ligando de los TLRs, y que regula grupos de genes específicos dependiendo de la intensidad del estímulo. También mostramos que NFAT5 facilita la accesibilidad de la cromatina en macrófagos, permitiendo el reclutamiento de reguladores transcripcionales como p65/NF-κB, c-Fos y p300 a sus regiones diana. Utilizando *Nos2* como un gen cuya inducción es más dependiente de NFAT5 a bajas dosis de LPS, demostramos que el NFAT5 controla el reclutamiento de p65 gracias a que facilita la actividad de las demetilasas de H3K27, pero sin influir en la unión del complejo Polycomb 2 ni JMJD3. En conclusión, esta tesis caracteriza al NFAT5 como un nuevo regulador del sistema inmunitario implicado en el control de la accesibilidad local de la cromatina en respuesta a baja carga de patógenos.

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INTRODUCTION

1. INNATE IMMUNITY

In vertebrates, the immune system is divided in two branches: innate and adaptive immunity. The innate immune system is the first line of defense of an organism and is evolutionarily conserved. It discriminates "self" from "non self" and it senses any signal of danger, not only pathogenic microbes, but also host-derived signals of cellular stress (Schroder and Tschopp, 2010). Innate immunity provides a rapid response without antigen-specificity and also activates the adaptive immune response. The innate immune system consists of soluble factors, like complement proteins, and cellular components. Cells of the innate immunity are macrophages, dendritic cells, natural killer cells, granulocytes (basophils, neutrophils, eosinophils) and mast cells.

When immune cells, such as tissue macrophages, encounter an antigen, they initiate the inflammatory response within few minutes. Inflammation has evolved as a rapid response to noxious insults, in which host cells recognize stimuli through different sensing mechanisms, in most cases requiring transmembrane receptors. Activation of these receptors transmits signals to the nucleus, leading to the expression of several genes (Kawai and Akira, 2006) that carry out many physiological functions, such as proinflammatory cytokines and chemokines, antimicrobial products, genes of pathogen recognition and targeting and also anti-inflammatory mediators (Foster et al., 2007).

1.1. MACROPHAGES

Leukocytes are a heterogeneous group of cells that mediate the immune response, circulating through the blood and the lymphatic system and being recruited to sites of infection and tissue damage. Leukocytes share a

common hematopoietic stem cell origin, but develop through distinct differentiation pathways in response to internal and external signals. Mononuclear phagocytes are a subgroup of leukocytes derived from bone marrow myeloid cells that circulate in the blood as monocytes and are found to be resident in tissues as macrophages (Geissmann et al., 2010). Monocytes are needed to replenish the pool of resident macrophages and dendritic cells (DCs) in tissues, both in steady state and during inflammatory response. Monocytes, macrophages and DCs, but also neutrophils and mast cells, are phagocytic cells that express a big number of receptors on their surface to detect signals of danger for the organism. These receptors have different functions, like recognition and binding of apoptotic and necrotic cells (scavenger receptors) and recognition of "non-self" (pattern recognition receptors-PRRs) (Murray and Wynn, 2011). PRRs specifically recognize pathogen-associated molecular patterns (PAMPs), which are unique to microbes and whose detection determines the discrimination between self and non-self by the immune system (Carpenter and O'Neill, 2007).

Macrophages were discovered by Mechnikov at the end of the XIX century. A main characteristic of macrophages is the ability to phagocyte microbes forming phagosomes that mature into phagolysosomes in which pathogens are trapped and digested by proteases, antimicrobial peptides and lysozyme (Garin et al., 2001). Macrophages and other phagocytic cells, like neutrophils, rapidly produce reactive oxygen species (ROS) as a critical weapon against bacteria (Nathan, 2006). This process is called oxidative (or respiratory) burst. The generation of microbicidal oxidants comes from the activation of the enzymatic complex NADPH oxidase, which catalyzes the formation of ROS (Roos et al., 2003). The importance of macrophage oxidative burst in innate immunity is demonstrated by a genetic disorder, the chronic granulomatous disease (CDG), characterized by defects in NAPDH oxidase. Patients suffering CDG present severe and recurrent bacterial infections and

develop granulomas formed by the fusion of macrophages that have phagocyted bacteria but are unable to destroy them with ROS (Heyworth et al., 2003).

Different macrophage subsets corresponding to different functions have been described (Martinez et al., 2008). M1 macrophages, or classically activated macrophages, are the responsible of the inflammatory response against pathogens and also have a role in antitumoral immunity. M2 macrophages, or alternatively activated macrophages, act as anti-inflammatory and regulate wound healing. Tumor associated macrophages (TAMs), as M2 macrophages, act as immune suppressors, and repress antitumor immunity (Murray and Wynn, 2011). Anyway, the different subsets of macrophages activation have to be considered like a spectrum with flexibility in reprogramming, not like stable subpopulations, as macrophages can switch from one phenotype to a different one in response to environmental signals (Mosser and Edwards, 2008).

In basal conditions, macrophages have anti-inflammatory functions (Martinez et al., 2008). Activation occurs when macrophages sense tissue damage or microbial infection. At this point, macrophages start to express a proinflammatory gene program that includes mediators such as tumor necrosis factor-alpha (TNF-α), nitric oxide (NO) and interleukin 6 (IL-6), that act through various anti-microbial mechanisms (Foster et al., 2007). Other mediators have the effect of recruiting cells of the adaptive immunity, as in the case of IL-12 production that is required by T helper 1 cells to produce interferon-γ (IFN-γ) (Hamza et al., 2010). At the same time, antigen stimulation induces also M2 macrophages to initiate an anti-inflammatory feedback loop, with the production of IL-10 or TGF-β, to avoid detrimental over-activation of inflammation (Martinez et al., 2008).

Macrophages recognize through PRRs signals associated with invading agents. Signaling PRRs, that include Toll-like receptors (TLRs), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), retinoic acid-inducible gene 1 (RIG1)-like receptors and cytoplasmic DNA receptors, detect microbial products and aberrant self on the cell surface or in the cytoplasm, and activate signaling cascades leading to the activation of transcriptional mechanisms in proinflammatory genes (Akira et al., 2006). TLRs will be presented in detail afterwards. NLRs are a family of intracellular PRRs whose members share a nucleotide-binding domain, a leucin-rich repeat domain and a caspase recruitment domain (CARD). NLRs sense various self and non-self molecules and activate important gene regulators like NF-κB (Dostert et al., 2008). RIG1-like receptors are another family of intracellular sensors. They recognize intracellular viral RNA through an RNA helicase and, as NLRs, they contain a CARD domain for the transmission of signaling, that ends with the activation of IRFs, NF-κB and AP1 transcription factors (Dostert et al., 2008). The recognition of cytoplasmic DNA is performed by multiple intracellular receptors. DNA-dependent activator of IRFs (DAI), absent in melanoma 2 (AIM2) and RNA polymerase III are some examples (Hornung and Latz, 2010).

1.2. TOLL-LIKE RECEPTORS (TLRs)

Among PRRs, Toll-like receptors are probably the best characterized immune sensors of offensive pathogens for many reasons: they are largely distributed on different cells of the immune system, they are evolutionarily well conserved and they play a fundamental role in the initiation of the innate immune response.

1.2.1. Structure and types of TLRs

There are 10 functional human TLRs (TLR1 to TLR10), 12 in mice (TLR1 to TLR9 and TLR11 to TLR13), with TLR 1-9 conserved in both species

(Lee et al., 2012). TLRs are transmembrane proteins that homo- or hetero-dimerize. All of them contain an extracellular leucine-rich motif responsible for the recognition of pathogens, and a transmembrane and cytosolic TIR (Toll/interleukin-1 receptor) domain that initiates intracellular signaling (Akira et al., 2006). The recognition of PAMPs by TLRs takes place in various cellular compartments, including the plasma membrane and endosomes (Akira et al., 2006). For all TLRs, except for human TLR10 and for mouse TLR12 and TLR13, ligands have been determined (Lee et al., 2012). In figure 1 the prototypical ligands of each TLR and its cellular localization are represented.

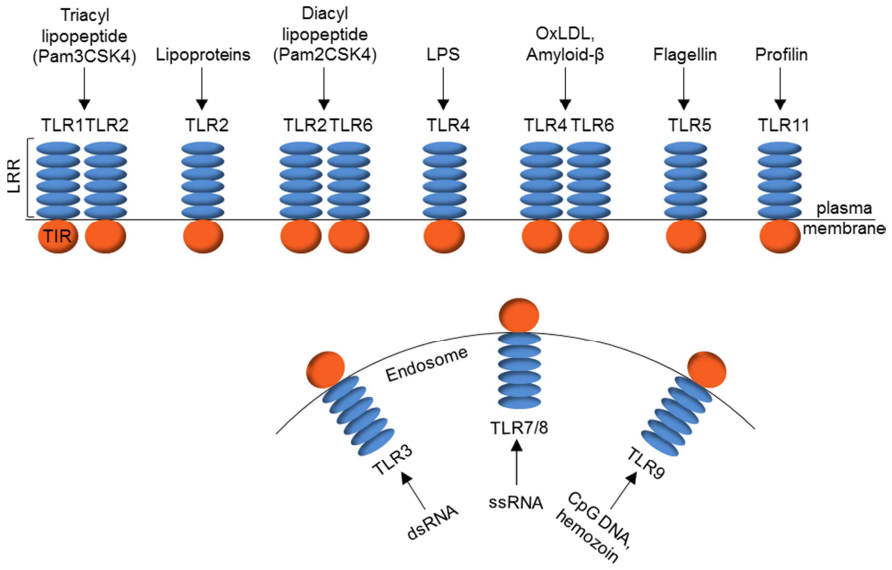


Figure 1. Schematic structure, subcellular localization and specific ligands of well-characterized murine TLRs. The ligand-binding portion of TLRs is constituted by leucine-rich repeats (LRR), represented in blue, while the cytoplasmic region constists on the Toll/Interleukin-1 receptor (TIR) domain, in orange.

1.2.2. Signaling of TLRs

Stimulation of TLRs gives rise to a potent proinflammatory response. For this reason, their signaling is tightly regulated to avoid the overactivation of pathways that could result in infectious and inflammatory disorders (Kawai and Akira, 2006; Kondo et al., 2012). Before ligand binding, TLRs are supposed to be preassembled in dimers with low affinity. When the ligand is bound, the receptors undergo a conformational change that brings two TIR domains on the cytoplasmic face into close proximity, forming a platform to build the signaling complex (O'Neill and Bowie, 2007). Signaling is initiated by adaptors containing TIR domain that are recruited to the TIR-TIR platform formed by dimerized receptors. Five TIR adaptors have been described: myeloid differentiation factor-88 (MyD88), MyD88 adapter-like protein (Mal or TIRAP), TIR domain-containing adaptor protein inducing IFNβ (TRIF), TRIF-related adaptor molecule (TRAM) and sterile α- and armadillo-motif-containing protein (SARM) (Kenny and O'Neill, 2008).

MyD88 is the first identified member of TIR family adaptors and it is required by all TLRs but TLR3 (O'Neill and Bowie, 2007). It leads to the activation of NF-κB transcription factors and of mitogen-activated protein kinases (MAPKs) to express proinflammatory cytokines. On the other hand, TRIF is one adaptor only used by TLR3 and TLR4 and induces pathways that activate IRF3 and NF-κB transcription factors, resulting in type I interferon and inflammatory cytokines production (O'Neill and Bowie, 2007). TRAM and TIRAP/Mal work as sorting adaptors that recruit TRIF to TLR4 and MyD88 to TLR2 and TLR4, respectively (Kawai and Akira, 2010). SARM expression is induced upon TRIF activation and it interacts with TRIF, interfering with its function, resulting in a negative feedback loop regulation of NF-κB and IRF activation (O'Neill and Bowie, 2007).

