UNIVERSITÁ DEGLI STUDI DI MILANO
SCIENZE BIOMEDICHE CLINICHE E SPERIMENTALI
DIPARTIMENTO DI MEDICINA TRASLAZIONALE

SCUOLA DI DOTTORATO IN
PATOLOGIA E NEUROPATOLOGIA SPERIMENTALI
CICLO XXIV

REGULATORY ROLE OF microRNAs ON INFLAMMATORY SIGNALING PATHWAYS

Settore scientifico disciplinare: MED/04

Dottorando: Mori Laura
Matricola: R08025

Tutore: Prof. Massimo LOCATI
Coordinatore del dottorato: Prof. Alberto MANTOVANI

A.A. 2010-2011

Table of contents

Abstract

Innate immunity is the primary defense mechanism that recognizes and respond to invading infectious microbes or their components, known as pathogen-associated molecular patterns (PAMPs), or to danger signals that comes from *self* components, known as danger-associated molecular patterns (DAMPs). These molecules are recognized in our body by the so called pattern recognition receptors (PRRs), whose activation quickly gives rise to an important cascade of events known as acute inflammatory response. Within few hours, pro-inflammatory cytokines, such as TNFα, IL-1β and IL6, and chemokine, such as CCL2, CCL3 or CXCL8, are released in the blood stream, and innate immune cellular components are induced to combat the pathogens to the site of injury. Circulating neutrophils and monocytes are implicated as essential players in defense against a range of microbial pathogens. The acute inflammation process is a double-edge sword. Normally, it terminated once triggering insult is eliminated, the infection is cleared and damage tissue is repaired. Misregulation of one or more step from initiation to resolution can significantly contribute to the pathogenesis of autoimmune, chronic inflammatory or infectious diseases. For this reason, the inflammatory response itself and its termination phase are active and highly regulated processes involving several key regulatory mechanisms.

MicroRNAs (miRNAs) are small noncoding RNAs recently emerged as powerful posttranscriptional regulators in various biological processes. A growing number of evidence suggests that the development and function of cells in the immune system is also subject to regulation by miRNAs, and in 2006 the first evidence on their potential involvement in inflammation control was provided by Taganov and colleagues, who reported a unique set of microRNAs (miR-146a, miR-155 and miR-132) overexpressed in the THP-1 monocytic cell line after TLR4 agonist engagement, and postulated that miR-146a may operate a negative feedback loop acting on TRAF6 and IRAK1, two keys adaptors in TLR4 signaling pathway. Moving from these information, this thesis project has characterized the complete microRNA expression profile induced by TLR4 activation in freshly purified human blood monocytes and neutrophils. Beyond the aforementioned miR-155, miR-146a and miR-132, a new set of molecules were first described as LPS-responsive miRNAs in monocytes, including miR-9, miR-187, and the miR-99b~7e~125a miR

cluster. This study also identified miR-9 as the only microRNA also up-regulated in neutrophils in a MyD88- and NF-κB-dependent manner and highlighted a new feedback regulatory loop acting at the NF-kB level, as miR-9 was demonstrated to directly target NFKB1 mRNA and post-transcriptionally modulating its expression.

Inflammatory response triggers an important number of events that bring not only to propagation (producing pro-inflammatory mediators such as TNFα or IL-1β) but also to resolution. Two fundamental anti-inflammatory mediators whose release is induced by inflammation itself are IL-10 and glucocorticoids (GC), that act in a autocrine/paracrine or systemic manner, respectively.

Our work has revealed that miR-187 and miR-99b~7e~125a miR cluster induction by LPS resulted by an IL-10-dependent loop. Bioinformatic tools and cellular and biochemical assays allowed us to uncovering the function of these new IL-10-dependent miRNAs in the modulation of several proinflammatory cytokines and chemokines (TNFα, IL-6, CCL3, CXCL8) acting at different steps of the signaling pathway that bring to their production. Indeed, both the transcription factor IκBζ and the signaling complex TLR4/CD14 were found to be targets of miR-187 and miR-99b~7e~125a miR cluster, respectively. These results underline a new mechanism for IL-10 in inducing pro-inflammatory genes silencing. In parallel, GC were shown to induce expression of miR-511 in a synchronized fashion with its host gene, the mannose receptor MRC1 (also known as CD206). Bioinformatic tools highlighted a relevant number of molecules involved in TGFβ signaling among miR-511 predicted target genes, and miR-511 was shown to block MAPK cascade and gene expression induced upon TGFβ stimulation.

Taken together, these results suggest that miRNA represent an emerging mechanism active in leukocytes to dampen inflammation and avoid exacerbated inflammatory mediators release by a multitargeting strategy affecting several key signaling pathways and transcription factors.

Introduction

Inflammation: from pathogen invasion to resolution

How inflammatory reaction starts

Inflammation is part of the complex biological response of tissues to harmful stimuli, such as pathogens, damaged cells, or irritants. It is a protective attempt by the organism to remove the injurious stimuli and to initiate the healing process.

A typical inflammatory response consists of four components: inflammatory inducers, the sensors that detect them, the inflammatory mediators induced by the sensors, and the target tissues that are affected by the inflammatory mediators (Medzhitov, 2010). The type of pathway induced under given conditions depends on the nature of the inflammatory trigger. Thus, bacterial pathogens are detected by receptors of the innate immune system, such as Toll-like receptors (TLRs) (Janeway and Medzhitov, 2002), and thereby the amplification of the inflammatory response starts, due in part to cytokines generation: within 1 to 2 h after endotoxin exposure, blood levels of TNFα increase acutely, accompained by modest increases in IL-1β levels (Michie et al., 1988; Preas et al., 1996); in parallel, cytokines that downregulate cytokine production or their effect (IL-10, IL-1ra, IL-6) are also detected in the blood (Granowitz et al., 1991; Michie et al., 1988); finally, growth factors that recruit and activate cells from the bone marrow (G-CSF, GM-CSF) and chemokines (CXCL8, CCL2, CCL3) that activate and direct the migration of leukocytes are detected within hours after endotoxin administration (Martich et al., 1991; Suffredini et al., 1995).

These inflammatory mediators then act on target tissues, including local blood vessels, to induce vasodilation, extravasation of neutrophils, and leakage of plasma into the infected tissue. Neutrophils recruited from the circulation, macrophages, and mast cells seek and destroy invading pathogens. This process is aided by plasma components, including antibodies and complement. In addition, IL-1β, TNFα, and IL-6 can have systemic effects when secreted in sufficient amounts (Suffredini et al., 1999). They induce liver cells to produce acute phase proteins such as C-reactive protein and coagulation factors, and they activate brain endothelium to produce prostaglandins, including the major proinflammatory prostaglandin, PGE2. Locally produced PGE2, in turn, induces specific populations of neurons in the

central nervous system to promote so-called sickness behavior: fever, anorexia, fatigue, sleepiness, and social withdrawal (Pecchi et al., 2009). Depending on the type of infection (bacterial, viral, or parasitic), the sensors, mediators, and target tissues vary such that the appropriate type of inflammatory response is induced.

The cellular poin of view – the innate immune system

Innate immunity covers many areas of host defense against pathogenic microbes, including the recognition of pathogen-associated molecular patterns (PAMPs) (Janeway, 1989). It is an evolutionarily ancient part of the host defense mechanisms: the same modules are found in plants and animals, meaning that it arose before the split into these two kingdoms (Hoffmann et al., 1999). Conversely, adaptive immunity is a relative newcomer on the evolutionary landscape. Because the mechanism of generating receptors in the adaptive immune system involves great variability and rearrangement of receptor gene segments, the adaptive immune system can provide specific recognition of foreign antigens, immunological memory of infection, and pathogen-specific adaptor proteins. However, no adaptive response can be initiated without a prompt activation of the innate immune system, that represent the firing line against pathogen invasion.

Innate immunity also lies behind most inflammatory responses, that are triggered in the first instance by neutrophils, monocytes, macrophages and dendritic cells through their innate immune receptors.

i. Neutrophils

Neutrophils are classically characterized by their ability to act as phagocytic cells, to release lytic enzymes from their granules and to produce reactive oxygen intermediates (ROI) with antimicrobial potential (Borregaard, 2010). Recently, neutrophils were observed as cells that survive much longer than first suggested (Colotta et al., 1992) and can be induced to express genes encoding key inflammatory mediators, including complement components, Fc receptors, chemokines and cytokines (Cassatella, 1999). Recent data have also suggested that neutrophils can be polarized towards distinct phenotypes in response to environmental signals (Fridlender et al., 2009). Neutrophils express a vast repertoire

of PRRs, including all members of the Toll-like receptor (TLR) family with the exception of TLR3 (Hayashi et al., 2003). The sensing of pathogens and tissue damage activates the effector functions of neutrophils. These include the production of ROI, lytic enzymes and antimicrobial peptides, as well as more recently described neutrophil extracellular traps (NETs) (Brinkmann et al., 2004). Indeed, neutrophils can extrude extracellular fibrillary networks composed mainly of DNA, but also contain proteins from neutrophil granules. NETs act as a mesh that traps microorganisms and, in turn, facilitates their interaction with neutrophil-derived effector molecules.

Cytokine production by neutrophils is controlled by regulatory mechanisms that act at different levels, including mRNA transcription (Cassatella, 1999), stability or translation (for example, through microRNA-mediated targeting, as in the case of mouse IFNγ (Yamada et al., 2011)), as well as protein secretion.

Although neutrophils do not proliferate and have an estimated half-life of approximately 10–12 hours under in vitro culture conditions, signals such as adhesion, transmigration, hypoxia, microbial products and cytokines (Colotta et al., 1992) can delay their programmed cell death and thus extend their survival in vivo. For example, macrophages can attract neutrophils to the site of injury and produce cytokines to control the lifespan and activity of the recruited cells (Soehnlein and Lindbom, 2010). On the other hand, as a key component of the inflammatory response, neutrophils make important contributions to the recruitment, activation and programming of APCs. Neutrophils generate chemotactic signals that attract monocytes and dendritic cells (DCs), and influence whether macrophages differentiate to a predominantly pro- or anti-inflammatory state. They also produce TNFα and other cytokines that drive DC and macrophage differentiation and activation.

ii. Monocytes

Neutrophils, macrophages, and dendritic cells (DCs) are important cellular mediators of innate immune defense. Circulating monocytes, however, are increasingly implicated as essential players in defense against a range of microbial pathogens. Monocytes represent 10% of leukocytes in human blood and 4% of

leukocytes in mouse blood. They are present in mammals, birds, amphibians, and fish (Hadji-Azimi et al., 1987; Herbomel et al., 1999; Kelly et al., 2000), and a related population of hemocytes (called plasmatocytes) is present in the fly (Williams, 2007), which does not have lymphocytes. Monocytes play an important role in development and homeostasis, in part via the removal of apoptotic cells and scavenging of toxic compounds (Williams, 2007). Strikingly, monocyte/macrophage specialization can already be observed among simple eukaryotic organisms, as phagocytes able to scavenge toxic compounds and kill bacteria differentiate inside colonies of social amoeba (*Dictyostelium discoideum*) (Chen et al., 2007a). Monocytes represent immune effector cells, equipped with chemokine receptors and adhesion receptors that mediate migration from blood to tissues during infection. The best known function of monocytes is as a considerable systemic reservoir of myeloid precursors for the renewal of some tissue macrophages and antigen-presenting dendritic cells (DCs) during inflammation and possibly, less efficiently, in the steady state (Randolph et al., 1999; Serbina and Pamer, 2006). Migration to tissues and differentiation to inflammatory DCs and macrophages are likely determined by the inflammatory milieu and pathogen-associated pattern-recognition receptors (Serbina et al., 2008). Monocytes have long been considered as a developmental intermediate between bone marrow precursors and tissue macrophages. It is now clear, however, that many DCs and tissue macrophages do not originate from monocytes in a steady state. Conversely, monocytes carry out specific effector functions during inflammation (Auffray et al., 2009). They produce inflammatory cytokines and take up cells and toxic molecules. Indeed, blood monocytes also represent a large pool of scavenger and potential effector cells inside blood vessels in homeostasis as well as during inflammatory processes (Auffray et al., 2007). Monocytes are equipped with a large array of scavenger receptors that recognize lipids and various microorganisms, and stimulated monocytes can produce large concentrations of ROI, complement factors, prostaglandins, nitric oxide (NO), cytokines such as TNFα, IL-1β, CXCL8, IL-6, and IL-10, vascular endothelial growth factor and proteolytic enzymes. They also have been involved in the defense against pathogen (Serbina et al., 2008). Antigen presentation has been described as a classical feature of monocytes, but since the identification of discrete subsets of DCs among monocyte cells, *bona fide*

monocytes have been found in most cases to be far less efficient than DCs for antigen presentation (Banchereau and Steinman, 1998).

A peculiar feature of monocytes is their plasticity, which holds that monocytes respond to their environment by differentiating into a variety of macrophages and DC-like cells (Taylor and Gordon, 2003). This concept of plasticity is largely based on the effects of cytokines on monocytes in vitro. Exposure to granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4 induces differentiation of human and mouse monocytes into DCs. Moreover, addition of transforming growth factor-β1 confers the phenotype of Langerhans Cells (Geissmann et al., 1998), whereas exposure to M-CSF induces monocytes to differentiate into macrophages. Addition of IFNγ and lipopolysaccharide to M-CSF-differentiated macrophages (resting macrophages) induces the differentiation of M1-like cells, whereas addition of IL-4 induces the differentiation of M2-like macrophages (Martinez et al., 2009).

iii. Mononuclear Phagocyte system: macrophages and dendritic cells

Macrophages and DCs form networks of phagocytic cells throughout most tissues, sometimes referred to as the Mononuclear Phagocyte System (MPS) (van Furth and Cohn, 1968), and play major roles in development, scavenging, inflammation, and antipathogen defenses. The MPS was initially defined as a population of cells, derived from a bone marrow progenitor, that differentiate and enter the blood as monocytes and then enter tissues to become resident tissue macrophages and antigen-presenting cells (van Furth and Cohn, 1968). However, it was soon recognized that DCs and macrophages have a remarkable heterogeneity related to their origin, phenotype, tissue localization, proliferative potential, and function (Geissmann et al., 2008).

Current models propose that blood monocytes, many macrophage subsets, and most DCs originate in vivo from hematopoietic stem cell-derived progenitors with myeloid-restricted differentiation potential. Successive commitment steps in the bone marrow include common myeloid progenitors (CMPs), granulocyte-macrophage precursors (GMPs), and macrophage/DC progenitors (MDPs). MDPs are a subset of proliferating cells in the bone marrow that share phenotypic

characteristics with myeloid precursor populations and give rise to many macrophages and DC subsets (Fogg et al., 2006) but largely cannot differentiate into granulocytes. Within the bone marrow, MDPs differentiate to monocytes and to the common DC precursors (CDPs).

Macrophages are resident phagocytic cells in lymphoid and nonlymphoid tissues and have a broad role in the maintenance of tissue homeostasis, through the clearance of apoptotic cells and the remodelling and repair of tissues after inflammation (Gordon, 1998). Macrophages are equipped with a broad range of pathogen-recognition receptors that make them efficient at phagocytosis and induce production of inflammatory cytokines (Gordon, 2002). They derived from a common anchestor, but once spread in the organism, show a high degree of heterogeneity. The heterogeneity reflects the specialization of function that is adopted by macrophages in different anatomical locations: the ability of osteoclasts to remodel bone (Quinn and Gillespie, 2005); the high expression of pattern-recognition receptors and scavenger receptors by alveolar macrophages, which are involved in clearing microorganisms, viruses and environmental particles in the lungs (McCusker and Hoidal, 1989); the gut is one of the richest sources of macrophages in the body, and isolation of macrophages from the lamina propria has highlighted a unique macrophage phenotype that is characterized by high phagocytic and bactericidal activity but weak production of pro-inflammatory cytokines (Smythies et al., 2005). Mirroring T helper type 1–T helper type 2 (T_H1-T_H2) polarization, two distinct states of polarized activation for macrophages have been recognized: the classically activated (M1) macrophage phenotype and the alternatively activated (M2) macrophage phenotype (Martinez et al., 2008). Bacterial moieties such as LPS and the T_H1 cytokine interferon-γ (IFN-γ) polarize macrophages toward the M1 phenotype. In contrast, M2 polarization was originally discovered as a response to the T_H2 cytokine IL-4 (Stein et al., 1992).