In the MyD88-dependent pathway (figure 2), binding of a PAMP to its respective TLR causes the recruitment of MyD88 to the TIR domains of the receptors and then the subsequent recruitment of the IL-1 receptor-associated kinase IRAK4 (Akira et al., 2006). After IRAK4 recruitment, IRAK1 and IRAK2 are activated sequentially and the result of this activation is their interaction with TRAF6, an E3 ligase that catalyzes the synthesis of polyubiquitin chains linked to lysine (K) 63 on target proteins, including TRAF6 itself and IRAK1. The polyubiquitin K63 then binds to TAB2 and TAB3, the regulatory components of the kinase TAK1 complex, to activate TAK1, which forms a complex with the IκB-kinase complex (IKK), allowing TAK1 to phosphorylate IKKβ and finally to activate NF-κB via degradation of its cytoplasmic repressors, the IκB proteins. TAK1 also phosphorylates the mitogen activated protein kinases (MAPKs) Erk1, Erk2, p38 and Jnk, which then activate transcription factors, such as AP1, and also regulate translation, leading to the expression of inflammatory cytokines (Kawai and Akira, 2010).

In the TRIF-dependent pathway (figure 2), TRAF6 is recruited by TRIF probably through mechanisms of ubiquitylation similar to those of the MyD88-dependent pathway, resulting in TAK1 activation and NF-κB-dependent gene expression. TRIF also recruits RIP1 adaptor via TRADD and Pellino-1; RIP1 undergoes K63-linked polyubiquitylation after TLR3 stimulation and this modification is required for TAK1 complex activation, which in turn activates NF-κB and MAPKs. Importantly, the TRIF-dependent pathway also leads to IRF3 activation and IFNβ transcription: TRIF requires TRAF3 to activate TBK1 and IKKι (IKKε), which catalyze IRF3 phosphorylation and induce its nuclear translocation, resulting in IFNβ transcription (Kawai and Akira, 2006; Kawai and Akira, 2010).

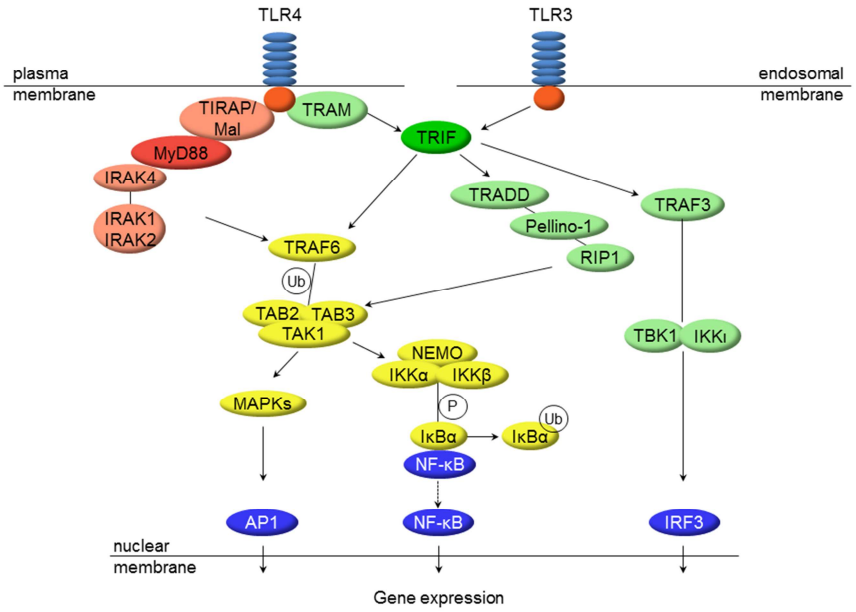


Figure 2. TLR4 and TLR3 signaling pathways. TLR4 stimulation induces both MyD88- (in red) and TRIF- (in green) dependent signaling pathways, while TLR3 stimulation only activates the TRIF-dependent pathway. The TRIF-dependent pathway can activate IRF3 via TRAF3. MyD88- and TRIF-dependent pathways converge in TRAF6-mediated (in yellow) activation of MAPKs and the IKK complex, leading to the activation of AP1- and NF-κB-driven transcription of inflammatory genes. Of note, TLR4 association with TRAM has been described to occur in endosomes.

Focusing on TLR4, it has been described that the signaling from this receptor needs cofactors for ligand recognition. As described in figure 1, TLR4 recognizes lipopolysaccharide (LPS), a component of Gram-negative bacteria outer membrane. One of the cofactors needed by TLR4 is LPS-binding protein (LBP), a soluble molecule that binds LPS with high affinity and facilitates its disaggregation and its presentation to CD14. CD14 acts as a cofactor for several TLRs, including TLR4. It is a glycoprotein receptor

present as soluble protein in the blood or as a glycosylphosphatidylinositol (GPI)-anchored membrane protein on myeloid cells. CD14 binds diverse microbial products, including LPS, and potentiates their capacity to activate TLRs. MD2 is a third cofactor needed by TLR4. It associates with the extracellular domain of TLR4 and it is necessary for the stabilization of the receptor on the cellular membrane, but also for LPS recognition by TLR4, since MD2-LPS complexes help the bridging of the two TLR4 molecules (Lee et al., 2012).

As previously mentioned, TLR signaling needs a controlled negative regulation to avoid detrimental and inappropriate activation that can lead to the development of autoimmune and inflammatory diseases. Negative regulation is achieved through different mechanisms: one of these is to prevent association between adaptors, blocking downstream signaling pathways. One example is the SARM adaptor, which, after LPS stimulation, binds TRIF, blocking the formation of subsequent signaling complexes (Kondo et al., 2012). Another example is A20, an LPS-inducible gene that works as a deubiquitylation enzyme to remove K63-linked polyubiquitin chains from TRAF6, blocking the NF-κB signaling (Kondo et al., 2012). In other cases, the expression of splice variants of signaling components of the TLR cascade competes with their homologs, such as IRAKM, which inhibits the dissociation of IRAK1 and IRAK4 from the TLR complex, and MyD88s, a splice variant of MyD88 that impairs IRAK1 phosphorylation (Han and Ulevitch, 2005). A different mechanism of negative modulation is the one used by suppressor of cytokine signaling (SOCS) proteins of the E3 ubiquitin ligase family, which promote degradation of TIRAP/Mal or TRAF via ubiquitylation (Kondo et al., 2012). TLR signaling can also be negatively modulated through transcriptional regulation; this is the case of IκBδ, a TLR-inducible gene that blocks the expression of IL-6 and IL-12p40, or cyclic AMP-dependent transcription factor (ATF3), which recruits histone

deacetylase 1 (HDAC1) to the regulatory regions of proinflammatory genes, silencing their transcription (Kondo et al., 2012). If negative regulation mechanisms fail, activation-induced apoptosis can ensure the elimination of hyper-responsive cells (Liew et al., 2005): in circumstances of hyperactivation, recruited MyD88 can interact with FAS-associated death domain (FADD) through their death domains and trigger apoptosis via caspase activation. The TRIF-dependent pathway can also trigger apoptosis when the TRIF signal results in the activation of PKR and IRF3 instead of NF-κB.

1.2.3. Effectors of TLRs

The effect of TLR activation, common among all TLRs, is the induction of gene products with antimicrobial and immunomodulatory properties (Jenner and Young, 2005). As previously described, the signaling cascades activated by TLR stimulation lead to the regulation of MAPKs, AP1, NF-κB and IRFs. An important contribution to the specificity of the response is given by the combination of transcriptional regulators, as in the case of NF-κB proteins, that can cooperate with other factors, like IRF3 (Wietek et al., 2003) or E2F1 (Lim et al., 2007). In addition, transcription factors such as NF-κB can also control the expression of other regulators, such as C/EBPδ (Litvak et al., 2009) or JMJD3 (De Santa et al., 2007).

The final output of TLR stimulation is the expression of genes belonging to different categories, although all associated with the immune response (Doyle and O'Neill, 2006; Honda and Taniguchi, 2006; Foster et al., 2007; Kawai and Akira, 2007). The most consistent group of genes upregulated downstream TLR are inflammatory mediators. This group is composed by cytokines, such as tumor necrosis factor-alpha (TNFα), IL-1β, IL-6, IL-12; chemokines, such as CCL2/MCP-1, CXCL2, CXCL3; enzymes, such as PTGS2 (cycloxygenase-2), which has a role in the production of

prostaglandins; genes related to the nitric oxide balance, such as Nitric oxide synthase-2 (*Nos2*) and arginase-2 (*Arg2*). Another class of genes induced in response to TLR stimuli is formed by genes whose products participate in signaling cascades. These are transcriptional regulators and proteins of the signaling cascades downstream TLRs, such as NF-κB family members, AP1 components (Jun, JunB, FosL2), factors involved in IFN production (IRFs, STATs), signaling adaptors and other proteins of signaling cascades, such as MyD88, TRAF1, TRAF6. This second group of genes indicates a positive feedback loop given by transcriptional activators that activate themselves and other genes. At the same time, inflammatory response is auto-regulated through the expression of genes such as IκBα and IκBε, that retain NF-κB protein in the cytoplasm, or the previously mentioned A20 and genes regulating apoptosis (Jenner and Young, 2005).

A special attention has to be given to the *Nos2* gene and its product, inducible nitric oxide synthase (iNOS). Murine macrophages produce iNOS in response to pathogen infection and this enzyme synthesizes nitric oxide (NO), a molecule responsible for the inhibition or the killing of a broad range of microorganisms (Fang, 2004). iNOS synthetizes NO from arginine, donor of the nitrogen. The catabolite of this reaction is citrulline, which can be reconverted in arginine by enzymes of the urea cycle (Mori and Gotoh, 2004). NO blocks essential microbial physiological processes, such as respiration (Stevanin et al., 2000) and DNA replication (Schapiro et al., 2003) by targeting protein thiols and metal centers. The antimicrobial activity of NO is important against extracellular bacteria like *Escherichia coli*, but also against intracellular pathogens such as *Mycobacterium tuberculosis, Salmonella* and *Leishmania major* (Chakravortty and Hensel, 2003). *L. major* is an intracellular parasite of macrophages whose clearance is strictly dependent on TLRs (Tuon et al., 2008) and iNOS production (Bogdan et al., 2000; Kropf et al., 2004). In mouse strains genetically resistant to *L. major*, like *129/sv* or

C57BL/6 mice, the infection is restricted to the site of inoculation and the first draining lymph node. Mice lacking iNOS have an important defect in the clearance of *L. major*, with parasite spreading from skin and lymph nodes to visceral organs (Diefenbach et al., 1998)

2. TRANSCRIPTION OF INFLAMMATORY GENES

The LPS-inducible gene expression program is a good model to understand the transcriptional control of the inflammatory response. In a window of a few hours of LPS-induced TLR4 stimulation, hundreds of genes are expressed, or repressed, in macrophages (Ravasi et al., 2007; Ramsey et al., 2008). Such a complex transcriptional response needs to be coordinately regulated at different layers: chromatin architecture and histone modifications, recruitment of many transcription factors, combination of transcriptional co-regulators (co-repressors and co-activators), and regulation of RNA polymerase II (Pol II) binding and activity (Medzhitov and Horng, 2009). This chapter presents an overview on the transcriptional regulation of inflammatory genes upon TLR stimulation.

It is necessary to point out that LPS-induced genes have been classified in different groups, depending on their kinetic of expression and their transcriptional requirements (Saccani et al., 2001; Ramirez-Carrozzi et al., 2006; Hargreaves et al., 2009; Ramirez-Carrozzi et al., 2009). TLR-signaling-responsive genes are expressed in waves, and the first responders are called early primary response genes (PRG) (Saccani et al., 2001). They were initially characterized by containing CpG-rich promoters (Ramirez-Carrozzi et al., 2009), although a more recent work of the same group shows that the kinetic of the expression of a gene is independent of its CpG content. In contrast, they describe a negative correlation between the CpG content and the magnitude of induction of gene expression, with CpG-rich promoters more prevalent among genes with a weaker induction (Bhatt et al., 2012). PRG expression is independent of *de novo* protein synthesis or SWI/SNF-induced nucleosome remodeling. Moreover, their regulatory regions present characteristics of activated genes, such as histone marks of active chromatin.

Genes such as *Tnf*, *Nfkbia*, and *Nfkbiz* belong to this category. *Ptgs2* also behaves as PRG, but it shows a second phase of induction with characterisctics of secondary response gene, as described below (Caivano et al., 2001). A second group of late primary response genes includes *Ccl5*, *Ccl2* and *Cxcl10*. They are still independent of *de novo* protein synthesis, but dependent on chromatin remodeling. Finally, secondary response genes (SRG) are the ones with the slowest kinetics of expression, due to the fact that they require both *de novo* protein synthesis and nucleosome remodeling. Their promoters are marked with repressive histone modifications that need to be removed for gene expression. This is the case of *Nos2*, *Il6*, *Il12b* and *Lcn2*, among others. It is interesting to note that many early PRGs encode for transcriptional regulators that have the ability to control the expression of certain SRGs. A detailed description on the mechanisms of transcription for the different classes of genes is provided below.

2.1. CHROMATIN DYNAMICS

Different mechanisms involved in chromatin dynamics upon TLR stimulation are already revealed. In general terms, chromatin influences transcription through the modulation of two different aspects: the first is the compaction of nucleosomes and the accessibility of DNA, that make more or less permissive the recruitment of the transcriptional machinery; the second is chromatin diversity in terms of histone residue modifications, especially acetylation and methylation, that mark transcription activation or repression. In the next chapter we will present a brief overview on the current knowledge of these aspects during the response to TLRs.

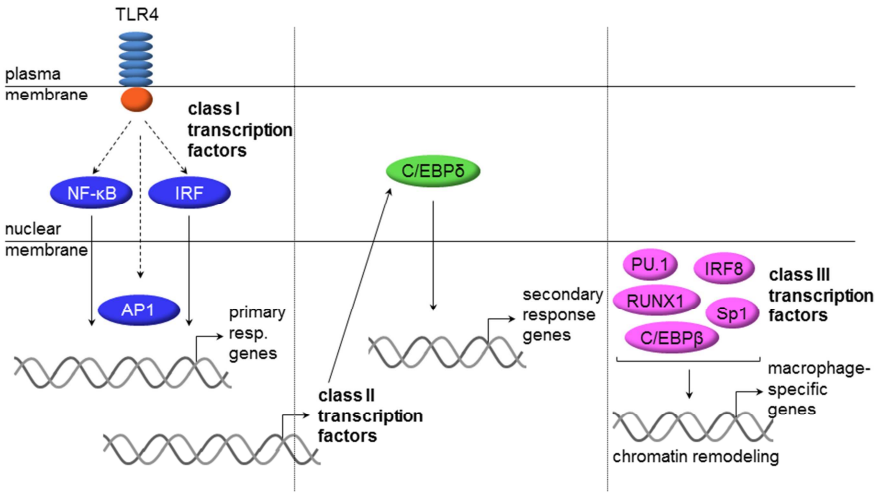
2.2. TRANSCRIPTION FACTORS

The complexity of the response to LPS is in part reflected by the compartmentalization of the transcription factors that participate in this

context. Medzhitov and Horng have classified transcription factors in three categories (figure 3), based on their mechanisms of activation and function (Medzhitov and Horng, 2009). Class I transcription factors are constitutively expressed in different cell types and only require signal-dependent post-translational modifications to be active. They are often retained in the cytoplasm by specific inhibitory mechanisms and upon signaling activation they translocate to the nucleus. For this reason, they are fundamental for the expression of genes that are induced rapidly after LPS challenging (primary response genes). Examples are NF-κB, AP1 and IRFs, among the best characterized and the most important factors participating in the inflammatory response.

Class II transcription factors are the ones that need *de novo* synthesis after LPS stimulation. They usually regulate subsequent waves of gene expression, that are the secondary response genes. They are often transcriptionally autoregulated, like the CCAAT/enhancer-binding protein-δ (C/EBPδ), which requires autoinduction (Litvak et al., 2009).

Class III transcription factors include lineage-specific factors expressed during macrophage differentiation, such as PU.1, C/EBPβ, RUNX, IRF8 and Sp1 (Valledor et al., 1998; Resendes and Rosmarin, 2004; Friedman, 2007). Their expression specifies the macrophage phenotype, although they are not exclusive to macrophages. During development, they activate constitutively expressed genes of macrophages and repress genes associated with other cell fates. In mature cells, they confer permissive chromatin configuration to LPS-inducible genes (Medzhitov and Horng, 2009). In this category BCL-6 can also be included, although it negatively modulates transcription. In fact, it works as negative regulator of proliferation in the monocytic lineage (Yu et al., 2005) and mediates binding of corepressors in quiescent macrophages (Dhordain et al., 1997).



Adapted from Medzhitov and Horng, 2009

Figure 3. Three categories of transcription factors regulate LPS-induce primary and secondary response genes.

In the response to LPS each class of transcription factors acts coordinately in regulatory circuits that control gene expression. This is demonstrated for example by the work of Aderem and collaborators, who identified a transcriptional network in a subset of inflammatory genes, in which NF-κB is the initiator of inflammatory response, C/EBPδ is the inducible amplifier and ATF3 the inducible repressor (Litvak et al., 2009). Another network of different factors is the one described by Evans and colleagues regarding the interchange between BCL-6-dependent repression and NF-κB-dependent activation of gene expression: LPS stimulation switches BCL-6 and HDAC3 occupancy in enhancers for NF-κB recruitment, resulting in activation of transcription (Barish et al., 2010).

2.3. COREGULATORS

Coregulators are transcriptional modulators that do not recognize specific sequences on DNA, unlike transcription factors. They can inhibit (corepressors) or promote gene expression (coactivators) mainly by providing enzymatic activities that modulate the function of transcription regulators or the structure of chromatin.

In the last years a model has emerged, in which both repression and activation are dynamic mechanisms: gene repression is not only maintained by the constitutive presence of repressors but also by histone repressive modifications actively deposited by intermittent corepressor activity (Wang et al., 2009). In addition, also coactivators are proposed to dynamically interchange with corepressors on DNA and these cycles would reset chromatin for subsequents rounds of transcription (Wang et al., 2009; Perissi et al., 2010).

2.3.1. Corepressors

One of the best characterized complex acting as corepressor in TLR4 target genes is the one containing histone deacetylase 3 (HDAC3) associated with nuclear receptor corepressor (NCoR) or with silencing mediator of retinoic acid and thyroid hormone receptor (SMRT). The core components shared by the two complexes are HDAC3, tranducin β-like 1 (TBL1), TBL-related 1 (TBLR1) and G-protein-pathway suppressor 2 (GPS2) (Perissi et al., 2010). HDAC3 is the exclusive enzyme that confers stable deacetylase activity to these corepressor complexes, because neither NCoR nor SMRT are found to stably associate with other enzymatic subunits (Perissi et al., 2010). However, the contribution of HDAC3 is a more complex issue since, as shown recently, it is also required for the transcriptional response to TLR4 stimulation (Chen et al., 2012).

Under basal conditions, many TLR4-regulated genes are occupied by NCoR and/or SMRT complexes that need to be removed for LPS-dependent gene activation (Ogawa et al., 2004; Ghisletti et al., 2009). These genes are normally activated by NF-κB and AP1, like *Nos2* (Pascual et al., 2005), *Mmp13*, *Ccl2* and *Il12b* (Ghisletti et al., 2009). NCoR is associated with its target regions by its interaction with c-Jun, while SMRT is doing so by its association with p50/NF-κB dimers and translocated ETS leukemia (TEL) (Ghisletti et al., 2009). Upon TLR4 activation, the adaptors TBL1 and TBLR1 recruit the ubiquitin-conjugating enzyme UBCH5, which ubiquitylates NCoR and SMRT, marking them for degradation by the 19S proteasome subunit (Ogawa et al., 2004; Perissi et al., 2004; Pascual et al., 2005). In addition, TLRs stimulation induces phosphorylation of TBLR1 and c-Jun, which also controls NCoR removal (Huang et al., 2009).

A different repressor complex, that uses HDAC1 as deacetylase, is the one formed by CoREST. CoREST/HDAC1 complexes are found in basal conditions bound to promoters of genes such as *Tnf*, *Il1a*, *Il1β* and *Cxcl2* and are displaced upon TLR stimulation (Hargreaves et al., 2009). Besides the activity of HDAC1, the mechanism through which CoREST silences gene expression was proposed to be its ability to recruit G9a, an H3K9 methyltransferase, and LSD1, an H3K4 demethylase (Cunliffe, 2008).

2.3.2. Coactivators

One of the best characterized coactivators of gene transcription in the response to pathogens is CBP-p300. CBP-p300 is a histone acetyl transferase, that acetylates histone residues and whose activity is coupled to the recruitment of the nucleosome remodeling complex SWI-SNF (Huang et al., 2003). In particular, p300 associates very well with enhancers of proinflammatory genes in response to LPS stimulation (Ghisletti et al., 2010). In addition, p300-CBP-associated factor (PCAF) and general control

of amino acid synthesis 5 (GCN5) are histone acetyltransferases that can direct elongation factors, such as P-TEFb, to target genes upon TLR4 activation (Hargreaves et al., 2009). Not only p300 and PCAF participate in the activation of transcription by modifying histones, but, as we will see later, they acetylate transcription factors, like NF-κB, increasing their activity (Chen et al., 2002; Huang et al., 2009).

A different mechanism of coactivation is the one controlled by IκBζ. This IκB family member is expressed upon TLR stimulation and promotes the switch between inhibitory p50 homodimers and transcriptionally active p50-p65 heterodimers occupancy on specific gene promoters (Yamamoto et al., 2004). Moreover, in a subset of secondary response gene promoters, IκBζ acts downstream of nucleosome remodeling to regulate H3K4 trimethylation and the assembly of the preinitiation complex (Kayama et al., 2008).

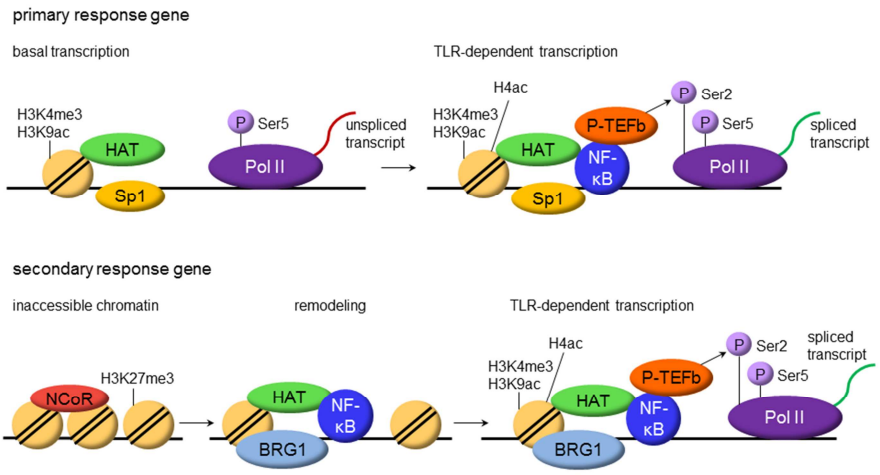
In general terms, any type of protein whose activity influences positively transcription can be considered as a coactivator. Now we will focus on other chromatin modifying enzymes that initiate or enhance transcription, such as chromatin remodeling complexes, histone methyltransferases or histone demethylases to describe the current knowledge on their role during the response to pathogens.