Dendritic cells represent a heterogenous cell population, residing in most peripheral tissues, particularly at sites of interface with the environment (skin and mucosae), where they represent 1-2% of the total cell numbers (Banchereau and Steinman, 1998). Dendritic cells (DCs) are unique APCs because they are the only ones that are able to induce adaptive immune responses, thus permitting

establishment of immunological memory (Banchereau and Steinman, 1998). In the absence of ongoing inflammatory and immune responses, dendritic cells constitutively patrol through the blood, peripheral tissues, lymph and secondary lymphoid organs. A signal from pathogens, often referred to as a danger signal, induces dendritic cells to enter a developmental program, called maturation, which transforms dendritic cells into efficient antigen presenting cells (APCs) and T cell activators (Gallucci and Matzinger, 2001). Numerous factors induce and/or regulate DC maturation, including pathogen-related molecules such as LPS (Rescigno et al., 1999), bacterial DNA (Akbari et al., 1999), and double-stranded RNA (Cella et al., 1999); the balance between pro-inflammatory and anti-inflammatory signals in the local microenvironment, including TNFα, IL-1β, IL-6, IL-10, TGFβ, and prostaglandins and T cell-derived signals.

Individual myeloid cell populations may share features of DCs and macrophages and can be difficult to ascribe to one or the other cell type.

Sensing pathogens – Toll-like Receptors family and related proteins

Microorganisms have various features that distinguish them from multicellular organisms. These features are known as "microbial patterns" and their detectors are defined as "pattern-recognition receptors" (PRRs) (Janeway, 1989). To initiate immune responses, PRRs recognize pathogen-associated molecular patterns (PAMPs) and induce several extracellular activation cascades such as the complement pathways and various intracellular signaling pathways, leading to inflammatory responses. The principal functions of pattern recognition receptors include opsonization, activation of complement and coagulation cascades, phagocytosis, activation of proinflammatory signaling pathways and induction of apoptosis.

The innate immune system utilizes PRRs present in three different compartments: body fluids, cytoplasm and cell membranes. The PRRs in body fluids play major roles in PAMPs opsonization, the activation of complement pathways and in some cases the transfer of PAMPs to other PRRs. Complement components C3 and C1q and a collectin family member MBL can activate complement pathways after PAMP recognition (Gasque, 2004). Pentraxin family members SAP, CRP and

PTX3 opsonize microbes for phagocytic clearance and/or activate the classical complement pathway (Garlanda et al., 2005; Gasque, 2004). Lipopolysaccharide-binding protein (LBP) binds LPS, a major gram-negative bacterial cell wall component, and transfers it to membrane-bound CD14, a GPI-anchored, leucine-rich repeat (LRR) protein (Ulevitch and Tobias, 1995).

Viruses and some bacterial pathogens can gain access to the intracellular compartments, such as the cytosol. Several pattern recognition receptors are expressed in the cytosol where they detect these intracellular pathogens and induce responses that block their replication. We can group cytoplasmic PRRs into three classes: interferon-inducible proteins, caspase-recruiting domain (CARD) helicases and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs). IFN-inducible proteins such as PKR and OASs (Stark et al., 1998) and CARD helicases such as RIG-I and MDA5 (Yoneyama et al., 2004) are involved in antiviral defense. In contrast, NLRs are mainly involved in antibacterial immune responses (Martinon and Tschopp, 2005).

PRRs located on the cell membrane have diverse functions, such as the presentation of PAMPs to other PRRs, the promotion of microbial uptake by phagocytosis, and the initiation of major signaling pathways. Several cell surface receptors expressed on macrophages function as pattern recognition receptors that mediate phagocytosis of microorganisms. Macrophage mannose receptor (MRC1) is a member of the C-type lectin family and interacts with a variety of gram-positive and gram-negative bacteria and fungal pathogens. The main function of the MRC1 is thought to be phagocytosis of microbial pathogens and their delivery into the lysosomal compartment where they are destroyed by lysosomal enzymes (Fraser et al., 1998).

TLRs are the best-characterized receptors among the PRRs. Their name reminds to their discovery. In *Drosophila*, Toll is a single-pass transmembrane receptor with an ectodomain marked by leucine-rich repeat motifs, originally identified for its essential role in development as the determinant of dorsoventral polarity in the fruitfly embryo. In addition, Toll was found to have a crucial role in immune defence. This second function of Toll was discovered by investigating the transcriptional regulation of antimicrobial genes that are induced by infection.

Subsequently, nuclear factor-κB (NF-κB) immune signaling, first identified in mammals, was suggested to occur in insects because NF-κB-binding motifs were identified in promoters of immunoresponsive genes in Drosophila (Sun et al., 1991). Surprisingly, the ligand for Toll in the immune response is not a microbial product, and so Toll is not a pattern-recognition receptor in fruitflys (Levashina et al., 1999).

When administered to most mammalian species, LPS causes the prompt development of fever, disturbances in the clotting of blood, hypotension and shock (Ulevitch et al., 1984). All these effects are mediated by the activation of macrophages (Galanos and Freudenberg, 1993), which in turn release toxic cytokines, such as tumour necrosis factor (TNFα), that is known to provoke the release of terminal constituents of the inflammatory cascade (Beutler et al., 1985), and is considered to be largely responsible for LPS-induced lethality. The pathway through which LPS signals has been investigated extensively at a biochemical level. The LPS is driven to the surface of cells by the acute-phase protein LBP (LPS-binding protein). There, it binds to the glycosylphosphoinositol-anchored membrane protein CD14, that lacks a transmembrane domain. How the LPS signal is transduced across membrane became clear only after analyis of C3H/HeJ and C57BL/10ScCr mice, whose genetic lesion, simply called Lps mutation, makes these mice unsusceptible to endotoxin shock after LPS systemic subministration. By a strict positional cloning approach, these mutations were found to affect the Tlr4 gene. In C3H/HeJ mice, a point mutation modified the cytoplasmic domain of the Tlr4 protein creating a dominant-negative effect (Du et al., 1999). Later, a knockout mutation of Tlr4 (Hoshino et al., 1999) proved to be an excellent phenocopy of the C57BL/10ScCr mouse in terms of LPS response, confirming that the gene is required for LPS signal transduction. During 1999 and 2000, knockout mutations of Tlr2 and Tlr9 revealed that the former acts as a specific transducer for bacterial lipopeptide and peptidoglycan signals (Takeuchi et al., 1999), whereas the latter acts to detect bacterial DNA (Hemmi et al., 2000). The general impression of TLR function, therefore, is one in which each of the TLRs recognizes a discrete subset of those molecules that are widely shared by microbial pathogens. In this way, TLRs collectively provide protection against an immense number of microorganisms.

All TLRs known in mammals are type I integral membrane glycoproteins

containing an extracellular domain with LRRs responsible for ligand recognition and a cytoplasmic Toll/IL-1R homology (TIR) domain required for initiating signaling (Akira and Takeda, 2004). Working as homo or heterodimers or with other PRRs, they recognize several ligands, consisting of bacterial cell wall components, bacterial genomic DNA, viral, fungal and parasitic products, and synthetic analogs of natural products. However, TLRs can also bind with autologous self-molecules such as heat shock proteins (HSPs), intercellular matrix products, and mammalian genomic DNA, revealing that the TLR immune system is concerned with damage signals from injured tissue, including endogenous ligands such as high mobility group box 1 protein (HMGB1), HSPs (HSP22, HSP60, HSP70, HSP96), hyaluronan, type III repeat extra domain of fibronectin, uric acid crystals, mouse mammary tumor virus envelope proteins and respiratory syncytial virus fusion protein, β-defensin and plant ligands (paclitaxel) (O'Neill et al., 2009). It is suggested that TLRs recognize not only PAMPs, but also stress- or damage-associated molecular patterns (DAMPs) (Seong and Matzinger, 2004).

TLRs 1, 2, 4, 5, and 6 are located mainly on the cell surface and primarily recognize bacterial components, but TLRs 3, 7, 8, and 9 are mostly on endocytic compartments and mainly recognize viral products (Akira et al., 2006).

The model currently used to describe initiation of signaling (Figure 1) involves ligand- induced dimerization of TLRs, creating a TIR-TIR interface, which acts to recruit adapters via their TIR domains (Weber et al., 2005). Specificity is evident in adaptor usage by different TLRs. TIR-containing TLRs recruit the TIR-containing cytosolic adaptors MyD88, TRIF (TIR domain–containing adaptor-inducing IFN-β), MAL (MyD88 adaptor-like; also known as TIRAP), TRAM (TRIF-related adaptor molecule) and SARM (sterile α and HEAT/armadillo motif protein) (Jenkins and Mansell, 2010; Kenny and O'Neill, 2008; O'Neill and Bowie, 2007). The canonical TIR pathway is dependent on MyD88, which is used by all TLRs except TLR3. The alternative pathway is controlled by another key adaptor, TRIF, the only TLR3 adaptor, with TLR4 binding both MyD88 and TRIF. The remaining three adaptors serve as coadaptors (MAL, TRAM) or as a negative regulator (SARM). The sorting adaptors MAL and TRAM are used by only some TLRs. MAL recruits MyD88 to TLR2 and TLR4, whereas TRAM recruits TRIF to TLR4 (O'Neill

and Bowie, 2007). Finally, SARM is supposed to be a negative regulator of TRIF (Carty et al., 2006). The TIR-containing adaptors recruit various molecules such as cytosolic kinases, including the IL-1R-associated kinase (IRAK) complex, leading to the sequential activation of IRAK4, IRAK1, and TNF receptor- associated factor 6 (TRAF6) (Akira and Takeda, 2004). Then, the activated TRAF6 as an E3 ubiquitin

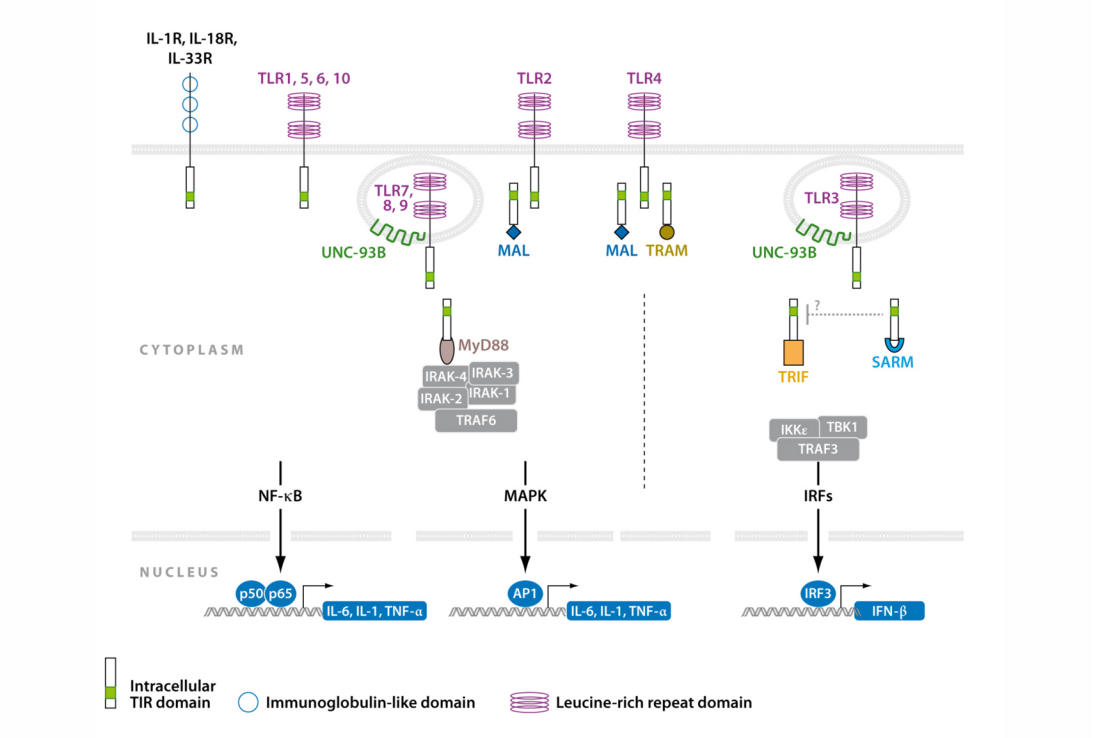

Figure 1. IL-1β/TLR signaling pathways. Activated TLRs and IL-1Rs recruit specific combinations of TIR-containing proteins (green spots). Specific TIR complexes activate specific kinase complexes (IRAKs or IKKε/TBK1), leading to the expression of specific genes via the NF-κB, AP1, and IRF transcription factors.

ligase, together with an E2 ubiquitin-conjugating enzyme complex, ubiquitinates TRAF6 and IκB kinase γ (IKKγ) (also called NEMO) (Deng et al., 2000). The ubiquitinated TRAF6 is recruited to the TGFβ-activated kinase 1 (TAK1)-TAB1/2/3 complex by binding to TAB2 (Chen, 2005). This promotes the activation of TAK1, a mitogen-activated protein (MAP) kinase kinase kinase (MAP3K), which in turn activates the canonical NF-κB activation pathway, leading to the expression of inflammatory cytokines (Chen, 2005). TAK1 also activates MAP kinases such as c-Jun N-terminal kinases (JNKs) and p38, leading to the activation of AP-1 and thus the regulation of inflammatory cytokine expression (Wang et al., 2001). Finally, TRIF leads to IRF3 activation via recruitment of the IKKε/TBK1 (IκB kinase

ε/TANK-binding kinase 1), and this signal, in the case of both TLR3 and TLR4, comes from the endosome, with TLR4 trafficking there after LPS recognition (Kagan et al., 2008; Tanimura et al., 2008). The TRIF-mediated activation of IRF3 was shown to be essential in defence against virus, as it regulates the production of type I interferons (Yoneyama et al., 2004). A schematic representation of TLR signaling pathway is given in Figure 6.

Although all TLRs signal through the conserved signaling cascade described above, the complexity of the TLR-induced cellular responses indicates that there must be additional regulatory mechanisms and signaling pathways downstream of TLRs. One example is provided by the existence of a Rac1-PI3K-AKT pathway activated by TLR2. This pathway leads ultimately to phosphorylation of NF-κB and is necessary for NF-κB transactivation activity (Arbibe et al., 2000). Because Rac1, PI3K and AKT regulate diverse cellular functions in other pathways, this study also raises the interesting possibility of links connecting the TLR pathway to other signaling pathways.

The inflammatory response as a genetic program

Innate immune cells respond very rapidly to the presence of specific ligands signaling infectious danger that they recognize through pattern recognition receptors, as described below. Activation of TLR induces the expression of cytokines, chemokines, and other inflammatory mediators within less than 1 h. Two major signaling pathways are activated downstream of the TLR-induced MyD88-IRAK-TRAF6 complex, which are essential for such a rapid response, and both involve the activation of the following latent transcription factors: 1) activation of the IκB kinase complex targets IκBs for degradation leading to nuclear translocation of active NF-κB and 2) the MAPK pathway, a cascade of phosphorylation events that primarily results in the post-translational activation of several transcription factors, such as AP-1. Both pathways synergize in inflammatory gene expression through coordinate binding of transcription factors to κB and AP-1 sites, often found together in the promoters of many genes up-regulated in response to TLRs engagement. A schematic representation of what will be discussed here is given in Figure 6.

NF-κB system

NF-κB is an eucaryotic transcription factor that exists in virtually all cell types. It was first described in 1986 as a nuclear factor necessary for immunoglobulin kappa light chain transcription in B cells (hence the name, nuclear factor-κB) (Sen and Baltimore, 1986).