2.4. RNA POLYMERASE II

Essential steps required for transcription are the recruitment and activation of RNA Polymerase II (Pol II). In primary response genes (figure 4), Pol II is already present in basal state and is responsible for gene transcription at low level (Hargreaves et al., 2009; Escoubet-Lozach et al., 2011). This form of Pol II is phosphorylated on serine 5 of its carboxy-terminal domain (CTD) heptapeptide repeats, but not in serine 2, whose phosphorylation controls the recruitment of the RNA processing and splicing machineries.

Therefore, the basal transcripts of primary response genes are not spliced. Upon TLR signaling, the promoters of these genes are acetylated in lysines 5, 8 and 12 of histone H4 by PCAF and GCN5 histone acetyltransferases. This acetylation forms a platform for the binding of the adaptor protein Brd4, which is necessary for the recruitment of P-TEFb (Huang et al., 2009), a complex composed by the kinase Cdk9 and the cyclin T1. P-TEFb then phosphorylates Pol II CTD at serine 2, leading to the generation of mature and spliced transcripts (Hargreaves et al., 2009).

Regarding secondary response genes, in unstimulated conditions they do not bind RNA Pol II (figure 4). After LPS challenging, the key regulatory step for this class of genes is the recruitment of Pol II prior to initiation. Once recruited, Pol II is phosphorylated at serine 5 of the CTD by TFIIH and transcription initiates (Sims et al., 2004). In these conditions, Pol II produces short transcripts, pausing about 40 base pairs downstream of the transcription start site (TSS) (Rasmussen and Lis, 1993), until the second phosphorylation in the serine 2 of the CTD occurs. This last phosphorylation, mediated by P-TEF with the same mechanism as in primary response genes (Hargreaves et al., 2009), allows transcription elongation also in secondary response genes (Sims et al., 2004).



Adapted from Medzhitov and Horng, 2009

Figure 4. Two distinct mechanisms of regulation of TLR-inducible genes. In the upper panel the promoter of a primary response gene is represented. TLR stimulation induces the switch from the basal transcription of unspliced mRNAs to the production of mature transcripts, which depends on the recruitment of P-TEFb. In the lower panel, a secondary gene promoter is shown, which initially requires nucleosome remodeling to allow the accessibility of histone-modifying enzymes and the transcription initiation machinery.

Transcript elongation has been associated also with other histone modifications, such as H3K36 and H3K79 trimethylation, (Kouzarides, 2007). However, these modifications occur downstream of Pol II phosphorylation (Kouzarides, 2007), suggesting that they might control transcription elongation. Indeed, H4K5/8/12 acetylation has an essential role for overcoming the serine 5 RNA Pol II checkpoint in proinflammatory gene expression.

3. MODULATION OF CHROMATIN STATUS IN MACROPHAGES

Different epigenetic marks are dynamically regulated in response to environmental stimuli, including TLR stimulation (Natoli, 2010; Ivashkiv, 2012). Epigenetic marks refer to modifications that do not alter the genetic code, but control how information encoded in DNA is expressed. Despite this dynamism, epigenetic marks are more stable than post-translational modifications of upstream signaling proteins, so they persist longer after the original stimulus, rendering cellular response more sustained. The epigenetic status of a cell determines the accessibility for transcription factors and cofactors, being the first level that needs to be regulated for activating or repressing transcriptional responses.

Different aspects of the chromatin status are modulated during the inflammatory response. Chromatin remodeling regulates histone density and the accessibility of the DNA to transcriptional regulators. In addition, enzymes that modify histone residues create a "histone code" that is coupled with transcription.

3.1. CHROMATIN COMPACTION AND ACCESSIBILITY

Chromatin remodeling is mediated by complexes that use ATP to slide nucleosomes on DNA or to alter nucleosome-DNA contacts (Clapier and Cairns, 2009). These actions render chromatin-associated DNA more or less accessible for transcription regulators (Becker and Horz, 2002). Remodeling complexes are often regulated at the level of their recruitment to target gene promoters. However, it has been described that in LPS-stimulated macrophages the complex SWI/SNF can also be regulated by calcium-calmodulin-dependent signaling after its recruitment (Lai et al., 2009).

Switching-defective-sucrose non-fermenting (SWI/SNF) is an ATP-dependent chromatin remodeling complex, firstly identified in yeast (Peterson and Tamkun, 1995). SWI/SNF complexes contain two ATPase subunits, BRG1 and BRM, and a number of BRG-associated factors (BAFs) (Martens and Winston, 2003). SWI/SNF interacts with several transcription factors, which position the remodeling complex into specific genes (Chi et al., 2004). Not all LPS-induced genes are dependent on SWI/SNF. As mentioned previously, secondary and late primary response genes show strong SWI/SNF dependence, whereas early primary response genes are constitutively accessible to nucleases and are essentially independent of the activity of SWI/SNF (Ramirez-Carrozzi et al., 2006). Although early primary response genes do not require nucleosome remodeling, the fact that they are constitutively associated with BRG1 (Ramirez-Carrozzi et al., 2006) opens the possibility that SWI/SNF complexes could actively participate in the maintenance of an open chromatin status also in primary response genes.

Mi-2/NuRD is another nucleosome remodeling complex that contains Mi-2α or Mi-2β ATPase subunits and other associated factors, including histone deacetylases (Feng and Zhang, 2003). It is associated with transcriptional repression, antagonizing SWI/SNF in LPS-stimulated macrophages (Ramirez-Carrozzi et al., 2006). Mi-2β is recruited to late primary and secondary response genes in a SWI/SNF-dependent manner, but it is constitutively associated with early primary response genes, coinciding with BRG1. Although the mechanisms of remodeling by the Mi-2/NuRD complex is not clear, it was proposed that Mi-2β association with promoters requires prior remodeling by SWI/SNF or at least corecruitment of both complexes (Ramirez-Carrozzi et al., 2006).

The net effect of the remodeling complexes described above is demonstrated by different works through experiments of micrococcal nuclease or restriction enzyme accessibility on the chromatin of TLR-responsive genes. Promoters of early primary response genes are accessible to restriction enzymes in unstimulated cells (Ramirez-Carrozzi et al., 2006), while late primary and secondary response genes show LPS-induced accessibility, demonstrating their dependence on nucleosome remodeling for their expression (Weinmann et al., 1999; Ramirez-Carrozzi et al., 2006; Foster et al., 2007). Moreover, while late primary response genes are independent of new protein synthesis for nucleosome remodeling, secondary response genes depend on it, as demonstrated by their sensitivity to cycloheximide treatment in experiments monitoring chromatin accessibility (Ramirez-Carrozzi et al., 2006).

3.2. HISTONE MODIFICATIONS AND ASSOCIATED ENZYMES

Apart of the degree of nucleosome compaction and the accessibility of chromatin-associated DNA, several histone post-translational modifications regulate the chromatin status of LPS-induced genes. The N-terminal tails of histones are subjected to a large number of covalent modifications, such as lysine methylation, lysine acetylation, serine phosphorylation and lysine ubiquitylation (Kouzarides, 2007). Histone modifications exist in precise patterns that define a histone code in which distinct combinations of modifications determine unique states of gene expression (Jenuwein and Allis, 2001). These patterns are recognized by reader proteins that discriminate and bind histones, coupling the recognition of modified residues to the regulation of gene transcription. Reader proteins can recruit other chromatin-modifying factors or the transcription machinery (Strahl and Allis, 2000).

In the last years, genome maps of histone modifications associated with transcription profiles allowed the identification of modifications that mark active or inactive chromatin. At the same time, the enzymes responsible for the deposition and clearance of each modification are being characterized. This chapter presents the current view of histone modifications and the enzymes modifying them, which act on inflammatory genes expressed by macrophages (table 1).

3.2.1. Acetylations

Lysine acetylation is a post-translational modification using acetyl CoA as the donor of an acetyl group for the ε-amino group of the lysine, therefore neutralizing the positive charge of this amino acid (Shakespear et al., 2011). Lysines of histones H2A, H2B, H3 and H4 can be acetylated by histone acetylatransferases (HATs), such as CBP/p300, PCAF and GCN5 (Kouzarides, 2007). Acetylation is linked to transcriptional activation (Strahl and Allis, 2000). Secondary response genes induced by LPS require H4 acetylation previous to NF-κB recruitment, but early primary response genes show high basal levels of acetylation (Saccani et al., 2001). Acetylation of lysines 9 and 14 of histone H3 (H3K9 and H3K14) is required for efficient recruitment of TFIID and transcriptional initiation (Agalioti et al., 2002), and for potentiation of TFIID binding to K4-methylated H3 tails (Vermeulen et al., 2007), while H3K9 is shown to be acetylated in basal conditions in some early primary response genes (Hargreaves et al., 2009; Escoubet-Lozach et al., 2011). Acetylation of H4K5, K8 and K12 deposited by inducible GCN5 and PCAF allows the recruitment of P-TEFb elongation factor in early primary response genes (Hargreaves et al., 2009). Active chromatin is also enriched in H3K27 acetylation by CBP/p300, especially at nucleosomes flanking enhancers (Rada-Iglesias et al., 2011; Calo and Wysocka, 2013).

Histone acetylation is antagonized by the activity of HDACs, which are generally associated to transcriptional repression. As mentioned previously, NCoR, SMRT and CoREST complexes contain HDACs to maintain inflammatory response genes in a repressed state under basal conditions (Ogawa et al., 2004; Perissi et al., 2004; Hargreaves et al., 2009). These complexes are also necessary during gene activation to counteract the action of coactivators, resetting chromatin for subsequent rounds of transcription (Perissi et al., 2010). In this regard, a defective activation of inflammatory gene program is shown in HDAC3-deficient macrophages, mainly due to the lack of basal and LPS-induced IFNβ expression, suggesting a positive role of HDAC3 in the inflammatory response (Chen et al., 2012). Deacetylation of molecules involved in TLR signaling could explain the positive regulation of this pathway by HDACs, such as the deacetylation of some IRF family members, like IRF7, that controls its nuclear translocation and DNA binding (Shakespear et al., 2011).

3.2.2. Methylations

N-terminal tails of histone H3 and H4 are also methylated, affecting both transcriptional activation and repression. The effect of this modification depends on the specific lysine residue, the degree of methylation and the position within the genome where it occurs (Black et al., 2012). Methylation is regulated by lysine methyltransferases (KMTs) and demethylases (KMDs). Knowledge regarding KMTs is expanding since the 2000, when the discovery of the first histone KMT, KMT1A took place (Rea et al., 2000). Since then, several KMTs have been identified through homology searches using their enzymatic SET domain (Black et al., 2012). On the other hand, the first KDM was identified in 2003 and was named LSD1/KDM1A (Shi et al., 2003; Shi et al., 2004). This enzyme works through an amine oxidase domain and is part of a repressor complex (Shi et al., 2004). It remains the only KDM that contains an amine oxidase domain, as a few years later

another class of KDMs was described. The Jumonji domain protein family, that is composed by 27 members, presents a JmjC domain that catalyzes demethylation through the oxidation of methyl groups, using Fe(II) and α-ketoglutarate as cofactors (Shi and Whetstine, 2007).

Several methylated lysine residues influence the inflammatory transcriptional program. An important chromatin modification associated with transcription repression of inflammatory genes in macrophages is H3K27me3 (De Santa et al., 2007). H3K27me3 was previously shown to silence developmental genes during differentiation (Lund and van Lohuizen, 2004). H3K27me3 is found at high levels in a subset of secondary response genes at basal conditions and TLR stimulation induces its clearance (De Santa et al., 2009). The enzymatic complex responsible for this modification is the Polycomb repressive complex 2 (PRC2) (Cao et al., 2002), which contains Ezh2, a protein with a SET histone methyltransferase domain and two other essential subunits, ESC/EED and Su(z)12 (Cao and Zhang, 2004). Polycomb group (PcG) proteins, through their repressor effect on transcription, and the antagonistic trithorax proteins, are responsible for the maintenance, during the development and the adulthood, of the correct patterns of homeotic gene expression in a spatially defined manner (Schuettengruber and Cavalli, 2009). PcG include the PRC1 and PRC2 complexes. H3K27 methylated by PRC2 can recruit the PRC1 component CBX, suggesting that PRC1 works downstream of PRC2 (Margueron and Reinberg, 2011). Although this concatenation of PRC complex activities is not universal for all genes, PRC1 and PRC2 are often both required for the maintenance of gene silencing. While PRC2 complex have methyltransferase activity, PRC1 monoubiquitylates H2AK119, resulting in transcriptional repression (Wang et al., 2004). It has been recently shown that the activity of PRC2 is increased with high density of its substrate chromatin, maintaining a silenced transcriptional state in compacted histone regions, whereas active chromatin

marks and low nucleosome density antagonize PRC2 activity (Yuan et al., 2012). Demethylation of H3K27 is performed by two KDM6 family members, JMJD3 and UTX (Agger et al., 2007). In particular, JMJD3 is induced by NF-κB in primary mouse macrophages in response to TLR stimulation, while its paralog UTX is expressed at low and constant levels (De Santa et al., 2007). Additional genome-wide studies of Natoli and colleagues showed that JMJD3 associates with the TSS of numerous active LPS-responsive genes, coinciding with the distribution of H3K4me3 and RNA Pol II, but a complete dependence on JMJD3 for the induction of LPS-responsive genes is not so prevalent. The effect of JMJD3 on transcription was proposed to be mainly independent of H3K27me3 (De Santa et al., 2009). In fact, data of H3K27me3 chromatin immunoprecipitation in proinflammatory gene promoters like *Nos2*, show that loss of this histone mark could be attributed to a loss of nucleosomes, rather than an enzymatic demethylation (De Santa et al., 2009). The same effect is seen in the *Tnf* promoter, where loss of H3K27me3 coincides with loss of total histone H3 in response to LPS (Kruidenier et al., 2012). However, since inhibition of JMJD3 induces H3K27me3 maintenance independently of nucleosome loss, this suggests a dynamic balance between H3K27 demethylation and methylation upon TLR activation. The result of JMJD3 activation has been proposed to facilitate transcription elongation driving elongation factors to their target genes (De Santa et al., 2009; Chen et al., 2012).

An additional mark of transcriptional silencing is the methylation of H3K9 (Martin and Zhang, 2005). In human dendritic cells this methylation negatively correlates with RNA Pol II recruitment and H3K9 is demethylated upon LPS stimulation in specific genes (Saccani and Natoli, 2002). The mechanism of H3K9me2 regulation requires Aof1 as an H3K9 demethylase (van Essen et al., 2010), which is recruited to target promoters,

such as *Il12b* and *Ccl22*, by interacting with NF-κB. The demethylase activity of Aof1 is also necessary for the stimulus-induced increased binding of NF-κB and the activation of transcription.

More recently, Glass and colleagues identified an additional mark of silenced chromatin, H4K20me3. It was previously suggested that H4K20me3 inhibits H4K16 acetylation, resulting in the pausing of RNA Pol II (Kapoor-Vazirani et al., 2011). A subset of TLR4-induced genes in macrophages presents H4K20me3 in unstimulated conditions due to the activity of the SMYD5 histone methyltransferase, which associates with NCoR complexes. Upon LPS stimulation, NF-κB recruits the Phf2 histone demethylase, leading to H4K20me3 demethylation and activation of transcription (Stender et al., 2012).

Despite the mentioned examples of inhibitory methyl marks, methylations can also result in activation of gene expression. One of the best studied histone modification with a positive effect on transcription is the trimethylation of H3K4 (H3K4me3). Human and mouse have at least six KMTs that can methylate H3K4, including MLL1-4 and SET1A/B (Smith and Shilatifard, 2010). H3K4me3 is highly enriched at the transcription start sites of active and poised genes (Escoubet-Lozach et al., 2011), and is detected by different recognition domains in transcriptional regulators. Although H3K4me3 has a high correlation with gene activity, its functional role remains unclear (Vermeulen et al., 2007). It is suggested that this histone modification would participate in the initiation of transcription since TAF3, a subunit of TFIID, binds H3K4me3 through its PHD finger, helping the recruitment of RNA Pol II (Vermeulen et al., 2007). H3K4me3 is demethylated by LSD1/KDM1 and the JARID1 family of demethylases (Agger et al., 2008).

In addition to H3K4me3, regions flanking the transcription start sites can also present H3K4me1/2 (Black et al., 2012). However, H3K4me1 is highly enriched in distal gene regulatory elements (enhancers), which also have low levels of H3K4me3 and are frequently bound by p300 (Ghisletti et al., 2010).

MLL4 methyltransferase, also known as Wbp7, has been linked to macrophage inflammatory function not only for regulating H3K4 methylation. In fact, MLL4 is also necessary for the expression of Pigp, a component of the enzymatic complex that catalyzes the first step of the glycosylphosphatidyl inositol (GPI) anchor synthesis, and is then required for CD14 function, which enhances LPS detection by TLR4 (Austenaa et al., 2012). Therefore, MLL4 contributes to both a biosynthetic pathway essential for innate immunity and the regulation of transcriptional response to LPS.

Histone modification	Effect on transcription	Writer enzyme	Eraser enzyme
H3K9ac	+	GCN5/PCAF	HDAC3
H3K14ac	+	CBP/p300 GCN5/PCAF	HDAC3
H4K5/8/12ac	+	CBP/p300 GCN5/PCAF	HDAC
H3K27ac	+	CBP/p300	HDAC1,2
H3K4me3	+	MLL1-4 SET1A/B	LSD1 JARID1
H3K4me1/2	+	MLL1-4	LSD1 JARID1
H3K36me3	+	SET2 SMYD2	LSD1
H3K79me3	+	DOT1	
H3K27me3	-	PRC2	JMJD3 UTX
H3K9me	-	RIZ1	Aof1
H3K20me3	-	SMYD5	Phf2

Table 1. Summary of the main histone lysine modifications involved in the regulation of the TLR-induced gene expression.

4. NF-κB TRANSCRIPTION FACTORS

4.1. THE NF-κB FAMILY

Nuclear Factor-κB is a family of transcription factors identified more than 25 years ago for its binding to the intronic enhancer of the kappa light chain gene in B cells (κB site) (Sen and Baltimore, 1986). Initially NF-κB was described to have a central role in innate and adaptive immune response, but it was soon observed a disregulation of this transcription factor in other biological scenarios, such as cancer, atherosclerosis and diabetes, unraveling its importance in the regulation of several processes, like cell proliferation, survival or differentiation (Gerondakis et al., 1999).

The NF-κB family consists of five members, all sharing an N-terminal Rel homology domain (RHD) necessary for DNA binding and dimerization. p50 (and its precursor p105), p52 (and its precursor p100), p65/RelA, c-Rel and RelB are the different subunits of NF-κB, but only p65, cRel and RelB present a transactivation domain (TAD) necessary for the activation of gene expression, while p50 and p52 act as repressors of transcription when they are not associated with a subunit of NF-κB that contains TAD (Hayden and Ghosh, 2004).

4.2. SIGNALLING AND ACTIVATION

In basal conditions, NF-κB dimers are retained in the cytoplasm by binding to IκB proteins. There are two main activation pathways for NF-κB, the canonical and the non-canonical pathways (figure 5). The canonical pathway is initiated by the majority of physiological NF-κB stimuli, for example the stimulation of tumor necrosis factor receptor (TNFR), IL-1 receptor, and TLRs. In this pathway the central point is the activation of the inhibitor of κB kinase β (IKKβ) subunit of the IKK complex, composed by IKKα,

IKKβ and IKKγ/NEMO. IKKβ phosphorylates IκBα bound to NF-κB in cytoplasm. The consequence of this phosphorylation is that IκBα is targeted for ubiquitylation and degraded by the proteasome, allowing NF-κB dimers, most of them p65-containing heterodimers, to translocate to the nucleus (Hayden and Ghosh, 2004).

On the other hand, the non-canonical pathway is dependent on IKKα activity that selectively phosphorylates p100 bound to RelB in the cytoplasm, leading to p100 processing to obtain p52/RelB heterodimers. This alternative NF-κB pathway is induced by some TNF family cytokines, for example lymphotoxin-β, CD40 and BAFF (Hayden and Ghosh, 2004).

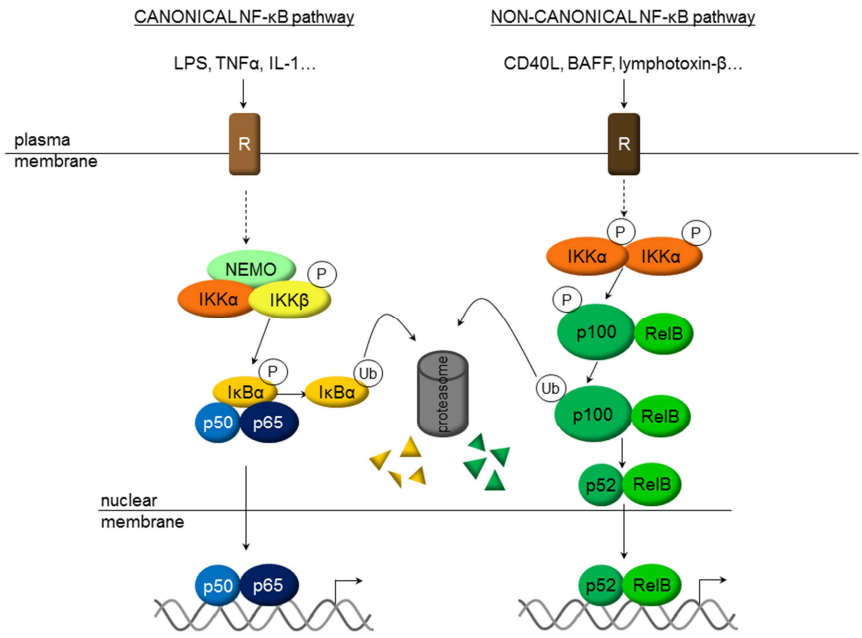


Figure 5. Canonical and non-canonical pathways of NF-κB activation. R: specific receptor.

To be fully functional, NF-κB undergoes tightly regulated post-translational modifications that modulate its association with IκB proteins, coactivators, corepressors, and other transcription factors. These modifications have been studied especially for p65 and the most relevant ones are summarized below.

Regarding phosphorylation, one of the first residues identified was p65 serine (S) 276, which is targeted by protein kinase A (PKAc) in the cytoplasm in response to LPS (Zhong et al., 1997) or by MSK-1 and MSK-2 in the nucleus in response to TNFα (Vermeulen et al., 2003). S276 phosphorylation is necessary for the recruitment of CREB Binding Protein (CBP) to p65-target genes and also regulates the transcriptional activity of p65 (Zhong et al., 2002). Other residues of p65 that also undergo phosphorylation are S311 by protein kinase C ζ (PKCζ) (Leitges et al., 2001); S468 by GSK3β, IKKβ and IKKε (Schwabe and Brenner, 2002; Buss et al., 2004); S529 and S536 by IKKα/β/ε, TBK1 and RSK1 (Sakurai et al., 1999; Wang et al., 2000; Bohuslav et al., 2004).