The basic scheme of NF-κB signaling consists of a series of positive and negative regulatory elements. Inducing stimuli trigger IKK activation leading to phosphorylation, ubiquitination, and degradation of IκB proteins. Released NF-κB dimers are further activated through various posttranslational modifications and translocate to the nucleus where they bind to specific DNA sequences and promote transcription of target genes. In its most basic form, therefore, the pathway consists

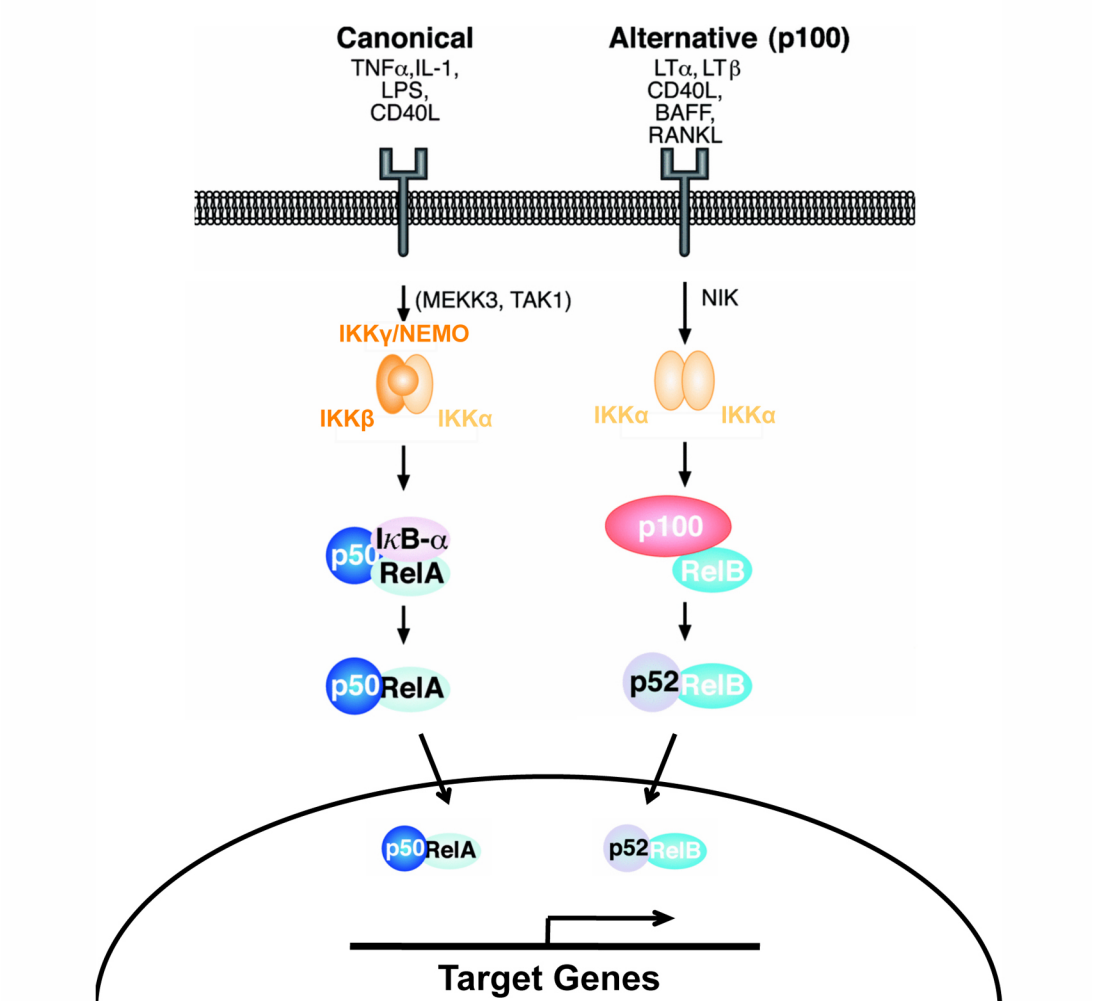

Figure 2. Model of different NF-κB signaling pathways. Activation of the canonical pathway (left) depends on the IKK complex (IKKα–IKKβ–IKKγ/NEMO), which phosphorylates IκBα to induce its rapid degradation (IκBβ and IκBε are similarly regulated by IKK). The alternative pathway requires NIK and IKKα and induces the slow processing of p100 to p52, resulting in nuclear translocation of p52/RelB heterodimers.

of receptor and receptor proximal signaling adaptor molecules, the IKK complex, IκB proteins and NF-κB dimers (Hayden and Ghosh, 2008) (Figure 2).

Mammals express five NF-κB proteins, namely RelA (p65), RelB, c-Rel, NF-κB1 p50 and NF-κB2 p52. NF-κB1 and NF-κB2 are synthesized as large precursors of 105 (p105) and 100 kDa (p100) respectively that are processed by the proteasome, to produce the active NF-κB1 p50 and NF-κB2 p52 subunits. A common feature of all NF-κB proteins is the Rel-homology domain (RHD), that contains a nuclear localization sequence (NLS) and is involved in dimerization, sequence-specific DNA binding and interaction with the inhibitory IκB proteins (Ghosh et al., 1998). Although p50 and p52 lack a transcription activation domain, such a domain is present in RelA, RelB and c-Rel. The NF-κB proteins form numerous homo and heterodimers that are associated with specific biological responses that stem from their ability to regulate target gene transcription differentially. For instance, p50 and p52 homodimers function as repressors, whereas dimers that contain RelA or c-Rel are transcriptional activators. RelB exhibits a greater regulatory flexibility, and can be both an activator (Ryseck et al., 1992) and a repressor (Ruben et al., 1992).

In its inactive state, NF-κB dimers are associated with one of three typical IκB proteins, IκBα (NFKBIA), IκBβ (NFKBIB), or IκBε (NFKBIE), or the precursor proteins p100 (NFKB2) and p105 (NFKB1). These IκBs maintain NF-κB dimers in the cytoplasm and are crucial for signal responsiveness. There are two inducibly expressed, atypical IκB proteins, Bcl-3 (BCL3) and IκBζ (NFKBIZ), that function quite differently in the regulation of NF-κB. The prototypical and most extensively studied member of the family is IκBα. IκBα is rapidly degraded during activation of canonical NF-κB signaling pathways leading to the release and activation of multiple NF-κB dimers, although the p65:p50 heterodimer is likely the primary target of IκBα. The nuclear NF-κB drives IκBα expression generating a negative feedback loop. IκBα, IκBβ, and IκBε, as traditional IκB proteins, sequester NF-κB dimers away from κB elements thus inhibiting transcription. The functional characteristics of IκBα, IκBβ, and IκBε are most likely a result of temporal differences in their degradation and resynthesis (Hoffmann et al., 2002). IκBβ and IκBε degradation and resynthesis occur with considerably delayed kinetics compared to that of IκBα.

NF-κB1 p105 and NF-κB2 p100, as precursors, can display IκB functions. Multiple reports have demonstrated that IKKβ-dependent phosphorylation of p105 leads to complete degradation of the protein analogous to IκBα (Hayden and Ghosh, 2004).

Processing of p100 to p52 requires IKKα and is predominantly stimulus-dependent (Senftleben et al., 2001). This process is recognized as alternative NF-κB activation pathway. This pathway is crucial for RelB activity, as RelB-containing dimers only associate with p100 and it has been suggested that they require p100 binding for stabilization (Solan et al., 2002).

The "Atypical" IκBs consist in two members, IκBζ and Bcl-3, that appear to regulate NF-κB signaling by a distinct mechanism. Bcl-3 is found in the nucleus associated with p50- and p52-containing homo and heterodimers. The mechanism of action of Bcl-3 is still not completely understood. Bcl-3 may mediate release of transcriptional repression by removing p50 homodimers from κB sites, thus acting as a traditional IκB but mediating activation by acting on repressive NF-κB dimers (Hayden and Ghosh, 2004). Alternatively, Bcl-3 may also stabilize repressive p50 homodimers (Carmody et al., 2007). As a result, the induction of Bcl-3 expression inhibits subsequent NF-κB activation and may con- tribute to LPS tolerance in macrophages.

IκBζ is not expressed constitutively but rather is upregulated in response to IL-1β and TLR4 ligands and upon expression localizes to the nucleus (Hayden and Ghosh, 2004). Most intriguingly in the absence of IκBζ, LPS- or IL-1-induced expression of a subset of NF-κB-regulated genes is lost (Yamamoto et al., 2004). IκBζ is inducibly expressed following NF-κB activation and once expressed associates primarily with p50 homodimers. Furthermore, IκBζ is found associated with p50 on the promoter of IL-6, which is not inducibly expressed in IκBζ knockout cells, and it is, therefore, hypothesized that IκBζ acts as a coactivator for p50 homodimers (Yamamoto et al., 2004). IκBζ has also been reported to negatively regulate p65-containing NF-κB complexes, and the slight elevation of NF-κB activity observed in IκBζ knockouts seems consistent with this (Motoyama et al., 2005; Yamamoto et al., 2004). Thus IκBζ, like Bcl-3, may also be capable of selectively inhibiting or activating specific NF-κB dimers.

Degradation of IκB is a rapidly induced signaling event that is initiated upon specific phosphorylation of these molecules by activated IKK. The IKK complex contains two highly homologous kinase subunits, IKKα/IKK1 (CHUK) and IKKβ/IKK2 (IKBKB), and a regulatory subunit NEMO (NF-κB essential modulator)/IKKγ (IKBKG) (Hacker and Karin, 2006). Receptor engagement results in IKK activation, and the activated IKK complex phosphorylates IκBs, leading to their polyubiquitination and subsequent degradation via the 26S proteasome, thereby inducing nuclear translocation of NF-κB dimers (Karin and Ben-Neriah, 2000).

A remarkable diversity of stimuli lead to activation of NF-κB. These include both endogenous and exogenous ligands as well as a plethora of physical and chemical stresses. Signaling to NF-κB proceeds through intracellular adaptor proteins that allows their incorporation into various receptor-induced signaling events. Although there are exceptions, it appears that both canonical and non-canonical pathways utilize TRAF family members for activation, while only canonical NEMO-dependent signaling to typical IκBs additionally requires RIP proteins (Hayden and Ghosh, 2008).

TRAFs are key intermediates in nearly all NF-κB signaling pathways. As discussed above, in TLR/IL-1R signaling TRAF6 is recruited to the receptor complex and is necessary for MyD88-dependent activation of NF-κB (Kawai and Akira, 2007). TRAF6 is recruited to MyD88 in a manner dependent on IRAK proteins and RIP1, another key adapter in at least canonical NF-κB activation (Kawai and Akira, 2007).

Receptor-interacting proteins (RIPs) appear to act both upstream of and with TRAF proteins to activate IKK. In fact, it is thought that RIPs may act as adapters and scaffolds in facilitating TRAF-induced IKK activation (Lee et al., 2004; McCarthy et al., 1998), as well as in TRIF-dependent NF-κB activation via TLR3 and TLR4 (Cusson-Hermance et al., 2005). Following recruitment by IRAKs, TRAF6 binds to the TAB1/2/3 complex, leading to TAK1-mediated IKK activation (Wang et al., 2001). In noncanonical pathways NIK is instead required for IKKα activation and p100 phosphorylation (Senftleben et al., 2001; Xiao et al., 2001). Despite the clear requirement for TAK1 in multiple signaling pathways to IKK, the mechanism of action of TAK1 in signaling to NF-κB remains unclear. It is generally

agreed that this complex is needed for JNK and p38 activation (Sato et al., 2005). Indeed, a TAK1:TAB1 fusion protein is constitutively active and capable of stimulating AP-1 activity (Sakurai et al., 2002).

MAP kinase cascade – AP-1 activation

Mitogen-activated protein kinases (MAPKs) are protein Ser/Thr kinases that convert extracellular stimuli into a wide range of cellular responses. MAPKs are among the most ancient signal transduction pathways and are widely used throughout evolution in many physiological processes. All eukaryotic cells possess multiple MAPK pathways, which coordinately regulate gene expression, mitosis, metabolism, motility, survival, apoptosis, and differentiation.

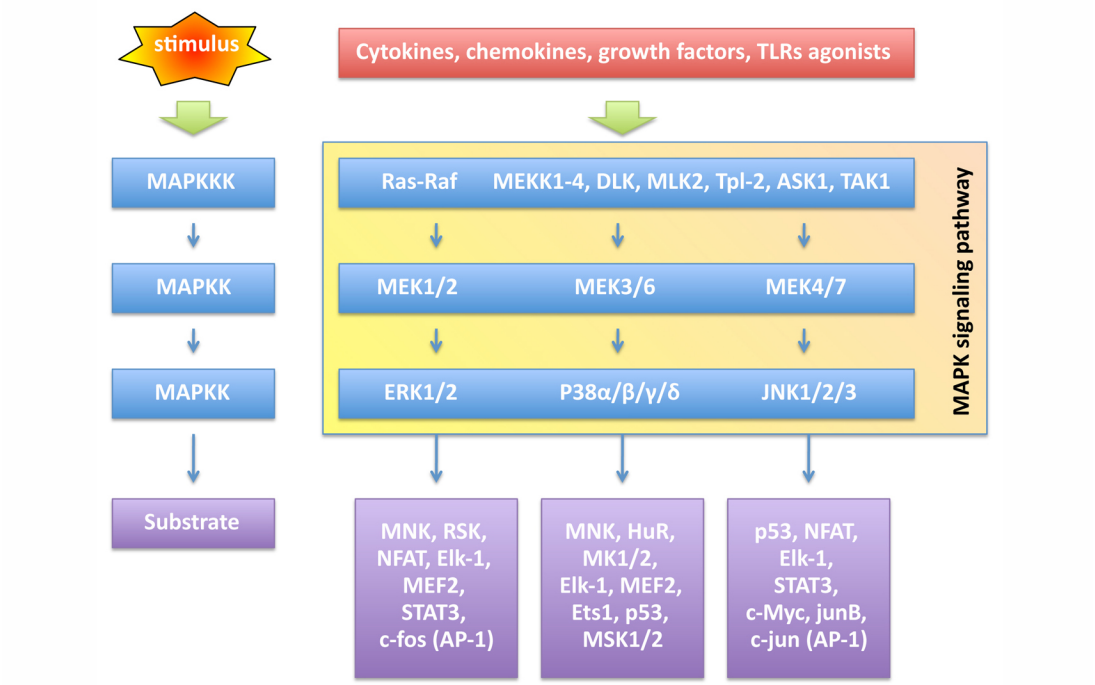

Figure 3. Mammalian MAP kinase pathways. The activation of MAPK signaling pathways is achieved through a triple kinase cascade: MAPKs are activated by dual phosphorylation on Thr and Tyr caused by specific MKKs. The MKKs are activated, in turn, by MKKKs. ERK1/2 can be activated by a specific signaling pathway that involve Ras downstream molecules, whereas p38s and JNKs share several MAPKKKs that selectively activate MEK3/6 or 4/7 with a ratio that determines the final outcome.

Three main families of MAP kinases (MAPKs) exist in mammalian species, grouped by their structures and functions: the extracellular signal-regulated protein kinases (ERKs), the p38 MAPK, and the c-Jun NH_2-terminal kinases (JNKs)

(Schaeffer and Weber, 1999). All the MAPKs consist of a Thr-X-Tyr (TXY) motif within their activation loop. The phosphorylation of both threonine and tyrosine within the activation loop is essential and sufficient for their activation. The ERK family contains a TEY (Thr-Glu-Tyr) activation motif (Cobb and Goldsmith, 1995). The p38 family has a TGY activation motif and includes α, β, γ and δ. There are three members in the JNK family which all contain Thr-Pro-Tyr (TPY) in their activation motif (Kyriakis et al., 1994). Each group of conventional MAPKs is composed of a set of three evolutionarily conserved, sequentially acting kinases: a MAPK, a MAPK kinase (MAPKK), and a MAPKK kinase (MAPKKK). The MAPKKKs, which are protein Ser/Thr kinases, are often activated through phosphorylation and/or as a result of their interaction with a small GTP-binding protein of the Ras/Rho family in response to extracellular stimuli. MAPKKK activation leads to the phosphorylation and activation of a MAPKK, which then stimulates MAPK activity through the dual phosphorylation described above (Cargnello and Roux, 2011) (Figure 3).

Multiple mechanisms present in the MAPK signaling pathways ensure the fidelity and efficiency of the signaling flow. These mechanisms include the interaction between the kinase catalytic domain and the substrate phospho-acceptor site, docking interactions between members of a particular MAPK cascade and scaffold proteins (Zhang and Dong, 2007).