In the nucleus, some lysine residues of p65 are also acetylated. Acetylation is regulated by histone acetyl transferases (HATs) and histone deacetylases (HDACs). Modulation of the acetylated status of lysines influences several functions of p65, especially DNA binding, transcriptional activity and its association with IκBα. K310 is a target of CBP/p300 acetyl transferase required for full transcriptional activity but not for DNA binding. AcK310 creates a docking site for two bromodomains of Brd4, which in turns recruits and activates Cdk9, responsible for the phosphorylation and activation of RNA Polymerase II (Chen et al., 2002; Huang et al., 2009). Additional amino acids in p65 that are acetylated are K122 and 123, targeted by both CBP/p300 and PCAF, and K218/221 or 314/315, which are targeted by CBP/p300 (Chen et al., 2002; Kiernan et al., 2003; Buerki et al., 2008).

Methylation of specific lysine residues in p65 has functional consequences that depend on the position and the state of methylation (mono-, di- or tri-). For example, SET9 methyl-transferase targets K37 in response to TNFα (Ea and Baltimore, 2009) to facilitate DNA binding, but the same SET9 enzyme also promotes methylation in K314/315, leading to ubiquitylation and degradation of p65 with the termination of NF-κB response (Yang et al., 2009). More recently, SETD6 has been identified as a methyltransferase that monomethylates chromatin-associated p65 at lysine 310 in unstimulated macrophages. p65K310me1 is inert and p65-target gene transcription results attenuated. The mechanism of this effect resides on the fact that p65K310me1 is recognized by G9a-like protein (GLP), a histone KMT that methylates H3K9 and represses chromatin. Upon stimulation, PKCζ-mediated phosphorylation of p65 on serine 311 blocks GLP binding to lysine 310 and overcomes gene repression (Levy et al., 2011).

In general terms, every class of post-translational modification of p65 can result in either promotion or dampening of transcription, making this level of NF-κB regulation fundamental to determine the strength, the duration and the transcriptional output of NF-κB activity.

4.3. NF-κB IN INNATE IMMUNITY

NF-κB proteins control a great number of processes linked to the development, activation or transformation of components of the immune response. They regulate the transcriptional induction of cytokines, growth factors and effector enzymes upon stimulation of many immune-related receptors, such as TLRs, TNF receptor (TNFR), RIG-I-like receptors, NOD-like receptors, C-type lectin receptors, T and B cell receptors (TCRs and BCRs) and CD40 (Medzhitov et al., 1997; Bonizzi and Karin, 2004; Hayden and Ghosh, 2004; Geijtenbeek and Gringhuis, 2009). In particular,

the p65 subunit has a crucial role in the response to LPS and TNFα signaling (Beg et al., 1995; Medzhitov et al., 1997). In this regard, while p65-deficient mice show embryonic lethality due to hepatocyte apoptosis when analyzed in the background of TNFα- or TNFR-deficient mice, the double knock-out p65-/TNFα-deficient (Doi et al., 1999) or p65-/TNFR-deficient (Alcamo et al., 2001) mice are viable and allowed the study of the relevance of p65 in processes such as bacterial infections.

4.4. COOPERATION WITH OTHER FACTORS

Due to the central role of NF-κB in the regulation of the immune response, it participates in the formation of enhanceosomes at the promoters of most proinflammatory genes. An important aspect of modulation of NF-κB-dependent transcription is the coordination and cooperation with heterologous transcription factors. This interaction can occur through occupancy at adjacent sites on DNA, with or without direct binding. One example is the crosstalk between NF-κB and IRFs. Either ISRE or κB sites are able to form complexes p65-IRF3 in response to TLR4 stimulation (Wietek et al., 2003), but the transcription factor indirectly recruited acts as a cofactor helping transcriptional activation (Taniguchi et al., 2001). In this scenario, it was suggested that glucocorticoid receptors, that inhibit a subset of NF-κB-dependent genes after binding their ligand, can displace IRF3 from p65 (Ogawa et al., 2005). In particular, enhanceosomes containing NF-κB and IRF have been studied for the enhancer of interferon-β gene: upon viral infection, assembly of NF-κB, IRF3/7 and ATF-c-Jun occurs (Thanos and Maniatis, 1995; Apostolou and Thanos, 2008). Therefore, interferon-β is selectively expressed in conditions that provide this combinatorial mechanism that confers great specificity to gene expression.

AP1 transcription factors also influence the activity of p65 to regulate gene expression in response to infection (Oeckinghaus et al., 2011). Both c-Jun

and c-Fos can interact with p65, increasing the ability of AP1 proteins for DNA binding and facilitating their activation through AP1 sites (Shaulian and Karin, 2002). Similarly, p65 transactivation can be promoted by c-Jun and c-Fos through κB sites (Stein et al., 1993).

Synergistic interaction with NF-κB is also described for Sp1, a ubiquitous transcription factor that, while it usually regulates constitutive expression of many genes, it also has a role in stimulus-dependent transcription (Oeckinghaus et al., 2011). NF-κB and Sp1 use binding sites often found in close proximity to cooperate in the induction of a subset of genes, like those encoding for IL-6 and TLR2 (Sanceau et al., 1995; Wang et al., 2001).

Regarding STAT proteins, cooperation between NF-κB and a complex formed by STAT1, STAT2 and IRF9, called ISGF3, has been described for *Nos2* gene expression in macrophages infected with *Listeria monocytogenes*. NF-κB helps Cdk7 recruitment and ISGF3 facilitates RNA Pol II recruitment to the *Nos2* promoter. Cdk7 is necessary for RNA Pol II CTD S5 phosphorylation, but while NF-κB alone could not recruit Pol II by itself, ISGF3 alone would not be able to provide CTD kinase activity (Farlik et al., 2010).

Cells can also take advantage of combinations of NF-κB with other transcription factors to discriminate between transient and persistent stimulation. This is the case of macrophages that induce C/EBPδ in a NF-κB-dependent manner to increase transcriptional activity after its initiation by NF-κB when the stimulus becomes persistent (Litvak et al., 2009).

Another example of NF-κB association with an auxiliary factor is the recruitment of E2F1 by p65, necessary for transcriptional activation of a subset of LPS-induced genes, such as IL-1β and CXCL9 (Lim et al., 2007).

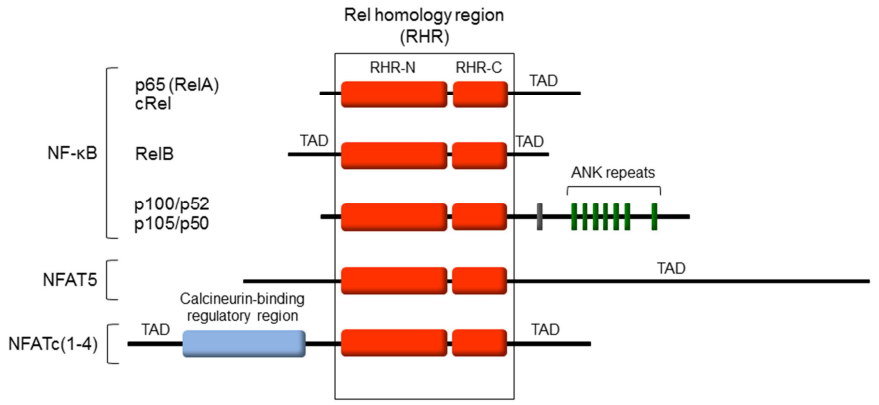
Moreover, in response to hypertonicity, NF-κB activation is proposed to be mediated by NFAT5, which associates with p65-containing heterodimers bound to DNA, enhancing NF-κB transcriptional activity (Roth et al., 2010).

NF-κB cooperation with histone modifiers has been reported to participate in the transcriptional response to TLR4. As mentioned in the previous chapter, NF-κB is needed for the recruitment of Aof1 demethylase to target genes, where it demethylates H3K9 (van Essen et al., 2010), and of Phf2 demethylase, which demethylates H4K20me3 (Stender et al., 2012).

5. THE TRANSCRIPTION FACTOR NFAT5

5.1. GENERAL CHARACTERISTICS

Nuclear factor of activated T cells 5 (NFAT5) is a transcription factor that was cloned in 1999 by López-Rodríguez and colleagues (López-Rodríguez et al., 1999) taking advantage of its structural homology with the calcium/calcineurin-regulated NFAT transcription factors (NFATc1-4). It is also known as tonicity-responsive enhancer-binding protein (TonEBP) or osmotic response element binding protein (OREBP) (Miyakawa et al., 1999; Ko et al., 2000). NFAT5 belongs to the Rel family of transcription factors, which also includes NF-κB and NFATc proteins (figure 6). It is the largest Rel domain-containing protein in vertebrates, with almost 1500 amino acids and a long carboxy-terminal transactivation domain of more than 900 amino acids (López-Rodríguez et al., 1999; Miyakawa et al., 1999). Rel-containing proteins have important roles in the regulation of stress response and also participate in the development and activation of immune cells. The first function attributed to NFAT5 was the response to osmotic stress (Miyakawa et al., 1999; Aramburu et al., 2006), but nowadays different functions independent of osmotic stress have been described, as it will be discussed later.



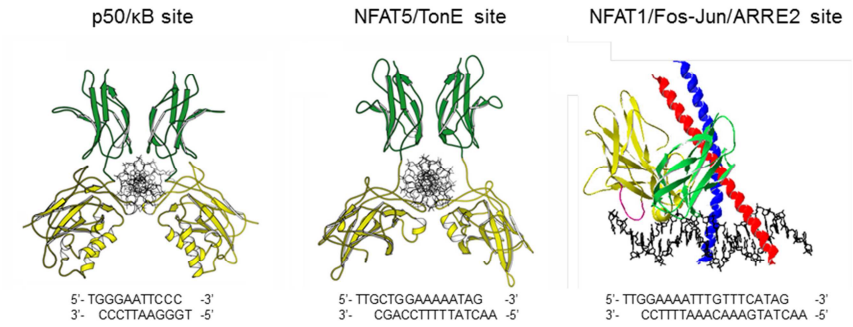
Adapted from Aramburu et al., 2006

Figure 6. Schematic diagram of mammalian Rel family of transcription factors All the Rel family members share the Rel homology region (RHR, in orange). The RHR amino (N) terminal contains the DNA-binding domain, while RHR carboxy (C) terminal comprises the dimerization domain. NF-κB members p100/p52 and p105/p50 present a proteolytic cleavage site (in grey) and ankyrin repeats (in green) in their C-terminal region. The calcineurin binding regulatory region of NFATc proteins is shown in blue. TAD: transactivation domain.

5.2. STRUCTURE

NFAT5 shares with all other Rel family members the Rel-like homology domain (RHR) that comprises the DNA-binding domain (DBD) and the dimerization domain (figure 6). As NF-κB transcription factors, but different from NFATc proteins, NFAT5 is a constitutive dimer and its dimerization surface is very similar to that of NF-κB proteins (López-Rodríguez et al., 2001). This surface, which is found in the carboxy-terminal region of the RHR, together with an additional dimer interface (E'F) loop in the amino-terminal half of the DBD, encircles DNA (Stroud et al., 2002). As for NF-κB, dimerization of NFAT5 is essential for DNA binding (López-Rodríguez et al., 2001). Although NFAT5 dimerizes like NF-κB proteins do, it does not recognize the same sequence on DNA, sharing only 20% of homology

between their consensus sites (figure 7). NFAT5 binds DNA elements similar to those recognized by NFATc proteins, with a consensus sequence for NFAT5 defined as (A/T)GGAAANN(C/T)N(C/T). This sequence contains the NFATc binding core (GGAA) and differs from the palindromic elements selected by NF-κB (GGGRNNYYCC, where R is A/G and Y is C/T) (López-Rodríguez et al., 1999).



Adapted from Hogan et al., 2003

Figure 7. Crystal structures of p50, NFAT5 and NFAT1/Fos-Jun complexes bound to consensus DNA binding sites (κB, TonE, ARRE2, respectively). The yellow structures are the amino-terminal regions of the Rel homology domain, the green ones are the carboxy-terminal regions of that domain. The DNA sequence bound by each complex is represented below each structure.

The calcineurin-docking sites in the amino-terminal region and the Jun contact residues that characterize NFATc proteins are not found in NFAT5, suggesting that it is not activated by the same pathway than NFATc and that it does not need AP1 to bind DNA cooperatively (López-Rodríguez et al., 1999).

The structural similarities of Rel family members indicate that they are evolutionarily related. *Drosophila melanogaster* expresses NF-κB and a single

NFAT-like factor, called dNFAT, whose DBD resembles the one of NFAT5 (Adams et al., 2000). For this reason, NFAT5 is considered the most ancient member of NFAT family, the first to diverge from Rel proteins during evolution (Aramburu et al., 2006). In fact, NFATc proteins are present only in vertebrates (Graef et al., 2001; Hogan et al., 2003).

5.3. IMMUNITY-UNRELATED FUNCTIONS

In cells exposed to hypertonicity, NFAT5 activates an osmoprotective gene expression program to counteract the stressful condition achieved. In particular, NFAT5 promotes the transcription of genes encoding for transporters of organic osmolytes (Ito et al., 2004; Burg et al., 2007), enzymes like aldose reductase (López-Rodríguez et al., 2004), heat-shock proteins (Woo et al., 2002), but also cytokines, such as TNFα, lymphotoxin-β (López-Rodríguez et al., 2001), CCL2 (Kojima et al., 2010), COX2 (Favale et al., 2009). This last group of NFAT5-target genes suggests a link between NFAT5 and pro-inflammatory immune responses, as it will be shown later. The adaptation to extracellular hypertonicity centrally regulated by NFAT5 is proposed to be controlled by many kinases (Aramburu et al., 2006).

As mentioned, NFAT5 plays important roles independently of osmotic stress. One of these is the regulation of embryonic development. NFAT5 expression is detected is most organs of murine embryos (Maouyo et al., 2002). Furthermore, reduced embryonic viability and increased perinatal lethality are shown by NFAT5-null mice (Go et al., 2004; López-Rodríguez et al., 2004). More recently, this embryonic lethality has been associated with defects in cardiac development (Mak et al., 2011).

NFAT5 was demonstrated to be a target of α6β4 integrin signaling, promoting cell migration (Jauliac et al., 2002). Carcinoma cell lines overexpressing α6β4 transfected with a dominant negative form of NFAT5

blocked cellular invasion. In cardiomyocytes, NFAT5 is important for protection against the cellular toxicity caused by the antitumor drug doxorubicin (Ito et al., 2007). Moreover, NFAT5 has been related to muscle differentiation (O'Connor et al., 2007) and to bind the long-terminal repeat of retrovirus in hepatocytes (Yamaguchi et al., 2003).

5.4. NFAT5 IN THE IMMUNE SYSTEM

It is well established that calcium-regulated NFATc proteins control the development and activation of cells of the adaptive immune response. Nonetheless different roles in innate immunity have been described recently. NFATc proteins do not respond to TLR stimulation, but activation of Dectin-1, a C-type lectin receptor, or CD14, induces calcium mobilization and NFATc-driven gene transcription in dendritic cells, macrophages and neutrophils (Goodridge et al., 2007; Zanoni et al., 2009; Greenblatt et al., 2010).

Regarding NFAT5, different roles in immunity have been reported since it was discovered. In T lymphocytes, activation through stimulation of the T cell receptor (TCR) or with mitogens induces NFAT5 expression, which is downmodulated by calcineurin inhibitors (Trama et al., 2000; López-Rodríguez et al., 2001). Furthermore, under hypertonicity, NFAT5 is required for the expression of CD24 through direct binding to its promoter, and this expression is necessary for T cell expansion (Berga-Bolaños et al., 2010).

In response to osmostress, NFAT5 is also important for differentiation of splenic B cells and for antigen-specific immunoglobulin production by these cells (Kino et al., 2009).

In primary macrophages, NFAT5 regulates transcriptional responses to hypertonic stress (Morancho et al., 2008), including the induction of *Vegfc*, encoding for the vascular endothelial growth factor C, which acts as osmosensor in salt-induce hypertension in the skin (Machnik et al., 2009). Independently of osmotic stress, NFAT5 interacts with a specific enhancer binding site of the human immunodeficiency virus (HIV) and it is needed for viral replication in human monocytes, which constitutively express it (Ranjbar et al., 2006).

As mentioned above, NFAT5 has the ability to induce the expression of proinflammatory cytokines, growth factors and surface receptors, although in response to hypertonicity. For this reason and for its similarities with NF-κB proteins, a role of NFAT5 in specific immune receptor-mediated responses could was proposed.

5.5. REGULATION OF NFAT5 EXPRESSION

In mice, the mRNA of *Nfat5* is expressed ubiquitously (López-Rodríguez et al., 1999; Miyakawa et al., 1999; Trama et al., 2000). Upon hypertonicity, there is a significative increase in NFAT5 protein levels, not reflected by an equivalent increase of mRNA levels (López-Rodríguez et al., 1999; Miyakawa et al., 1999). The same is seen in the expression of NFAT5 in different subsets of T lymphocytes, showing big fluctuations in protein expression during their maturation, but little changes in mRNA (Berga-Bolaños, under revision). Overall, this suggests that increase in NFAT5 protein expression does not depend only on enhanced transcription.

The regulation of NFAT5 protein expression is in part still unknown, but some advance has been made studying the stabilization of its pre-existing mRNA, which is mediated by its 5'UTR (Cai et al., 2005). *In silico* analysis revealed that the 3'UTR of NFAT5 could also be target for different

miRNAs, suggesting a likely regulation of mRNA stability (Asirvatham et al., 2008). Up to date, five distinct miRNA regulating NFAT5 expression have been described. In melanoma cells, NFAT5 mRNA levels are downregulated by overexpression of miRNA-211 (Levy et al., 2010); in colon cancer cells miRNA-22 inhibition facilitates NFAT5 expression (Alvarez-Diaz et al., 2012); in myoblasts miRNA-206 downregulates the mRNA in NFAT5 of differentiating cells (Goljanek-Whysall et al., 2012); and finally, in the renal medulla, miRNA-200b and miRNA-717 inhibit NFAT5 expression and act as osmoregulators, that are downregulated in response to osmotic stress (Huang et al., 2011).

5.6. NFAT5-DEFICIENT MICE

To study NFAT5 function *in vivo*, two distinct total knock-out mouse models have been generated. The first NFAT5-deficient mouse model was obtained by Cristina López-Rodríguez and colleagues in 2004 by deletion of the exon 6 of NFAT5 gene, that produces a premature stop codon, preventing protein expression (López-Rodríguez et al., 2004). These mice are in a mixed C57BL/6 and 129sv background and present high rates of perinatal lethality, with only a small proportion of them surviving to adulthood. NFAT5-deficient mice weight about half of wild-type littermates and suffer kidney abnormalities, increased apoptosis in the renal medulla, defective activation of NFAT5 osmoprotective target genes (López-Rodríguez et al., 2004), hypernatremia and immunodeficiency (Berga-Bolaños et al., 2010). A conditional knock-out mouse for NFAT5 was also reported and allowed for the study of NFAT5 in T lymphocytes (Drews-Elger et al., 2009; Berga-Bolaños et al., 2010), but no conditional NFAT5 mice are described for cells of the innate immunity, such as macrophages.

In the same year, William Go and co-workers generated an independent NFAT5-deficient mouse model obtained by deletion of exons 6 and 7

resulting in a protein bearing an internal deletion, which is transcriptionally inactive in homozygosis (Go et al., 2004). These mice suffer late gestational lethality, for this reason the heterozygous *Nfat5*$^{+/-}$, which survives to adulthood, has been further analyzed. *Nfat5*$^{+/-}$ mice suffer lymphoid hypocellularity and impaired antigen-specific antibody responses.

OBJECTIVES

OBJECTIVES

Rel-like transcription factors, including NF-κB and NFATc proteins, are essential players in the response to pathogens. NFAT5 is a distinct type of Rel-like protein that is expressed in numerous cell types, including leukocytes, and regulates the expression of several proteins with immunomodulatory activity in response to osmotic stress. This observation led us to consider that, similarly to other Rel family members, NFAT5 might play a role in specific innate immune receptor-mediated responses. As Toll-like receptors (TLRs) are central modulators of the response to pathogens, it is of great relevance to characterize novel transcriptional regulators that can fine-tune the specificity of TLR-induced responses.

Specific objectives of our work are the following:

1. To dissect the mechanisms that regulate NFAT5 expression and activity in the response of primary macrophages to TLRs.

2. To characterize the impact of NFAT5 in the expression of TLR-responsive genes.

3. To analyze the effect of NFAT5 on chromatin configuration and recruitment of other transcription regulators to its target genes in response to TLRs.

RESULTS AND METHODS

RESULTS AND METHODS

Results and methods are described in the following articles:

- ARTICLE 1: Maria Buxadé, Giulia Lunazzi, Jordi Minguillón, Salvador Iborra, Rosa Berga-Bolaños, Margarita Del Val, José Aramburu, and Cristina López-Rodríguez. Gene expression induced by Toll-like receptors in macrophages requires the transcription factor NFAT5. *J. Exp. Med.* 2012 Feb 13;209(2):379-393.

- ARTICLE 2: Giulia Lunazzi, Silvia Gaggero, José Aramburu, and Cristina López-Rodríguez. Transcriptional responses to a low input from Toll-like receptors require NFAT5-dependent local H3K27 demethylation. *Submitted.*

ARTICLE 1.

Gene expression induced by Toll-like receptors in macrophages requires the transcription factor NFAT5

Maria Buxadé, Giulia Lunazzi, Jordi Minguillón, Salvador Iborra, Rosa Berga-Bolaños, Margarita del Val, José Aramburu, and Cristina López-Rodríguez

J Exp Med. 2012 Feb 13;209(2):379-93. doi: 10.1084/jem.20111569. Epub 2012 Feb 6.

Buxadé M, Lunazzi G, Minguillón J, Iborra S, Berga-Bolaños R, del Val M, et al. Gene expression induced by Toll-like receptors in macrophages requires the transcription factor NFAT5. J Exp Med. 2012 Feb 13;209(2):379–93. DOI: 10.1084/jem.20111569

ARTICLE 2.

Transcriptional responses to a low input from Toll-like receptors require NFAT5-dependent local H3K27 demethylation.

Giulia Lunazzi, Silvia Gaggero, José Aramburu, and Cristina López-Rodríguez

Submitted

Lunazzi G, Buxadé M, Riera-Borrull M, Higuera L, Bonnin S, Huerga Encabo H, et al. NFAT5 Amplifies Antipathogen Responses by Enhancing Chromatin Accessibility, H3K27 Demethylation, and Transcription Factor Recruitment. J Immunol. 2021 Jun 1;206(11):2652–67. DOI: 10.4049/jimmunol.2000624

DISCUSSION

DISCUSSION

The immune response to infections is among the main causes of inflammation, a complex mechanism of adaptation involving changes in the expression of hundreds of genes. In this context, Toll-like receptor (TLR) stimulation by pathogen-associated molecular patterns (PAMPs) is one of the principal events that occur. Regulation of the TLR-induced gene expression program takes into account parameters such as the nature of danger, the duration of stimulus and the strength of signal. The TLR-controlled network of transcriptional regulators is still expanding and in the last few years transcription factors known to act in other biological contexts have been related to the TLR response (Martinon et al., 2010). In this thesis, NFAT5 is identified as a key regulator of proinflammatory gene expression in macrophages in response to mild stimulation of TLRs. Moreover, NFAT5 is shown to be necessary for the modulation of chromatin accessibility in a subset of gene promoters and the consequent recruitment of other transcriptional regulators.