Downstream of the MAPK, a large number of substrates that are serine/threonine-phosphorylated have been defined, including transcription factors of the ATF/CREB and AP-1 family, kinases such as Mapkapk2/MK2 and RSK, and proteins controlling mRNA stability and translation. Although there is some overlap in the target proteins of MAPK, prototypic-specific downstream mediators have also been defined using specific pharmacological inhibitors. These studies demonstrated the importance of MAPK activation in cytokine and chemokine gene expression in general, and provided many specific examples of genes that are regulated preferentially by one or the other MAPK. For example, IL-10 production was inhibited by the Map2k1/MEK1 inhibitor U0126, whereas IL-12 expression was suppressed by inhibition of p38 with SB203580 (Yi et al., 2002). Furthermore, higher IL-12 production from DC than from macrophages after stimulation was

inversely correlated with differences in the amount of ERK activation between the cell types (Hacker et al., 1999). The concept that the pattern of MAPK activation may determine the type of cytokine output was further supported by investigations into the activation of MAPK by different TLR ligands inducing reciprocal patterns of secretion of the immunosuppressive cytokine IL-10 and the Th1-driving IL-12. High levels of IL-10 along with low IL-12 production in response to TLR2 stimulation were shown to correlate with strong ERK activation, whereas TLR4, TLR5, or TLR9 ligands preferentially activated p38 and induced more IL-12 (Agrawal et al., 2003; Dillon et al., 2004). Through the use of ERK1- and Fos-deficient macrophages, a pathway could be delineated that controls the ratio of IL-10 vs IL-12 production with strong ERK activity stabilizing and enhancing the transcription of Fos that in turn supports IL-10 production and inhibits IL-12 (Pulendran, 2005). Similar conclusions could be made from studies showing Th-2 type adjuvant activity of TLR2 ligands in vivo (Redecke et al., 2004) and IL-10-promoting effects of ERK-activation by Leishmania phosphoglycans (Feng et al., 1999). Thus, the MAPK pathway is used in innate immunity not only to deliver the alarm signals from TLR on fast-track to the nucleus, but also it provides a means to translate the nature of the stimulus into appropriate responses by balancing the strength of individual MAPK signals.

Firefighting: from inflammation to resolution

Detection of pathogens by innate immune cells triggers a robust and essential inflammatory reaction, known as acute inflammation, that can promote tissue repair, but it can also damage host tissues. The detrimental effects are further exacerbated in a chronic inflammatory state, which has been linked to a diverse range of diseases, including inflammatory autoimmune diseases, atherosclerosis, and cancer (Foster and Medzhitov, 2009). Chronic inflammation arises as a result of the continual presence of a stimulus or to genetic or physiological alterations that disrupt normal feedback mechanisms for attenuating the response.

Thanks to its stereotypic features acute, systemic inflammation can be divided into phases that generate distinct clinical phenotypes: an initiation (proinflammatory) phase, an adaptive (anti-inflammatory and reparative) phase, and a resolution (restoration of homeostasis) phase (Kimbrell and Beutler, 2001). These phases are reflected by changes from hyperinflammation to hypoinflammation to resolution. As already described, to sense pathogens, our immune system virtually always uses TLRs to incite transcription of multiple proinflammatory genes, such as TNFα and IL-1β, whose products generate the initiating phase (Cavaillon et al., 2005). This early phase is rapid and transient, terminating within hours by events that disrupt acute phase signaling transactivation through post-translational protein deactivation (Saccani et al., 2003), proteosome-dependent degradation (Saccani et al., 2004), and increased mRNA degradation (Hao and Baltimore, 2009), giving way to a well known adaptive phase called endotoxin tolerance (ET) (Beeson, 1947). During this phase, cells or organisms exposed to low concentrations of endotoxin (first demonstrated using LPS) enter into a transient unresponsive state and are unable to respond to further challenges with endotoxin. In other words, they develop a kind of "tolerance" to endotoxin. This phenomenon was firstly observed *in vivo*. Prior injection of mice with a sublethal dose of LPS protected them from a subsequent dose of LPS. These studies with mice demonstrated monocytes/macrophages as the principal cells responsible for the induction of ET *in vivo* (Cavaillon and Adib-Conquy, 2006). Subsequently, *in vitro* ET models confirmed that mouse macrophages as well as human monocytes exposed to

suboptimal levels of endotoxin show an inability to respond to further LPS challenge. The key readout for ET in these cells was the drastic reduction of TNFα

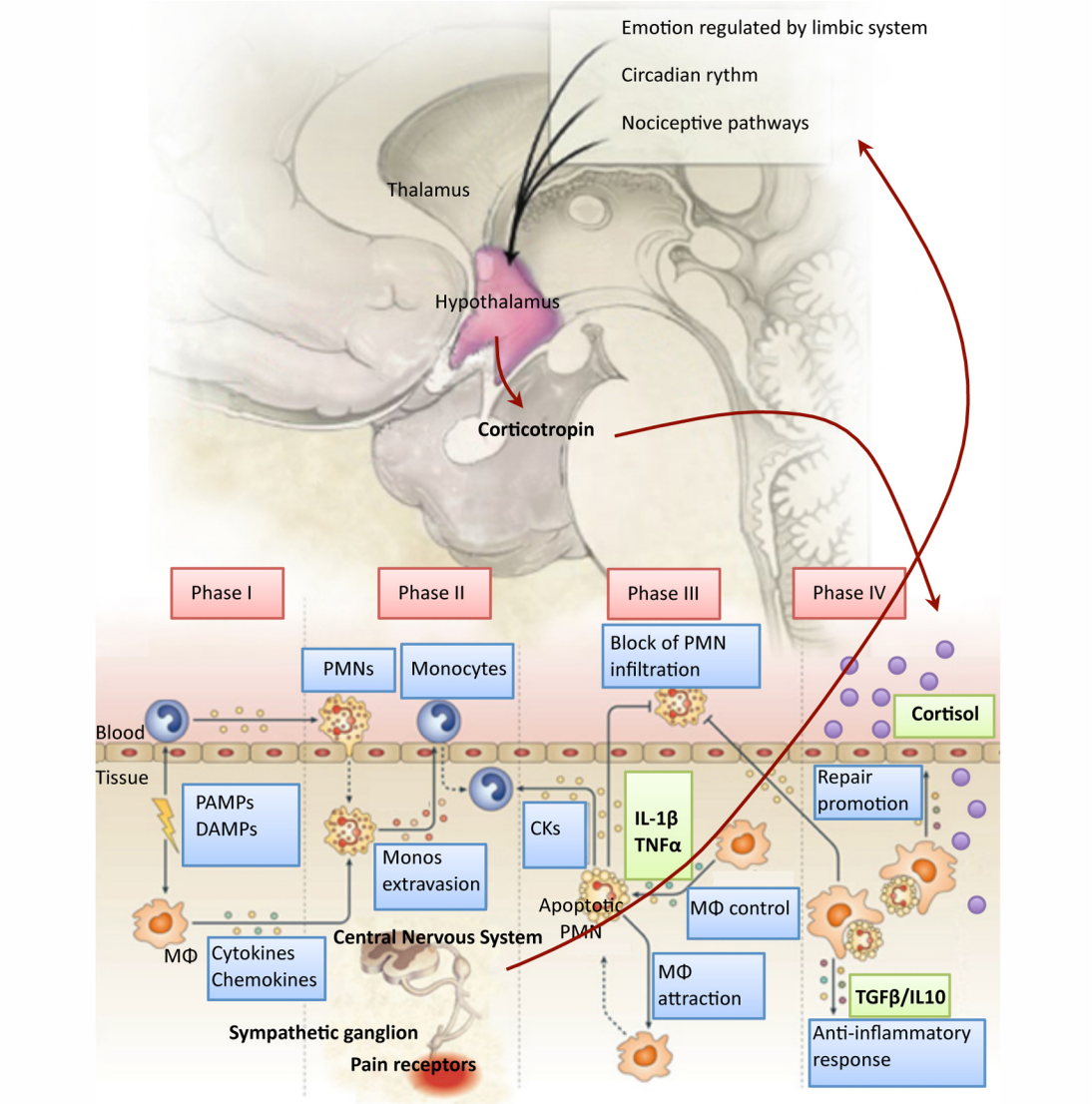

Figure 4. Local and systemic contribution in inflammation development and resolution. Phase I: patrolling monocytes and resident macrophages are the first cells to sense a disturbance in tissue homeostasis. They rapidly produce cytokines and chemokines to alert the immune system and to recruit neutrophils. Phase II: neutrophils invade the site of injury and release granule contents that promote the extravasation of inflammatory monocytes. Exudate formation, tissue swelling, and inflammatory mediators are responsible for "inflammatory pain," and nociception complements inflammatory sensors in monitoring tissue homeostasis. Phase III: the life-span of emigrated neutrophils is rather short and is subject to modification by pro- or anti-apoptotic signals, some of which are produced by macrophages. Macrophages and apoptotic neutrophils prevent further infiltration of neutrophils, but signals from apoptotic neutrophils promote continued monocyte influx. Phase IV: the clearance of apoptotic neutrophils, anti-inflammatory cytokines production and increasing amount of cortisol released by surrenal gland in response to Hypothalamic-Pituitary-Adrenal axis promotes an anti-inflammatory programme in monocytes and macrophages, which leads, ultimately, to the reconstitution of tissue homeostasis.

production as compared to the cells exposed to endotoxin only once (del Fresno et al., 2009; Dobrovolskaia and Vogel, 2002; Foster et al., 2007).

This "silencing" phase does not globally deactivate monocytes and macrophages, because expression of anti-inflammatory mediators and antimicrobial effectors is not inhibited and has been termed "TLR reprogramming" (McCall and Yoza, 2007). Poor inflammatory capacity coupled with upregulation of anti-inflammatory cytokines would contribute to protection against septic shock and increased phagocytosis would allow efficient bacterial clearance.

As already affirmed, an excessive inflammatory response is detrimental due to its negative effect on tissue function, as it can result in tissue damage. However, also a dysregulation of the resolution of inflammation may similarly contribute to tissue pathology. While ET has been thought as a protective mechanism against septic shock and ischaemia, its incidence is associated with high risks of secondary infections. For example, in sepsis, mortality due to secondary infection is associated with the incidence of a tolerant state (Monneret et al., 2008). Similarly, in acute pulmonary syndromes and cystic fibrosis, ET relates to an increased susceptibility to nosocomial infectious (del Fresno et al., 2009). Therefore, exactly like inflammation establishment, also its resolution phase must be finely regulated.

Anti-inflammatory cytokines such as IL-10 and TGFβ and glucocorticoid hormones are considered as important agents in supporting endotoxin tolerance (Parrillo, 1993) and, in general, inflammation resolution (Figure 4).

TGFβ and IL-10 – anti-inflammatory cytokines with autocrine and paracrine effects

SMAD-dependent and SMAD-independent TGFβ-induced cellular response

TGFβs are regulatory molecules with pleiotropic effects on cell proliferation, differentiation, migration and survival that affect multiple biological processes, including development, carcinogenesis, fibrosis, wound healing and immune responses (Blobe et al., 2000). The TGFβ system originated before the divergence of arthropods from vertebrates, developing several and complex functions in higher organisms (Newfeld et al., 1999). The first observation of TGFβ regulation of immune cell functions was made by Kehrl and colleagues (Kehrl et al., 1986) in

1986, as the generation and analysis of TGFβ1-/- mice established a central role for this cytokine in inhibiting inflammation and autoimmune diseases.

TGFβs belong to the TGFβ superfamily, with additional members including bone morphogenetic proteins, activins, and growth differentiation factors (Chang et al., 2002). There are three homologous TGFβ isoforms in mammals, TGFβ1, TGFβ2, and TGFβ3, encoded by different genes. TGFβ1 is the predominant isoform expressed in the immune system, but all three isoforms have similar properties *in vitro* (Govinden and Bhoola, 2003).

TGFβ exerts the greatest impact on T lymphocytes and, in general, on adaptive immunity. In parallel, it has contrasting effects on innate immunity, in particular on monocytes/macrophages lineage, whose regulation appears to depend on the differentiation stage of the cells. Generally, TGFβ stimulates cells at the resting state (monocytes), whereas differentiated/activated cells (macrophages) are inhibited (Ashcroft, 1999). In fact, TGFβ recruits monocytes to the site of injury or inflammation via multiple mechanisms: it acts as a chemoattractant for monocytes (Wahl et al., 1987); it induces adhesion molecules, including LFA-1 and the fibronectin receptor on monocytes, enabling their attachment to extracellular matrix (Wahl et al., 1993); it induces matrix metalloproteinases (MMPs), which can dissolve vascular membranes and facilitate monocyte transmigration (Wahl et al., 1993). In addition, TGFβ potentiates inflammation through induction of pro-inflammatory cytokines, such as IL-1β and IL-6, in monocytes (Turner et al., 1990; Wahl et al., 1987). These observations reveal a proinflammatory function for TGF-β on monocytes. Once monocytes differentiate into macrophages, TGFβ functions mostly as an inhibitory molecule. In fact, TGFβ can negatively modulate expression of scavenger receptors and it inhibits its phagocytosis capacity. In vitro, TGFβ inhibits the expression of several LPS-induced inflammatory mediators such as TNFα and MMP-12 as well as chemokines including CCL3 and CXCL2 (Bogdan et al., 1992). Moreover, TGFβ can affect macrophage response to LPS down modulating CD14 expression (Imai et al., 2000) and promoting MyD88 degradation (Naiki et al., 2005). In addition, TGFβ inhibits expression of the costimulatory molecule CD40 and the inflammatory cytokine IL-12p40, which collectively results in the inhibition of the antigen-presentation function of macrophages (Takeuchi et

al., 1998). This inhibition may play an important role in resolving an ongoing immune response by diminishing secondary stimulation of T cells at the site of infection.

A TGFβ ligand initiates signaling by binding to and bringing together type I and type II receptor serine/threonine kinases on the cell surface. This allows receptor II to phosphorylate the receptor I kinase domain, which then propagates the signal mainly through phosphorylation of the Smad proteins. There are eight distinct Smad proteins, constituting three functional classes: the receptor-regulated Smad (R-Smad), the Co-mediator Smad (Co-Smad), and the inhibitory Smad (I-Smad). R-Smads (Smad1, 2, 3, 5, and 8) are directly phosphorylated and activated by the type I receptor kinases and undergo homotrimerization and formation of heteromeric complexes with the Co-Smad, Smad4. The activated Smad complexes are translocated into the nucleus and, in conjunction with other nuclear cofactors, regulate the transcription of target genes. The I-Smad, Smad6 and Smad7, negatively regulate TGFβ signaling by competing with R-Smads for receptor or Co-Smad interaction and by targeting the receptors for degradation (Shi and Massague, 2003).

The receptor serine/threonine kinase family in the human genome comprises 12 members, 7 type I and 5 type II receptors, all dedicated to TGF signaling (Manning et al., 2002). Binding to the extracellular domains of both receptor types by the dimeric ligand induces a close proximity and a productive conformation for the intracellular kinase domains of the receptors, facilitating the phosphorylation and subsequent activation of the type I receptor.

Among the three classes of Smads, only R-Smads are directly phosphorylated and activated by the type I receptor kinases. Smad2 and Smad3 respond to signaling by the TGFβ subfamily and Smads 1, 5, and 8 primarily by the BMP subfamily (Manning et al., 2002). Phosphorylation occurs also subsequently to interaction with other signaling pathways. Smads activity can be positively regulated by MAP kinases. Both Erk and JNK module target R-Smad enhancing heterodimerization with Smad4, nuclear translocation and transcription activation. On the contrary, ubiquitination of both R-Smad and Smad-associated TGFβ receptors, that occurs through TGFβ-activated Smurf family protein, designs them to degradation by the 26S proteasome (Derynck and Zhang, 2003).

Cell stimulation with TGFβ leads immediately to positive and negative changes in the expression of several hundred genes (Kang et al., 2003). Both activation and repression of gene expression use the same set of activated Smad proteins. Further compounding this complexity, many of these gene responses depend on the cell type and other conditions affecting the cell at the time of TGFβ stimulation. A general hypothesis for how cells read TGFβ signals posits that Smad access to target genes and the recruitment of transcriptional coactivators or corepressors to such genes depend on cell-type specific partner proteins (Massague, 2000) and on Smad-independent signaling activated by TGFβ itself (Derynck and Zhang, 2003). Indeed, besides Smad-mediated transcription, TGFβ activates other signaling cascades, including MAPK pathways.

TGFβ can activate the Erk, JNK and p38 MAPK kinase pathways. Activation with slow kinetics in some cases may result from Smad-dependent transcription responses, but the rapid activation (5–15 min) in other cases suggests independence from transcription. Studies using Smad4-deficient cells or dominant-negative Smads, support the possibility of MAPK pathway activation that is independent from Smads (Engel et al., 1999). In addition, mutated TGFβ type I receptors, defective in Smad activation, activate p38 MAPK signaling in response to TGFβ (Yu et al., 2002). Rapid activation of Ras by TGFβ in epithelial cells may implicate Ras in TGFβ-induced Erk MAPK signaling (Yue and Mulder, 2000). JNK and p38 MAPK signaling are activated by various MAPK kinase kinases (MAPKKKs) in response to many stimuli. Both TGFβ and BMP-4 can activate TGFβ-activated kinase 1 (TAK1), a MAPKKK family member (Sakurai et al., 2002). MEKK1 may also function upstream of TGFβ-mediated activation of MAPKKs; thus, MEKK1 and TAK1 could activate JNK through MAPK kinase 4 (MKK4), and p38 MAPK through MKK3 or MKK6, in response to TGFβ. Because TAK1 can phosphorylate and activate IkB kinase, thus stimulating NF-κB signaling, TGFβs induce also NF-kB signaling. As described above, TGFβ-induced activation of the Erk and JNK pathways can result in Smad phosphorylation and regulate Smad activation. Activation of MAPK pathways by TGFβ may also affect transcription responses through direct effects on Smad-interacting transcription factors, such as the JNK substrate c-Jun or the p38 MAPK

substrate ATF-2 (activating transcription factor 2), allowing convergence of TGFβ-induced Smad and MAPK pathways.

IL-10 and the JAK/STAT system – translating cytokines production in a transcriptional program

IL-10, which was identified by Mosmann and colleagues (Fiorentino et al., 1989) in 1989, is the founding member of the class II family of α-helical cytokines that is composed of the type I interferons, interferon γ and interleukin 10. It is produced by various leukocytes. It is expressed by cells of the innate and the adaptive immune system, including dendritic cells (DCs), macrophages, mast cells, natural killer (NK) cells, eosinophils, neutrophils, CD4 and CD8 T cells, and B cells (Pulendran, 2005). IL-10 production can be triggered in vitro in monocytes, macrophages and DCs by stimulation with certain PRR ligands, in particular TLRs (Boonstra et al., 2006). TLR2 ligands appear to be particularly potent inducers of IL-10 production (Agrawal et al., 2003) but also TLR9 and TLR4 ligation trigger IL-10 production from macrophages and DCs (Boonstra et al., 2006).

IL-10 is recognized by the IL-10R1/IL-10R2 complex, which primarily activates the receptor-associated Janus tyrosine kinases Jak1 and Tyk2, resulting in STAT1 and STAT3 activation. The Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway is one of a handful of pleiotropic cascades used to transduce a multitude of signals for development and homeostasis in animals, from humans to flies. In mammals, the JAK/STAT pathway is the principal signaling mechanism for a wide array of cytokines and growth factors.

Mechanistically, JAK/STAT signaling is relatively simple, with only a few principal components. STATs (signal transducers and activators of transcription) comprise a family of seven structurally and functionally related proteins: Stat1, Stat2, Stat3, Stat4, Stat5a and Stat5b, Stat6. JAKs (janus kinases) represent a family of four non-receptor tyrosine kinases, Jak1, Jak2, Jak3 and Tyk2. Jak1, Jak2 and Tyk2 are expressed ubiquitously, whereas the expression of Jak3 is restricted to cells of the myeloid and lymphoid lineages (Leonard and O'Shea, 1998). These kinases selectively phosphorylate STATs, leading to their activation. Once activated, STATs play a critical role in regulating innate and acquired host immune responses. STATs

transduce signals for the large hematopoietin subfamily of cytokines, and the conserved family of the receptors they bind. This includes the interferon (IFN) family (IFNα/β, IFNγ, IL-10, IL-19, IL-20, IL-22), the gp 130 family (IL-6, IL-11, OSM, LIF, CT-1, IL-12, IL-23, Leptin, CTNF, NNT-1/BSF-3), the γC family, (IL-2, IL-4, IL-7, IL-9, IL-15, IL-21) and the single chain family (Epo, GH, PRL, Tpo) of the receptors (Schindler and Strehlow, 2000).

Signaling through the JAK/STAT pathway is initiated when a cytokine binds to its corresponding receptor. This leads to conformational changes in the cytoplasmic portion of the receptor, initiating activation of receptor associated members of the JAK family of kinases. Upon ligand stimulation, receptors undergo the conformational changes that bring JAKs into proximity of each other, enabling activation by trans-phosphorylation (Remy et al., 1999). Phosphorylated JAKs tyrosine kinase domain allow recruitment and association with STATs and other signaling molecules.

Once recruited to the receptor, STATs also become phosphorylated by JAKs, on a single tyrosine residue. Activated STATs dissociate from the receptor, dimerize, translocate to the nucleus and start their transcriptional program.

Recent studies have determined that STAT signals are downregulated at several points in the signaling cascade including the receptors, JAKs and the STAT molecules themselves. Generation of soluble receptors, which compete for limiting quantities of ligands, were observed. These isoforms are generated either by proteolysis or alternate RNA splicing. Ubiquitin-proteosome dependent degradation may also play a role in the downregulation of cytokine signaling. Moreover, JAK activation is dependent on tyrosine phosphorylation. It is, therefore, not surprising that two related SH2 containing phosphatases, SHP1 and SHP2, have been found to negatively regulate JAK activity. Finally, JAK/STAT system activates a negative feedback loop through the suppressors of cytokine signaling (SOCS), a family of STAT target genes that directly antagonize STAT activation (Kile et al., 2001).

While the IL-10R2 chain is ubiquitously expressed, IL-10R1 chain is mainly expressed on leukocytes. Thus IL-10 acts primarily on leukocytes. IL-10 is a major regulator of innate immunity (Mege et al., 2006). It interferes with the production of inflammatory mediators by polymorphonuclear neutrophils, monocytes, and

macrophages as well as upregulating the expression of molecules that amplify the anti-inflammatory effect of IL-10 itself (Moore et al., 2001). IL-10 is a general suppressive cytokine. It inhibits pro-inflammatory responses from innate and adaptive immunity, and it prevents the lesions in tissues caused by exacerbated adaptive immune responses. IL-10 is thus a central cytokine during the resolution phase of inflammation.

All the major TLR signaling pathways (such as those involving NF-κB, p38, ERK, and PI-3K) are implicated as the targets for suppression by IL-10, except for the JNK pathway downstream of TLR4 signaling (Williams et al., 2004). Moreover, it seems that several mechanisms are involved in suppressing different genes and even the same gene (Williams et al., 2004). For example, IL-10 was demonstrated to inhibit TNFα mRNA and protein expression induced by TLR stimulation via SOCS3 during the early phase of its signaling, but later it may use Bcl-3 (upregulated by IL-10 in a STAT3-dependent manner), which interacts with NF-κB at the TNFα promoter, to antagonize the formation of functional NF-κBp50/p65 heterodimers and thus to inhibit TNFα transcription (Kuwata et al., 2003; Qasimi et al., 2006).

Consistently with *in vitro* datas, blocking the IL-10 pathway in mice causes spontaneous development of inflammatory bowel disease (IBD). However, evolutionarily pathogens have exploited the functions of IL-10 to repress the normal host inflammatory responses during infections, thus establishing chronic infectious states. Increased IL-10 expression has been associated with many chronic bacterial and viral infections. Furthermore, some viruses can produce their own version of IL-10 (vIL-10) to directly suppress the immune responses of the host (Moore et al., 1990). The induction of IL-10 in DCs and macrophages represents a powerful mechanism of immune evasion used by various pathogens.

Blocking or enhancing IL-10 efficacy can thus be considered a therapeutic approach for the treatment of different kinds of infections.

Glucocorticoids – systemic master tuners of inflammation

Central nervous system-released hormones and glucocorticoids (GCs) in particular are essential in limiting and resolving the inflammatory process (Webster et al., 2002). The hypothalamic-pituitary-adrenal axis plays a central role in

regulating signaling through the glucocorticoid receptor, which is expressed in virtually all cells. Briefly, acute inflammation signals converge at hypothalamus, where periventricular nucleus controls the secretion of corticotropin-releasing hormone (CRH) into the hypophyseal portal system (Webster et al., 2002). In turn, CRH stimulates anterior pituitary gland in releasing corticotropin, that induces the synthesis and secretion of cortisol by the adrenal cortex.

In many ways, glucocorticoids lead to termination of inflammation by enhancing the clearance of foreign antigens, toxins, microorganisms, and dead cells. They do so by enhancing opsonization and the activity of scavenger systems, and by stimulating macrophage phagocytotic ability and antigen uptake (Liu et al., 1999). Glucocorticoids stimulate the expression of the mannose receptor (MRC1) or the scavenger receptor CD163, promoting clearance of microorganisms, dead cell bodies and antigens (Hogger et al., 1998; Piemonti et al., 1999). At the same time, they prevent inflammation from overshooting by suppressing the synthesis of many inflammatory mediators, such as several cytokines and chemokines, prostaglandins, leukotrienes, proteolytic enzymes, free oxygen radicals, and nitric oxide (Franchimont, 2004).

A great number of cytokines (including IL-1β, TNFα, IL-6, IL-8, IL-12 and IL- 18, etc.) is broadly downregulated by glucocorticoids. Similarly, secretion of many chemokines is strongly suppressed, whereas anti-inflammatory cytokines such as IL-10 and TGFβ are upregulated by glucocorticoids (Batuman et al., 1995; Elenkov et al., 1996). Soluble or decoy receptors, inhibiting or further enhancing the inflammatory process, are also regulated by glucocorticoids. For example, the decoy receptor IL-1RII, which binds IL-1β without driving any signaling, is enhanced by glucocorticoids (Re et al., 1994). These represent several anti-inflammatory mechanisms of action of glucocorticoids.

It has been proposed that ~1% of the genome might be modulated by glucocorticoids (Rhen and Cidlowski, 2005). Glucocorticoids-induced transcriptional regulation or glucocorticoids-dependent inhibitory mechanisms are mediated by the GC receptor (GR), a member of a large family of nuclear hormone receptor transcription factors (Rhen and Cidlowski, 2005). Glucocorticoid receptor is expressed in virtually all cells. Human GR messenger RNA (mRNA) has alternative

splice variants (Lu and Cidlowski, 2004). Whereas exons 2 through 8 are constant components of GR mRNA, there are two exon 9 isoforms that can be spliced to produce mature mRNA. Splicing of exon 9a produces GRα mRNA, which is translated into a protein with a unique sequence of 50 amino acids at its carboxy end. The glucocorticoid receptor α isoform binds cortisol, DNA, and other transcription factors, thereby modifying transcriptional activity of target genes. Exon 9b produces GRβ mRNA, which is translated into a protein with 15 distinct amino acids at its carboxy end. Although glucocorticoid receptor β protein forms homodimers that bind DNA, it does not bind any ligands examined so far and fails to activate transcription. Glucocorticoid receptor β can also form heterodimers with glucocorticoid receptor α and interfere with the function of this protein. The relative levels of glucocorticoid receptor α and β in a cell influence the cell's sensitivity to glucocorticoid, with higher levels of glucocorticoid receptor β leading to glucocorticoid resistance (Pujols et al., 2001). TNFα and IL-1β can selectively up-regulate the levels of glucocorticoid receptor β, suggesting its role in inflammation (Webster et al., 2001).

Alternative translation-initiation sites within exon 2 produce additional isoforms of the glucocorticoid receptor (Lu and Cidlowski, 2004). Translation from the first methionine codon in GRα and GRβ mRNA produces proteins that consist of 777 aminoacids (glucocorticoid receptor α-A) and 742 aminoacids (glucocorticoid receptor β-A). Translation from a second methionine produces proteins with 751 aminoacids (glucocorticoid receptor α-B) and 716 aminoacids (glucocorticoid receptor β-B), respectively. Glucocorticoid receptor α-B has roughly twice the biologic activity of glucocorticoid receptor α-A in gene-expression studies in vitro (Yudt and Cidlowski, 2001). The finding that the two isoforms are expressed at different ratios in various types of cells and tissues also suggests that they may have distinct functions in vivo (Lu and Cidlowski, 2005). Moreover, the human glucocorticoid receptor has five serine residues that are phosphorylated under different conditions by cyclin-dependent kinases and MAP kinases (Ismaili and Garabedian, 2004). The glucocorticoid receptor is found primarily in the cytoplasm and is inactive when phosphorylated at serine 203, but it actively transcribes DNA when phosphorylated at serine 211 (Ismaili and Garabedian, 2004).

Unliganded GRs form large heterocomplexes with heat shock protein 90 (hsp90) and other heat shock proteins. On hormone binding, the hormone-receptor complex rapidly undergoes activation, in the course of which the heterocomplex dissociates to an activated hormone-receptor complex monomer and translocate in the nucleus (Pratt and Toft, 1997). Once there it can positively regulate gene expression by dimerizing and binding to palindromic GC response elements (GREs) in the promoters of target genes. GR can also interact with other transcription factors such as NF-κB and AP-1, impairing their ability to activate gene expression, a process known as transrepression. Most importantly, transrepression is both rapid and direct (mediated by pre-existing factors). GC can also block indirectly MAPK

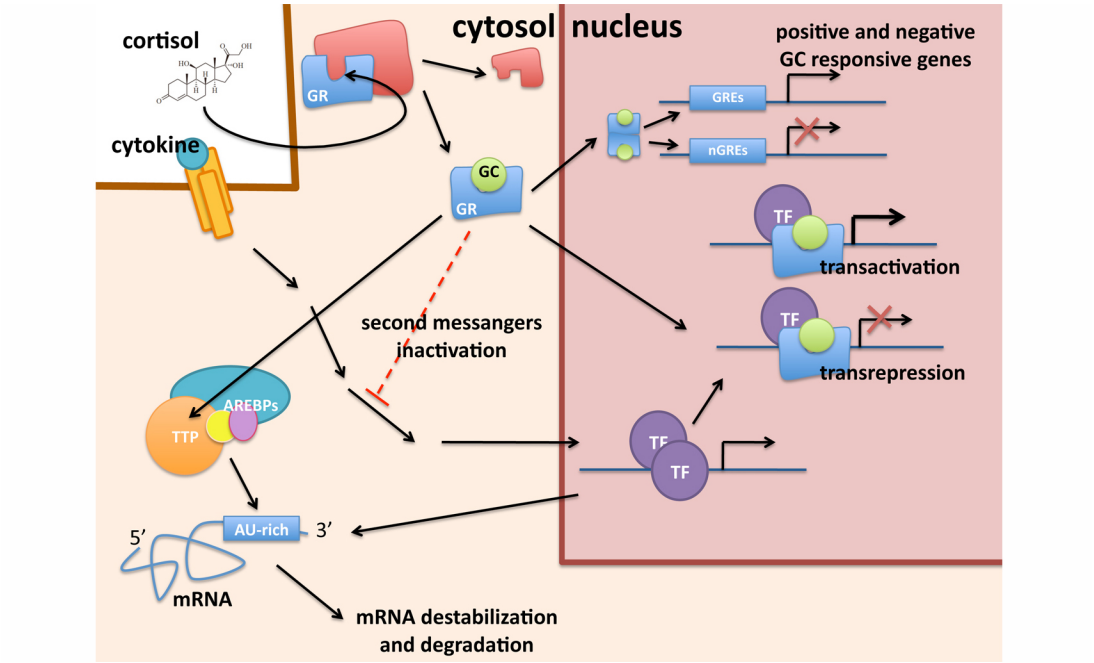

Figure 5. Mechanisms of action of glucocorticoids in inhibiting inflammation. The efficacy of GC in alleviating inflammatory disorders results from the pleiotropic effects of GC on multiple signaling pathways. Classically, the powerful activity of GC is explained through 3 levels of action. 1) Direct genomic effects (transcription factor activity): GC couple to their receptor (GR) and move to the nucleus as homodimer to bind GRE sequences, recruiting both trancription repressors and activators; 2) Indirect genomic effects (transactivation or transrepression): GR-GC complex interacts with other transcription factors, such as NF-κB, AP-1, STATs, SMAD4 and others, inhibiting or enhancing their activity; 3) Non-genomic effects (post-transcriptional activity and direct activity on signaling pathway): these actions do not require de novo protein synthesis and it can be explained by a GC-dependent inactivation of signaling cascade adaptors or by their activity in modulating RNA-binding proteins such as TTP.

cascade by transcription induction of MAPK phosphatase 1. GCs-induced MAPK phosphatase 1 dephosphorylates and inactivates JNK, thereby inhibiting c-Jun-mediated transcription, and p38 family members (Abraham et al., 2006).