NFAT5 is a distinct NFAT protein that regulates TLR-induced gene expression. Rel-family proteins (NF-κB and NFATc proteins) are essential regulators of the immune response. While NF-κB proteins are major players of the innate immunity, as they are activated by various families of pathogen receptors, the calcium-regulated NFATc proteins are activated by selective pathogen receptors that, such as CD14 or Dectin-1, are coupled to calcineurin activation (Goodridge et al., 2007; Zanoni et al., 2009; Greenblatt et al., 2010). However, no role for NFATc proteins in TLR-induced gene expression has been described. NFAT5 is a member of the Rel family of transcription factors that shows characteristics similar to both NF-κB and NFATc proteins and we now show that it is positioned downstream TLR signaling. The regulation of TLR responses by NFAT5 is independent of

osmotic stress, although some of the target genes regulated by NFAT5 upon LPS stimulation, such as *Tnf* and *Ccl2*, are also targets for this factor in macrophages under hypertonicity (Roth et al., 2010). An additional proof that the role of NFAT5 in the response to pathogens is unrelated to osmotic stress is the fact that signaling pathways leading to NFAT5 activation in these two scenarios are distinct. In this regard, while p38 seems to positively regulate NFAT5 activation in response to hypertonicity (Ko et al., 2002; Morancho et al., 2008), we observe that upon TLR activation it does not promote NFAT5 activity or expression. It would be interesting to study whether NFAT5 has a role independently of osmotic stress also in response to other pattern recognition receptors (PRRs), such as RIG-like receptors, C-type lectin receptors, NOD-like receptors or cytoplasmic DNA receptors (Geijtenbeek and Gringhuis, 2009; Hornung and Latz, 2010; Takeuchi and Akira, 2010).

According to the literature, primary and secondary response genes present different kinetics of induction and also different transcriptional requirements for their expression (Saccani et al., 2001; Ramirez-Carrozzi et al., 2006). While primary response genes are prepared for transcription since they have regulatory regions already accessible for the binding of transcriptional regulators, secondary response genes show inaccessible chromatin conformation and need nucleosome remodeling to allow the recruitment of the transcription machinery (Ramirez-Carrozzi et al., 2006; Hargreaves et al., 2009; Escoubet-Lozac et al., 2011). Along with these observations, we show that NFAT5 has a different behavior corresponding with these two different classes of target genes. NFAT5 is constitutively bound to early primary response gene promoters, such as *Tnf*, *Ccl2*, *Il1a* and *Traf1*, and its binding to *Tnf* and *Ccl2* promoters is not dependent on new protein synthesis. On the other hand, we found that NFAT5 is recruited to the regulatory regions of a late primary response gene (*Ccl5*) and secondary response genes, such as

Nos2, *Il6*, *Il12b* and *Ptgs2*, upon TLR stimulation, and this recruitment is sensitive to protein synthesis inhibition and seems to be dependent on chromatin remodeling.

Our findings with primary response gene targets suggest that NFAT5 could participate in the maintenance of a pre-activated state of the chromatin in early primary response genes, together with other factors, like BRG1 and RNA Pol II phosphorylated in serine 5 (Ramirez-Carrozzi et al., 2006; Hargreaves et al., 2009; Escoubet-Lozac et al., 2011). The observation that the inhibition of HDACs alone allows NFAT5 recruitment to secondary response genes without the need of TLR signaling raises the possibility that chromatin accessibility controls NFAT5 binding to its target genes. In addition to overcome the chromatin barrier, NFAT5 could require a newly synthetized regulator induced by TLR to be recruited to its secondary response target genes. In this regard, since we report that binding of NFAT5 to the *Nos2* promoter requires IKKβ activity, the IKKβ and the NF-κB axis could participate in the *de novo* expression of a transcriptional regulator needed by NFAT5, such as a chromatin modifier, a nucleosome remodeler or another primary response gene product. As reported in literature, several regulators present these characteristics, for example JMJD3, IKKε, IκBβ and IkBζ (De Santa et al., 2007; Kayama et al., 2008; Huang et al., 2009; Rao et al., 2010). Finally, we cannot exclude that the dependence of NFAT5 activity on HDAC inhibition, protein synthesis and IKKβ reflects a common mechanism: a chromatin regulator expressed upon TLR stimulation in a NF-κB-dependent manner that controls the NFAT5-driven transcription of a group of secondary response genes. Further research would explain the mechanism by which NFAT5 regulates secondary response target genes. In this regard, and as discussed below, our work expands the knowledge on secondary response gene expression positioning NFAT5 as a modulator of chromatin accessibility to other transcription regulators.

We observed that NFAT5 is constitutively expressed in macrophages and its protein levels do not increase at short time points after TLR stimulation. However, the strong increase in NFAT5 protein expression after long stimulation time (8-24 hours) has not been investigated in this work. We observe that this increase only corresponds to a minor increase in mRNA expression. This can be explained by the high stability of pre-existing NFAT5 mRNA and its regulation via miRNA (Huang et al., 2011). In addition, results from our group also suggest that the substantial increase in protein levels after persistent stimulation might reflect an equilibrium between its degradation by the proteasome and its TLR-induced transcription and translation (Giulia Lunazzi, data not shown). The increase in NFAT5 expression after sustained TLR stimulation requires NF-κB-driven transcription, as was predicted by the presence of conserved κB consensus sites in the *Nfat5* promoter. We observe direct binding of p65 to these putative sites, and also that suppression of IKKβ signaling prevents NFAT5 accumulation. Further studies would be necessary to clarify the role of NFAT5 in sustained TLR stimulations. We focused our work on the events occurring within the first hours after macrophage activation, because an efficient inflammatory response requires a rapid response.

A central point in this work is the fact that NFAT5 is required for the expression of TLR-responsive genes, like *Tnf* and *Il6*, especially under low doses of stimulus. The majority of the studies of the regulation of inflammatory gene expression in primary macrophages use amounts of LPS around 100 times higher than ours, corresponding to a high pathogen load. We study the effect of a milder stimulation, as it reflects the contribution of NFAT5 in the response to pathogens. The ability of a macrophage to detect small quantities of bacterial products is fundamental to activate an efficient immune response that controls the pathogen load and also primes immune

responses for subsequent waves of pathogen invasion (Zhang and Morrison, 1993; Hirohashi and Morrison, 1996). While higher doses of stimulation would lead to gene expression independently of NFAT5, we have seen that this factor is important in conditions of mild TLR stimulation for the induction of a specific subset of genes that is enriched in NF-κB-target genes (Barish et al., 2010). Low input signals from TLRs are sufficient to activate NF-κB at levels similar to stronger signals, as demonstrated by the magnitude of p65 recruitment to a group of proinflammatory genes, but the absence of NFAT5 prevents the recruitment of p65 and c-Fos to these genes mainly in conditions of mild stimulation.

An additional key aspect observed in our work is that mild stimulation activates mechanisms of transcription different from those used in response to a stronger stimulation. While stimulation with high doses of LPS did not cause a significant H3K27 demethylation in *Nos2* and *Il6* promoters, low amounts of LPS induced an NFAT5-dependent early H3K27 demethylation in both genes. Since this is observed in the absence of significant histone eviction, it suggests that the equilibrium between Polycomb repressive complex 2 (PRC2) and H3K27 demethylases could be skewed towards the latter in the response to mild LPS stimulation. This is supported by works showing that the net amount of H3K27me3 marks per histone does not change in the response to a strong TLR stimulation in the *Nos2* and *Tnf* promoters (De Santa et al., 2009; Kruidenier et al., 2012). Moreover, as inhibition of H3K27 demethylases revealed an equilibrium between the activities of these enzymes and PRC2 (Kruidenier et al., 2012) even at high strength of stimulation, our current view is that upon a low input from TLRs this dynamic equilibrium must be shifted towards H3K27 demethylases, such as JMJD3 or UTX, in an NFAT5-dependent manner. This differential recruitment at low or high LPS doses might reflect that a mild activation of transcription regulators would require mechanisms facilitating the binding to

their targets in chromatin. In this regard, it is relevant to mention that Polycomb group proteins are regulated by the compaction of chromatin and, in turn, facilitate chromatin compaction and oppose the binding of transcription regulators (Zink and Paro, 1995; Yuan et al., 2012). This thesis proposes that NFAT5 controls the expression of its target genes at reduced TLR stimulation strength by facilitating the dominance of H3K27 demethylases over PRC2. In addition, our work also raises the question of whether non-NFAT5 targets might be controlled by other factors whose role is similar to the one proposed here for NFAT5 at mild stimulation strength. Since the structure of the chromatin in primary response genes has various characteristics of active chromatin, a possibility exists that the role of H3K27 demethylases counteracting the activity of PRC2 would be more relevant for the expression of secondary response genes upon low stimulation strength. In this regard, ChIP-seq analysis for H3K27me3 in NFAT5-deficient macrophages stimulated with low LPS doses would be useful to expand our knowledge on genes whose mechanism of expression resemble the one presented in this thesis.

It has been described that NF-κB regulates the status of histone methylation in response to TLRs. NF-κB proteins recruit specific histone demethylases to its target genes, as reported for Aof1 H3K9 demethylase (van Essen et al., 2010) and for PHF2 H4K20me3 demethylase (Stender et al., 2012). Our work reports that H3K27 demethylation is necessary for p65/NF-κB binding to *Nos2*, an observation that is in line with the finding that JMJD3-regulated genes are enriched in NF-κB targets (Das et al., 2012). In this context, NFAT5 could be the mechanistic link between H3K27me3 demethylation and p65 binding in a specific set of genes and in conditions of mild TLR stimulation. Furthermore, in addition to p65, not only c-Fos, but likely other transcription factors would rely on the proposed NFAT5-assisted local H3K27 demethylation. Therefore, our work uncovers a new

hierarchy of transcription modulators in the regulation of gene expression in macrophages responding to pathogens.

In summary, our work has unravel a new role for pre-existing NFAT5 in the control of a set of TLR-induced genes in primary macrophages activated by mild LPS doses. While NFAT5 is pre-bound to primary response target genes, it is recruited to secondary response target genes in a stimulus-dependent manner. NFAT5 regulates local chromatin accessibility and allows for the TLR-dependent recruitment of certain elements of the transcription machinery, such as p65/NF-κB, c-Fos, p300 and RNA Pol II. Finally, we suggest that NFAT5 facilitates the induction of its target genes by controlling the balance between trimethylation and demethylation of a specific histone residue, K27 in histone H3.

CONCLUSIONS

CONCLUSIONS

1. NFAT5 is required for the expression of a subset of TLR-responsive genes in macrophages in a hypertonicity-independent manner.

2. NFAT5 is particularly relevant in the response to low doses of TLR agonists.

3. NFAT5 is constitutively bound to its primary response target genes and is recruited to its secondary response target genes in a TLR-dependent manner.

4. NFAT5 recruitment to *Nos2* requires IKKβ activity, protein synthesis, and is sensitive to HDACs activity.

5. TLR stimulation induces a delayed and NF-κB-dependent accumulation of NFAT5.

6. NFAT5 is necessary for the recruitment of p65/NF-κB, c-Fos and p300 to a subset of proinflammatory gene promoters in response to mild TLR stimulation.

7. Stimulation of macrophages with low doses of LPS elicits an NFAT5-dependent increase in chromatin accessibility in its target genes, but NFAT5 does not influence TLR-induced changes in the density of nucleosomes.

8. The repressive chromatin mark H3K27me3 is erased from *Nos2* in an NFAT5-dependent manner in response to a mild TLR stimulation.

9. The binding of Polycomb repressive complex 2 and JMJD3 to *Nos2* is independent of NFAT5.

10. NFAT5-dependent local H3K27 demethylation in response to mild LPS stimulation is required for p65/NF-κB recruitment to *Nos2*.

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