Although GR alone has low affinity for sites that diverge from the GRE consensus, high affinity binding and transcriptional activation can be restored in the presence of multiple sub-optimal GR binding sites such as half GREs (Lechner et al., 1997) or adjacent binding sites for other transcription factors (Guido et al., 1996). GR has been shown to cooperate with transcription factors of many different classes, including the zinc finger transcription factor, stimulatory protein 1 (Sp1), homeobox proteins, ets-related proteins, interferon response factors, helix-loop-helix factors, members of the CCAAT/enhancer-binding protein (C/EBP), forkhead box (Fox), STAT families and even NF-κB itself (Hofmann and Schmitz, 2002).

In experimental settings GCs are capable of blocking gene expression even if added some time after a pro-inflammatory stimulus, whilst inhibitors of transcription are ineffective. GCs have long been known to inhibit inflammatory gene expression at a post-trancriptional level, via destabilization of mRNA or inhibition of translation (Ing, 2005). Targets of such regulation include COX-2, TNFα, interferon, several chemokines and others. The majority of these mRNAs have in common the presence of adenylate/uridylate-rich elements (AREs) in their 3′ untranslated regions (UTRs). GCs may influence pro-inflammatory gene expression by altering the expression of AREBPs, by modulating the activity of the signaling pathways that control them, or both (Ing, 2005).

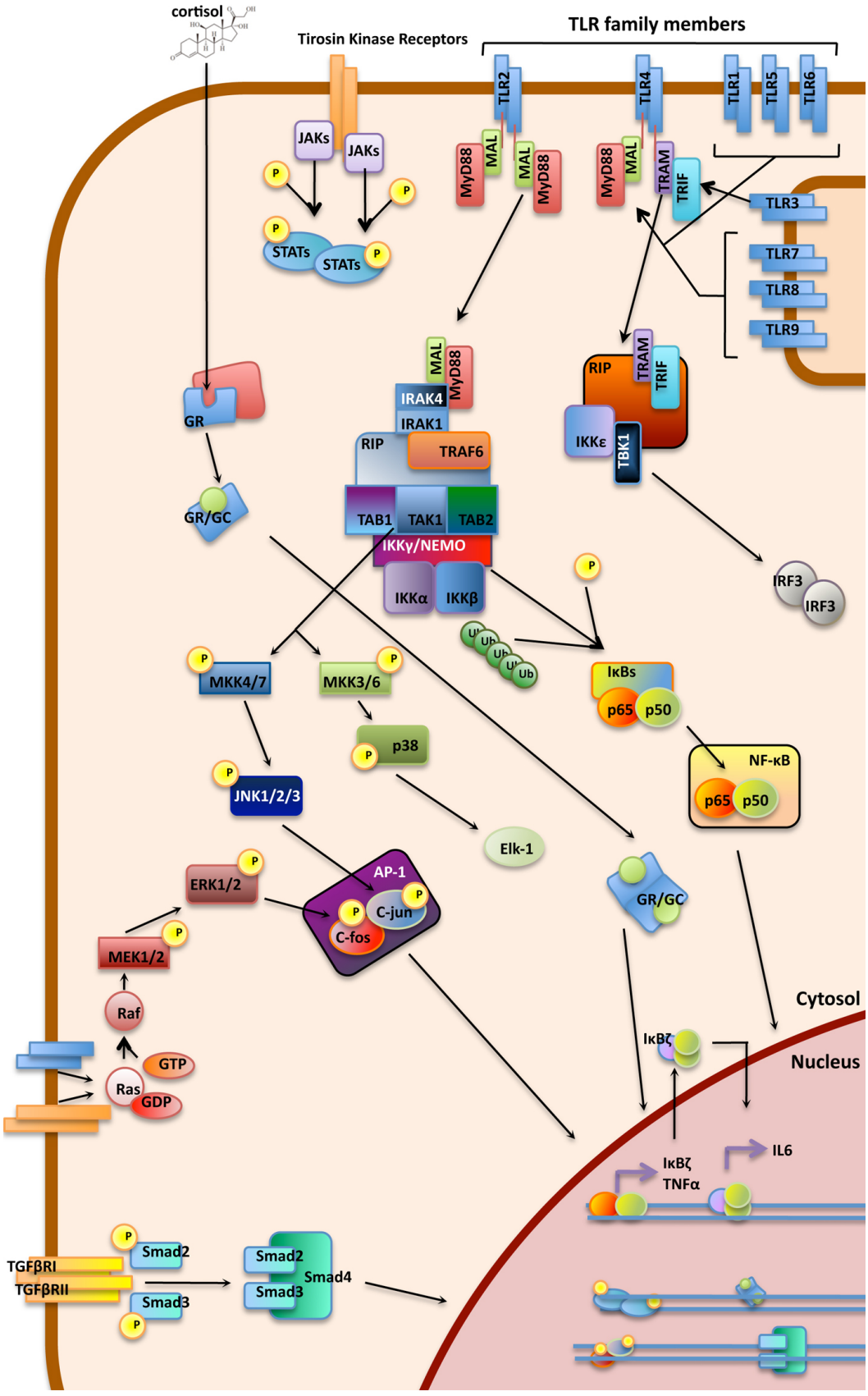

Figure 6. A schematic representation of described pro and anti-inflammatory pathways

Second wave of control: post transcriptional regulation

Signaling pathways described above are commonly activated during acute immune response. Transcription factors programme a transcriptome that induces the expression of a family of mRNAs encoding proteins involved in a specific immune function. Although transcription is an essential first step in the regulation of gene expression, it is a complicated process that cannot be rapidly turned off or redirected. Long after transcription has ceased, transcribed mRNAs can continue to synthesize proteins. Fortunately, the conversion of DNA into mRNA is only the first step in a process that directs the expression of the cytokines, chemokines and survival or differentiation factors that define immune cell function. The second step, the conversion of mRNA into protein, is a highly regulated process that often has a dominant role in coordinating the overall immune response (Shaw and Kamen, 1986). Factors that promote mRNA degradation or inhibit mRNA translation can rapidly repress protein expression despite on-going gene transcription. Most post-transcriptional control mechanisms target the 3′ untranslated region (UTR) of mRNAs to repress the expression of the target transcript. In the immune system, post-transcriptional dampening of protein expression can actively promote the resolution of inflammation to prevent unintended tissue damage (Chen and Shyu, 1995). In this way, post-transcriptional control mechanisms link the initiation phase and the resolution phase of inflammation.

AU-rich regions and ARE-binding proteins

The AU-rich mRNAs are a class of mRNAs that bear AU-rich elements (ARE) in their 3′ untranslated regions (3′UTR). Estimated now to be in the vicinity of 10–15% of all transcripts (Halees et al., 2008), the ARE-mRNAs comprise a functionally diverse group including inflammatory and immune response, transcription, cellular proliferation, RNA metabolism, development, and signaling (Bakheet et al., 2006). Many cytokines, chemokines and proinflammatory proteins are subject to ARE-mediated decay (AMD) (Stoecklin and Anderson, 2006).

The basic units of the ARE are pentamers of AUUUA, nonamers of UUAUUUAUU, and AU-rich 'clusters' composed of linked pentamers and/or

nonamers. AREs have been subcategorized into various classes on the basis of their sequence and deadenylation kinetics (Chen and Shyu, 1995).

The ARE recruits several different ARE-binding proteins (ARE-BPs) that can positively or negatively regulate mRNA stability and/or translation. Because many ARE-BPs shuttle between the nucleus and the cytoplasm, binding to specific mRNA molecules may occur in either compartment and the composition of individual messenger ribonucleoproteins may be modified as the transcript moves through the cell. Each of these proteins can individually affect the translation and/or decay of ARE-containing mRNA molecules: TIA-1, TIAR15, FXR1P16 and CUGBP2 inhibit translation; TTP18, BRF1, BRF2 and KSRP20 promote decay; AUF1 either promotes or inhibits decay; and HuR inhibits decay and either promotes or inhibits translation. HuR has a central location in this model, and it seems to be pivotal in the regulation of cytokine production by orchestrating the binding and activity of other ARE-BPs. The complexity of these interactions may explain how HuR can both stabilize and either promote or inhibit the translation of ARE-containing mRNA molecules (Anderson, 2008).

Fine tuning of inflammation exherted by microRNA

Discovered more than a decade ago, microRNAs (miRNAs) are a new class of small molecular regulators, generated as single-stranded non- coding RNAs of 19-23 nucleotides in length. These mediators regulate gene expression post-transcriptionally by binding complementary sequences within the 3' untranslated region of their target mRNA and inducing their degradation or translation inhibition (El Gazzar and McCall, 2011). More than 700 miRNAs have been identified in mammalian cells (miRNA registry at http://microrna.sanger.ac.uk/sequences; http://www.targetscan.org/; http://pictar.mdc-berlin.de; http://www.microrna.org/microrna/home.do).

Consistent with their roles in specifying cells, miRNAs have been implicated in regulating a variety of physiological processes, including hematopoietic cell growth, development, activation, differentiation and apoptosis.

The biogenesis of miRNAs

MicroRNAs (miRNAs) are very small (~22 nt) single-stranded non-coding RNA (ncRNA) molecules, processed from hairpin precursors of ~70 nt (pre-miRNA), that comes from primary transcripts (pri-miRNA). miRNAs have gained much interest, as recent genome-wide studies have shown that they are widespread in a variety of organisms and are conserved in evolution. In fact, they are now considered one of the largest gene families, and a growing number of biological processes involving miRNAs are continuously discovered (He and Hannon, 2004).

miRNAs in animals are found in diverse genomic locations, as exemplified in Figure 7. Most miRNAs are encoded in intergenic regions, but there are also many miRNAs that are hosted within the introns of pre-mRNAs or encoded within ncRNA genes (Rodriguez et al., 2004). Interestingly, it was observed that there are miRNA genes, both hosted and non-hosted, which are clustered. Clustered miRNA genes may show high similarity in sequence or not, but for sure they are transcribed as polycistrons and have similar expression patterns (Bartel, 2004).

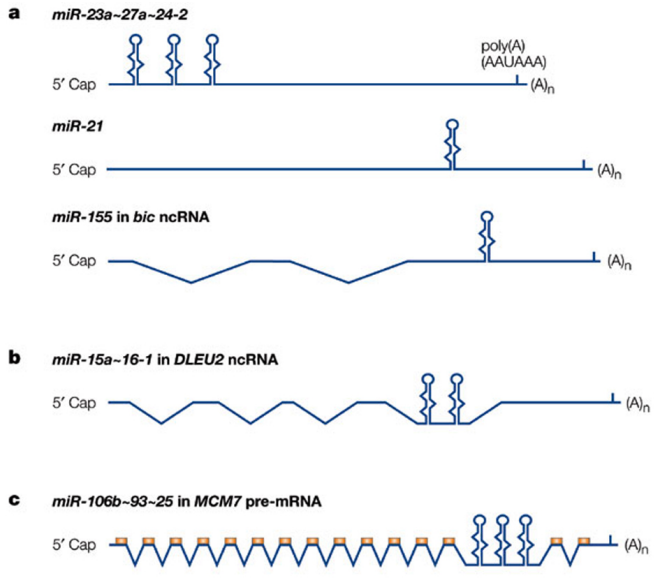

Figure 7. miRNA primary transcripts. Pri-miRs contain 5' cap structures as well as 3' poly(A) tails. miRNAs can be categorized into three groups according to their genomic locations. a) Exonic miR in non-coding transcripts such as an miR-23a~27a~24-2 cluster, miR-21 and miR-155. miR-155 was found in a previously defined ncRNA gene, bic. b) Intronic miR in non-coding transcripts. miR-15a~16-1 cluster was found in the 4th intron of a non-coding RNA gene. c) Intronic miR in protein-coding transcripts. miR-106b~93 ~25 cluster is embedded in the thirteenth intron of MCM7 transcript.

miRNAs are transcribed as primary hairpin transcripts by RNA polymerase II. These transcripts are first 5′ 7-methyl-guanosine (m7G) capped and 3′ polyadenylated before further processing occurs. Primary miRNA (pri-miRNA) transcripts containing one or more local hairpins are cleaved by the nuclear RNase III enzyme Drosha and its dsRNA-binding partner DGCR8. In Drosophila, however, some

intronic miRNA precursors, termed ‘Mitrons’, are processed in the nucleus by the usual RNA splicing machinery and not by the Drosha endonuclease (Ruby et al., 2007). The pre-miRNAs are then actively transported to the cytoplasm by exportin-5 in a RAS-related nuclear protein-guanosine triphosphate (RAN-GTP)-dependent manner and are further processed into 20-22-nucleotide duplexes by the cytoplasmic RNase III enzyme Dicer (Lee et al., 2002). One strand is preferentially incorporated into ribonucleoprotein (RNP) complexes called micro-RNPs (miRNPs) or miRNA-induced silencing complexes (miRISCs). The key components of miRNPs are proteins of the Argonaute (AGO) family. In mammals, four AGO proteins (AGO1 to

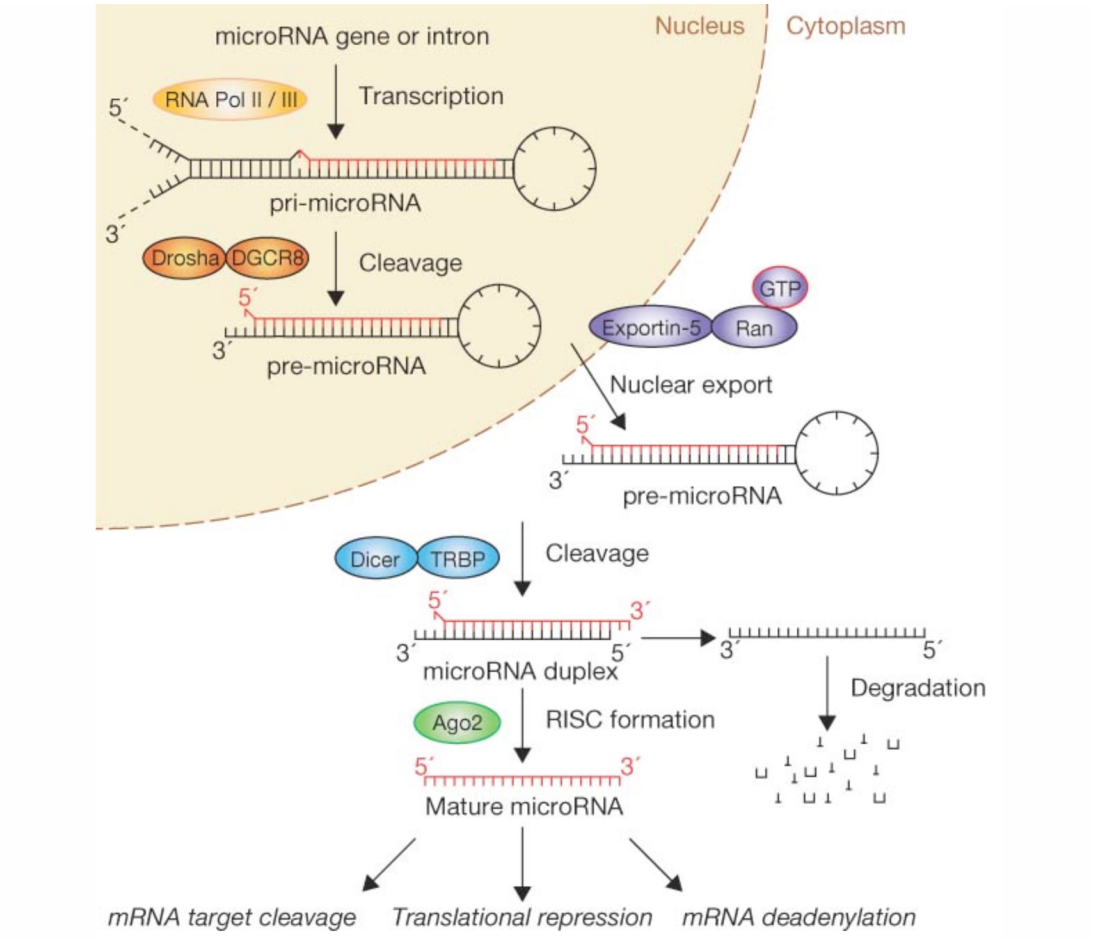

Figure 8. microRNA processing and activity. miR biogenesis includes the production of the primary transcript (pri-miR) by RNA polymerase II or III and its cleavage by the microprocessor complex Drosha-DGCR8 (Pasha) in the nucleus. The resulting precursor hairpin (pre-miR) is exported from the nucleus by Exportin-5-Ran-GTP. In the cytoplasm, the RNase Dicer-containing complex cleaves the pre-miRNA hairpin to its mature form. The functional strand of the mature miRNA is loaded together with Argonaute (Ago2) proteins into the RNA-induced silencing complex (RISC), where it guides RISC to silence target mRNAs through mRNA cleavage, translational repression or deadenylation.

AGO4) were described but only AGO2 seems to be functional. Apart from AGOs, miRNPs can contain further proteins that function as regulatory factors or effectors mediating inhibitory function of miRNPs (Sontheimer, 2005) (Figure 8).

miRNA-mRNA interactions

Animal miRNAs can repress targets via surprisingly short base-pairing of 6-8 nt in the 5' ends of miRNAs (preferentially nucleotides 2–8) with the 3' untranslated region of targets mRNA, inducing its destabilization and/or inhibiting productive translation (Fabian et al., 2010). Both transcriptome and proteome studies provide experimental evidence that individual miRNAs can directly repress hundreds of target genes (Selbach et al., 2008).

In the case of animal miRNAs, translational repression has been proposed to occur in three distinct ways: cleaving of target mRNAs, repressing mRNA translation or promoting its degradation (Figure 8). The cleavage of target mRNAs in eukaryotes seems an unusual mechanism, as only one case has been observed (Yekta et al., 2004). Target mRNAs are usually found in citoplasm in complex with RNPs and miRNA function and mechanism of action depend on the particular proteins constituting miRISC (Farazi et al., 2008). Notably, depending on environment and stimulation that cell received, microRNAs demonstrate both negative and positive regulation capacity of particular transcripts. Cell starvation has been recently reported to induce microRNA-dependent transcript protection of TLR4 and TNFα mRNAs (Tserel et al., 2011; Vasudevan et al., 2007).

microRNAs and the innate immune system

The first indication that miRNAs regulate the immune response came in 2004 with a report which showed selective expression of miR-142a, miR-181a and miR-223 in immune cells (Chen et al., 2004). miRNAs are now known to be involved in the regulation of maturation, proliferation, differentiation and activation of immune cells of both the innate and adaptive systems. This has emerged from studies that revealed selective expression of miR-181a in the thymus and miR-223 in the bone marrow and indicated their involvement in the differentiation of pluripotent

hematopoietic stem cells (HSCs) into the various blood cells lineages including B and T cells. Subsequent reports have identified functions for individual miRNAs such as miR-150, miR-181a and miR-17~92 cluster during T and B cell differentiation, whereas miR-17~92 and miR-223 are implicated in myeloid production. Experimentation has also revealed roles for miRNAs during the activation of the innate and acquired immune response. Thus, miR-146a are miR-155 are thought to be the most important miRNAs in regulation of the acute inflammatory response after the recognition of pathogens by the Toll-like receptors (TLR), whereas miR-155 and miR-181a are implicated in B and T cell responses (Lindsay, 2008).

microRNAs expression and activity after TLRs engagement

In the last years, several studies demonstrated that microRNAs, exactly like every others genes, can be up-regulated or down-modulated by TLR-dependent transcription factors or regulators with different kinetics in innate immune cells, with a consensus emerging that miR-155 and miR-146a are particularly ubiquitous. Their expressions seem NF-κB and AP-1-dependent, with the latter that is particularly indispensable for miR-155 (O'Neill et al., 2011).

Similar to other TLR-responsive genes, it is also important that the induction of TLR-responsive miRNAs is negatively regulated by anti-inflammatory molecules. For example, recently McCoy and colleagues characterized an IL-10 dependent miR-155 inhibition (McCoy et al., 2010). Less is known about microRNAs activity inhibition, but emerging evidences, in particular in tumours, suggest that the same stimulus that induce miRNA over-expression might also induce particular transcripts, usually long non-coding RNA (ncRNA), that act as miR "sponge" and because of their function are called competitor endogenous RNAs (ceRNAs). They contain multiple copies of miR seed regions, becoming suitable for microRNAs binding and therefore sequestering them avoiding interaction with target mRNAs (Cesana et al., 2011; Karreth et al., 2011; Sumazin et al., 2011; Tay et al., 2011). Moreover, some proteins have been described as inhibitors of miRNA biogenesis avoiding their processing from pri to pre-miR or from pre to mature miR (Winter et al., 2009).

TLR signaling control by miRNAs

As previously described, TLR signaling pathway is a very complex cascade of events that includes a great number of molecules: receptors to sensing pathogens, at least four different adaptors molecules that induce several signaling cascade that collide in NF-κB, AP-1 and other transcription factors activation and gene expression.

This kind of complexity was described as particularly suitable for microRNA-induced regulation (Inui et al., 2010). miR-dependent protein inhibition is usually not more than 20-30% and therefore *per se* not significative. Contextualizing this small inhibition in an elaborate pathway, such as TLRs system, allow us to highlight the magnitude of microRNA impact on acute inflammatory contest and, in general, on cell biology.

In the last years, researchers described innumerable negative but also positive interaction between transcriptome and micrornaome induced after TLR engagement. Here, we will give the example of miR-155 and miR-146a impacts on TLR response.

i. Ying and yang activity of miR-155

miR-155 has been extensively researched as a cancer-associated miRNA or "onco-miR". However, miR-155 also has significant role in the immune response. This was firstly noted in a key paper by Baltimore et al. (Taganov et al., 2006), who have observed miR-155 up-regulation as a continual feature of the mammalian inflammatory response. The complexity of miR-155-dependent regulation of inflammatory responses was soon evident, as Tili and colleagues suggested a miR-155-induced down-modulation of several proteins involved in LPS cellular response, such as FADD, IKKε and RIP-1, in concomitance with TNFα augmented release (Tili et al., 2007). As for TNFα, also CXCL8 and IL-6 protein hyper-expression were observed after miR-155 over-expression due to its activity on SHIP-1 and SOCS1 inhibitory proteins, respectively (Bhattacharyya et al., 2011; Lu et al., 2009).

In parallel, miR-155-dependent negative feedback circuits were observed. Firstly, miR-155 appears as a negative regulator of its own production, as it requires AP-1 transcription factor to be induced but it directly targets one of the two componend of AP-1 heterodimer, c-fos (Gottwein et al., 2007). Moreover, miR-155

seems to act on TLR/IL1R inflammatory pathway directly targeting TAB2, inhibiting activation of TAK1 and hence NF-κB and MAPK activation (Ceppi et al., 2009). miR-155-dependent inhibition of TGFβ-induced IL-1β gene transcription from monocytes was observed as consequence of direct targeting of Smad2 exherted by miR-155 (Louafi et al., 2010). A schematic representation of miR-155 impact on inflammation-related signaling pathway is given in Figure 9.

Both the positive and negative aspects of these control mechanisms implicate it as a highly interesting and significant player in downstream inflammatory pathways, and further research in this area will elucidate its main role in the immune response. The key to solve this enigma meybe come from observations on the kinetic of activation of this interesting miR and its interaction with effects of others miR (O'Neill et al., 2011).

The observation that dysregulation of miR-155 can lead to both cancerous phenotypes and inflammatory diseases, provides a clear need for miR-155 itself to be stringently regulated. A recent finding in this field that the potent anti- inflammatory cytokine IL-10 can down-regulate miR-155 is of note here. This study found that IL-10 suppresses LPS- induced miR-155 in a STAT3-dependent manner, leading to an increase in the miR-155 target SHIP1 (McCoy et al., 2010; Quinn and O'Neill, 2011).

ii. miR-146a inhibition of TLR signaling mediators

The miR-146 family is composed of two members, miR-146a and miR-146b that are located on chromosomes 5 and 10, respectively. In 2006, Taganov and colleagues pubblished a milestone paper in which not only they observed a TLR-induced set of microRNAs, among which miR-146a, in THP-1 monocytic cell lines, but they also proposed the first observation of a negative feedback loop involving microRNA-146a, induced by TLR4 activation, that in turn down-modulate TRAF6 and IRAK1 adaptors expression extinguishing TLR4-dependent signaling (Taganov et al., 2006 and Figure 9). They firstly introduce the concept of fine-tuning of the immune response, and the same group, recently, strenghtened this concept pubblishing their observations on miR-146a knock-out mice (Boldin et al., 2011).

Subsequent studies confirmed that over-expression of miR-146a in THP-1 monocytic cell line makes these cells refractory to any TLR stimulation, mimiking endotoxin tolerant state and implicating this microRNA in tolerance induction (El Gazzar et al., 2011; Nahid et al., 2011).

The high expression of miR-146a has been shown to be up-regulated in many inflammatory diseases such as osteoarthritis and rheumatoid arthritis (RA), the latter involving up-regulation following stimulation with inflammatory cytokines such as TNFα and IL-1β. Interestingly, a polymorphism in the 3'UTR of the mRNA encoding the miR-146a target IRAK1 is associated with a susceptibility to RA (Chatzikyriakidou et al., 2010a) and psoriatic arthritis (Chatzikyriakidou et al., 2010b).

Another study implicated miR-146a in regulation of type 1 interferon responses (Tang et al., 2009). This study again noted that miR-146a was present at reduced levels in SLE patients, and further investigation established that this resulted in increased IFNα and IFNβ. miR-146a acts as a negative regulator of interferon production in PBMCs, a fine-tuner in maintaining homeostasis.

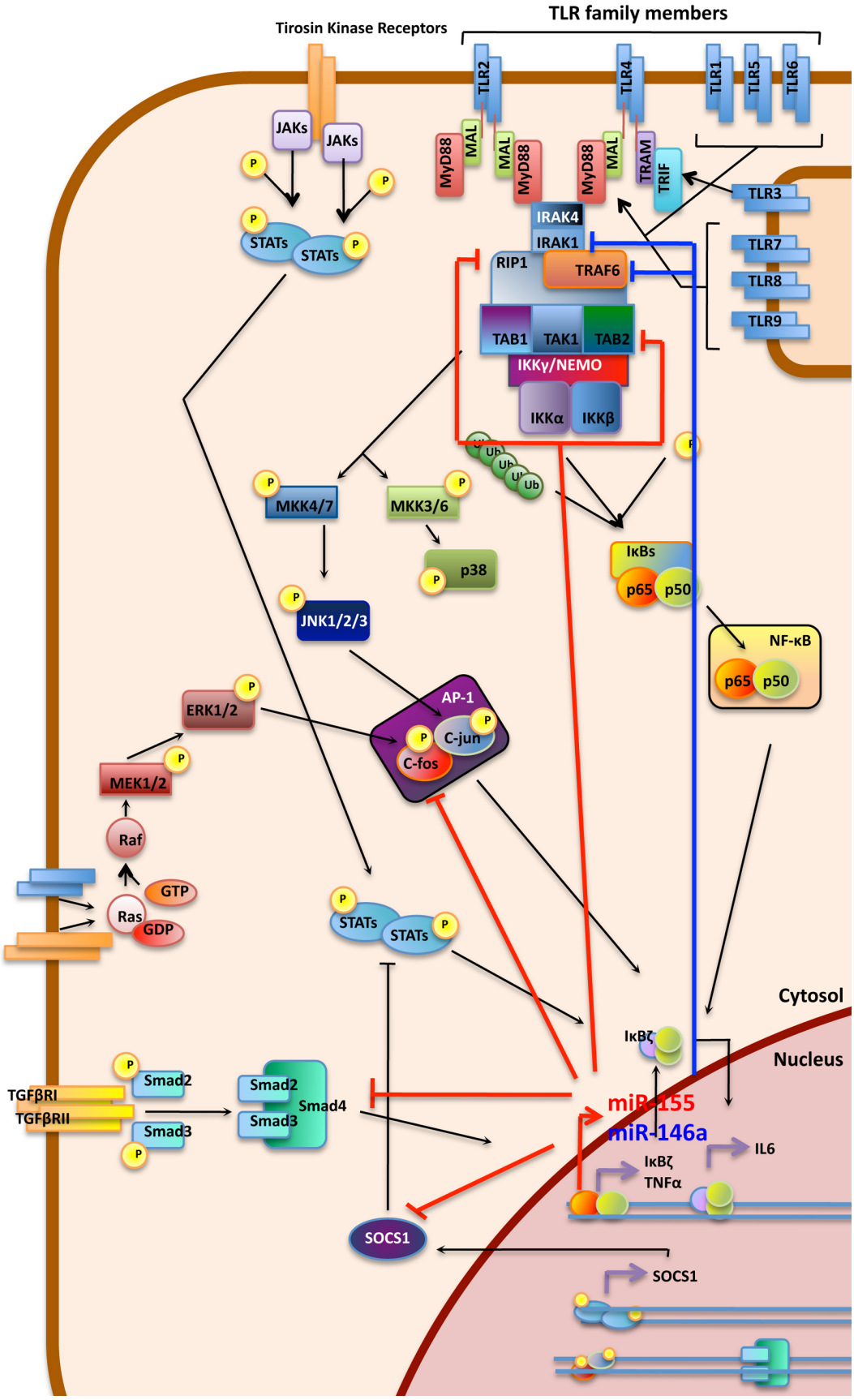

Figure 9. miR-155 and miR-146a impact on inflammation-induced signaling cascade.

Aim of the study

In 1993, Victor Ambros, Rosalind Lee and Rhonda Feinbaum reported a post-transcriptional regulation exerted by a short RNA product encoded by lin-4 gene on LIN-14 protein in C. elegans. Several years passed before a second confirmation of this mechanism led to the establishment of miRNAs as a new class of post-transcriptional regulators, which bind to complementary sequences on target messenger RNA transcripts (mRNAs) and induce translational repression or target degradation. Nowadays, miRNA have a well recognized key role in several contests, from development to pathogenesis of cancer, but their relevance for inflammation is still largely unknown.

The inflammatory response is a robust and essential reaction quickly triggered by detection of pathogens, endotoxins or, in general, injurious stimuli. Uncontrolled inflammation leads to extensive tissue damage and manifestation of pathological states like sepsis, autoimmune diseases, metabolic diseases and cancer. For this reason, this process is finely regulated by several mechanisms acting both at cellular and systemic level, including injury-induced molecules that inhibit ongoing inflammatory reaction, such as dominant negative proteins of TLR signaling adaptors, or activate anti-inflammatory programs, such as IL-10 or glucocorticoids. In this contest, microRNAs biology perfectly matched with the necessity of fine-regulation of the entire process, and in 2006 Taganov and colleagues reported the first observation of miRNA involvement in the control of inflammation describing a negative feedback loop exerted by the LPS-responsive miR-146a on TRAF6 and IRAK1, two foundamental adaptors in TLR signaling pathway, in the THP-1 monocytic cell line. The first aim of this work was to confirm this observation in human primary cells of the innate immune system, such as monocytes and neutrophils, and to eventually uncover other regulatory feedback circuits exerted by microRNAs after TLR4 activation.

Anti-inflammatory stimuli, such as IL-10 or glucocorticoids, are known to induce post-transcriptional regulation of pro-inflammatory molecules, such as TNFα and IL-6, by means of the activation of tristetraprolin proteins, which bind AU-rich elements in the 3’ untranslated region of target transcripts and promote their decay.

The second aim of our study was to investigate whether the anti-inflammatory autocrine/paracrine activity of IL-10 or systemic activity of glucocorticoids also included a microRNA-dependent regulation of the pro-inflammatory cascade.

Materials and methods

Materials

Ultra Pure E. coli lipopolysaccharide (LPS, 0111:B4 strain), palmitoyl-3-cysteine-serine-lysine-4 (Pam_3CSK_4), and polyinosinic:polycytidylic acids (poly(I:C)) were purchased from Invivogen. TNFα, IL-1β, and TGFβ were from Peprotech, IL-10 and IFNγ from R&D System, IFNβ from Betaferon (Schering) and Dexamethasone from Sigma-Aldrich. Monoclonal antibodies against human TNFα (B154.2) were a kind gift from Professor Giorgio Trinchieri (Laboratory of Experimental Immunology, National Cancer Institute at Frederick, Frederick, MD) whereas monoclonal antibodies against human IL-10R come from Biolegends. Brefeldin A, MG-132, BAY-117082, pyrrolidine dithiocarbamate (PDTC) were purchased from Sigma-Aldrich, Resiquimod (R848), SP-600125, and SB-203580 from Alexis (Axxora LLC).

Cell purification and culture

Human PMN and monocytes were separated by centrifugation on Ficoll-Paque Plus under endotoxin-free conditions from buffy coats of healthy donors. PMN (>99.9% pure) and monocytes (>99.8% pure) were obtained by negative magnetic selection performed with the EasySep Enrichment Custom Mixture (StemCell Technology) for PMN and with MACS Human Monocyte Isolation Kit II (Miltenyi Biotec) for monocytes. The purity of both leukocyte populations was assessed by flow cytometry. PMN (6×10^6/ml) and monocytes (2×10^6/ml) were then resuspended in RPMI medium 1640 (Cambrex) supplemented with 10% low endotoxin FBS (Cambrex) and 2 mM L-glutammine (Cambrex), plated and treated as described in Results.

HEK 293T cells were from ATCC (Manassas, VA) and maintained in DMEM (Cambrex) containing 100 U/ml penicillin/streptomycin (Cambrex), and 2 mM L-glutammine (Cambrex), and 10% FBS (Euroclone). THP-1 cells were obtained from ATCC and maintained in RPMI 1640 (Cambrex) supplemented with 100 U/ml penicillin/streptomycin (Cambrex), 2 mM L-glutammine (Cambrex), 5×10^{-5} M β-mercaptoethanol (Cambrex), and 10% FBS (Euroclone). Infected THP-1 were cultured for no more that 2 months in the same medium and conditions of wild-type cells.

Quantification of miRNAs expression level

PMN and monocytes were stimulated with 100 ng/ml LPS for 8 h and the RNAfraction that is highly enriched for small RNA species (< 200 bp) was isolated by using the mirVana isolation kit (Ambion, Applied Biosystems), according to the manufacturer's protocol. The small RNA fractions were reverse transcribed and the analysis of the expression level of 365 miRNA was performed using a TaqMan-based Low Density Array. One hundred nanograms of the small RNA fraction were reverse transcribed using the microRNA multiplex RT primers and the TaqMan microRNA reverse transcription kit (both form Applied Biosystems). The reaction was incubated for 30 min at 16 °C, 30 min at 42 °C, and 5 min at 85 °C. Each RT reaction was diluted to 0.5 ng/µl in the TaqMan Universal PCR Master Mix (No AmpErase UNG, Applied Biosystems) and subsequently distributed into the preloaded 365-well micro fluidic card of the TaqMan Human microRNA Array (Applied Biosystems). The reaction was incubated at 50 °C for 2 min, followed by 95 °C for 10 min, and then by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The TaqMan PCR reactions were performed on an ABI Prism 7900HT qPCR system equipped with a TaqMan Array Upgrade (Applied Biosystems).

Real time RT-PCR (RT-qPCR)

The expression of miR-9 precursors (pri-miR-9-1, pri-miR-9-2 and pri-miR-9-3), CROC-4a, CROC-4b, NFKB1, NFKBIZ, IL-6, TNFα, CD206, IL-1β, Serpine1 and SMAD2 genes was quantified by RT-qPCR. Total RNA from any cell type used was purified using Trizol reagent. 1 µg of total RNA samples were reverse transcribed by using the high capacity cDNA Reverse Transcription kit (Applied Biosystems) according to the manufacturer's protocol. Genes expression was quantified by RT-qPCR performed in duplicate from 20 ng cDNA in the presence of the SYBR green PCR Master Mix (Applied Biosystems) and 400 nM of relative primer pairs (Table 1). RT-qPCR data were analyzed with SDS 2.2.2 software and normalized to the expression of GAPDH (primers are listed Table 1). The expression of indicated miRNAs was quantified by RT-qPCR. Briefly, cDNA was sythesized from 100 ng of total RNA fraction using individual miRNA-specific RT primers contained in the TaqMan microRNA Human Assays according to manifacturer

instructions. Each generated cDNA was amplified in duplicate by qPCR with sequence-specific primers from the TaqMan microRNA Human Assays together with TaqMan Fast Advanced Master Mix (Applied Biosystems). The ubiquitous snRNA U6 was chosen as the internal control. Both RT-qPCR analysis were conducted using the Applied Biosystems 7900HT Fast Real-Time PCR System. Termal cycler conditions were the following: 95 °C for 20 s, 40 cycles of 95 °C for 1 s and 60 °C for 20 s.

Constructs and lentiviral particles generation

Expression cassettes encoding miR-9, miR-155, miR-187, entire cluster or miR-125a and let-7e alone, and miR-511 were constructed by amplifying from genomic DNA from healthy donors the hairpin sequence (primers in Table 2) and flanking regions of respective precursors and cloning them into pcDNA3 vector using pCR2.1 as subcloning vector and HindIII and XhoI restriction enzymes.

For reporter assays, the 3'Untranslated Regions were amplified from genomic DNA from healthy donors into the psiCHECK vector 2 (Promega). The pCR2.1 vector (Invitrogen) was used as subcloning vector. Mutated version of the constructs were generated by site-directed mutagenesis using the QuikChange II XL Site-Directed Mutagenesis Kit from Agilent Technologies (USA) in accordance with data sheet instructions or without in the case of 3'NFKB1-mut-luc construct. Primers used in inserting mutated basis or deleting seed region-forming basis are indicated in Table 3.

To generate lentiviral particles used to overexpress microRNA of interest in THP-1 cell line, packaging plasmids from Invitrogen ViraPower Lentiviral Expression Systems were used. pRRLSIN.cPPT.PGK-GFP.WPRE (Addgene) carrying miRNA hairpin sequence were obtained as described in results. Briefly, miRNA cassettes cloned in pcDNA3 vector were subcloned together with eukaryotic constitutive Human cytomegalovirus (CMV) promoter in lentiviral multiple cloning site oriented tail-to-tail with respect to EGFP expressing cassette. 11,7 μg of Lentiviral vector, 5,85 μg pLP1, 2,93 μg pLP2 and 3,52 μg pVSV-g plasmids were co-transfected in low passages HEK293T packaging cell line in 10-cm plates using Lipofectamine 2000 according to manufacturer's protocol. The day after, medium

was replaced with fresh DMEM with 10% FBS to allow lentiviral particles release in fresh medium. After 24 h, the cell free supernatant was collected and ultracentrifuged 25000 rpm at 4 °C for 2 hours. 1×10^4 THP-1 cells were plated in 96-well in 100 µl of complete RPMI 1640 and 20 µL of concentrated lentiviral particles were added to the cells. THP-1 infection were assessed by FACS analysis after 1 week.

Luciferase reporter assay

For the Luciferase Reporter Assay, HEK293T cells were plated in 24-well plates in DMEM supplemented with 10% FCS, 2 mM L-glutamine without antibiotics to avoid possible interference with lipofectamine 2000 (Invitrogen) action used as liposome carrier in cell transfection. 8×10^4 cells were plated in each well 24 h before transfection. To clarify microRNAs action on indicated 3'UTRs, cells were co-transfected with 100 ng of psiCHECK-2 vector together with miRNA precursors or miRNA negative control (Ambion, Applied Biosystems) to a final concentration of 100 nM. The day after, cells were lysed and both firefly and Renilla luciferase activities were determined using the Dual-Glo Luciferase Assay System (Promega). The enzymatic activities of both luciferases were quantified using a MultiDetection Microplate Reader Synergy 2 luminometer (BioTek). The use of psiCHECK-2 vector enables the detection of changes in the expression of the target gene (3'UTR) fused to a primary reporter gene (Renilla luciferase, R-luc). The second reporter gene (firefly luciferase, Luc) serves as an internal control that allows normalization of R-luc expression and activity. Results are expressed as mean ± SEM of the ratio between Renilla luciferase and firefly control luciferase activities (RLU) adjusted to 1. Data represented in the bar graphs derive from 3 experiments each one conducted in triplicate.

Monocytes transfection

Freshly purified monocytes (10^7) were transfected with 5 µg of plasmid DNA (pcDNA3 empty vector, pcDNA3-miR-155 or pcDNA3-miR-9) or with the indicated amount of miRNA mimic (Ambion, Applied Biosystems), miRCURY LNA miRNA Power Inhibitor (Exiqon) or onTARGETplus siRNA (Dharmacon) using the Amaxa Nucleofector and the Human Monocyte Nucleofector kit (Amaxa), according to the

manufacturer's protocol and plated in Recovery medium (Amaxa) supplemented with 2mMglutamine and 10% FCS, and stimulated 18 h later as indicated or harvested 48 h after transfection and total RNA or whole cell extracts were prepared as described in the respective sections.

Western blot

For immunoblot experiments, THP-1 cells or transfected monocytes were lysed in Protein Lysis Buffer containing 50 mM Tris-HCl (pH 7.5), 2 mM EDTA, 100 mM PMSF, 10 μg/mL Aprotinin, 5 μg/mL Leupeptin, 1% Triton X-100 and a cocktail of protease inhibitors (cOmplete, EDTA-free Protease Inhibitor Cocktail Tablets, Roche Applied Science, Canada). 35 μg of total proteins from each sample were electrophorased in denaturing conditions on SDS-PAGE gel at different percentage (depending on proteins analyzed) and transferred to nitrocellulose membrane (Bio-Rad). NFKB1 and actin were simultaneously detected using anti-NFKB1 p105/p50 polyclonal Abs (H-119, Santa Cruz Biotechnology) and anti-actin polyclonal Abs (Sigma-Aldrich). Detection was carried out with Alexa Fluor-680 goat anti-rabbit secondary antibody (Molecular Probes, Invitrogen). Blots incubated with anti-IκBζ, anti-STAT3 or anti-IκBα were then probed with goat anti-rabbit or anti-mouse antibodies conjugated to Alexa Fluor 680 (Molecular Probes, Invitrogen) or Irdye 800 (Rockland Immunochemicals) secondary antibodies, respectively. Blotted proteins were detected and quantified using the Odyssey infrared imaging system (LI-COR Biosciences). Quantification was performed with the analysis software provided by the manufacturer. Anti-p38α, anti-phospho-p38α, anti-phospho-ERK1/2, anti-SMAD2, anti-phospho-SMAD2 and anti-phospho-SMAD3 were obtained from Cell Signaling. The secondary antibodies were horseradish peroxidase-conjugated donkey anti-rabbit IgG secondary antibodies (GE Healthcare Bio-Sciences AB). ECL reagents were from Millipore. Luminescence detection and analysis were conducted with ChemiDoc XRS (BioRad) and relative densitometry analysis of Westerns blots using Image J software program from NIH.

Cytokine detection in cell-free supernatants

Supernatants were recovered and centrifuged after stimulation and time

points indicated in results section. Cytokines and chemokines concentration was measured by specific human ELISA kits for TNFα, IL-6, CCL3, CXCL8 and CXCL10 (R&D Systems).

FACS analysis

THP-1 cells were resuspended in PBS containing 1% Bovine Serum Albumin (FACS buffer) and were incubated for 45 min in the dark at 4°C with Allophycocyanin (APC)-conjugated monoclonal TLR4 (eBioscience, SanDiego, CA, USA) or CD14 (Biolegend) antibodies or respective Isotype control. Cells were then washed three times with 1 ml of FACS buffer before analysis.

Apoptosis assay

5×10^5 over night-starved THP-1 cells were plated in 12-well in RPMI with or without 1% FBS with or without 10 ng/mL TGFβ. 48 h after stimulation, cells were collected and resuspended in Binding Buffer 1X. 5 µL of APC-Annexin-V and 5 µL of 7-Amino-Actinomycin D were added to the cells and analyzed with FACS Canto II. All reagents were obtained from BD Pharmingen. Datas are presented as percentage of Annexin V positive and 7-AAD negative cells (apoptotic but not necrotic cells). Bar graphs presents data from 3 independent experiments.

Table 1. Primers used in RT-qPCR analysis

Gene	pri-miR-9–1
Sense primer	GGCTGGATTCCCTCTGATAA
Antisense primer	TGCTAGAGCCTAGCCTCATCTT
Gene	pri-miR-9–1/CROC-4a
Sense primer	TTCCAGCTTTGGGAGTCAAG
Antisense primer	TGGCTCTATCGTCCACACG
Gene	pri-miR-9–1/CROC-4b
Sense primer	GGTGCTGGATGTGGCTCTAT
Antisense primer	GGGCTCGATCTTCTCACCT
Gene	pri-miR-9–2
Sense primer	GGAGGTTCAATTAAGGCAATAAGA
Antisense primer	TGACTTCATTGAGTGCTTTCAGTA
Gene	pri-miR-9–3
Sense primer	ACTTTGCCCCAGCTTCAA
Antisense primer	GTCTCGGCCATTGTCTTCA
Gene	18 s
Sense primer	CTCAACACGGGAAAGGTCAC
Antisense primer	CGCTCCACCAACTAAGAACG
Gene	NFKB1
Sense primer	CCTGAGACAAATGGGCTACAC
Antisense primer	TTTAGGGCTTTGGTTTACACGG
Gene	GAPDH
Sense primer	AACAGCCTCAAGATCATCAGC
Antisense primer	GGATGATGTTCTGGAGAGCC
Gene	NFKBIZ
Sense primer	GAGACAGGGTCTTGCTCTGG
Antisense primer	CCCAGCATTTTGGGAGACTA
Gene	TNFalpha
Sense primer	GCTGCACTTTGGAGTGATCG
Antisense primer	GAGGTACAGGCCCTCTGATG
Gene	IL-6
Sense primer	TACCCCCAGGAGAAGATTCC
Antisense primer	TTTTCTGCCAGTGCCTCTTT
Gene	CD206/MRC1
Sense primer	GGGCAGTGAAAGCTTATGGA
Antisense primer	CCTGTCAGGTATGTTTGCTCA
Gene	IL-1beta
Sense primer	AGTCTGCCCAGTTCCCCAAC
Antisense primer	GTTATATCCTGGCCGCCTTTG
Gene	SERPINE1
Sense primer	AACCCAGCAGCAGATTCAAG
Antisense primer	GGAACAGCCTGAAGAAGTGG

Gene	SMAD2
Sense primer	CTCCAGGTATCCCATCGAAA
Antisense primer	GTCGGGGCACTAATACTGGA

Table 2. Primers used in generating plasmids

Plasmid	miR-9–1/pcDNA3
Sense primer	TGTCCCTTCCCTCCTACTCC
Antisense primer	ATCCTCTGGTGCTGGTCAGT
Plasmid	miR-155/pcDNA3
Sense primer	CTTTCTCTCTTGCAGGTGGCACAAAC
Antisense primer	AGGTTGAACATCCCAGTGACCAGA
Plasmid	miR-187/pcDNA3
Sense primer	AGCCAAGACTCCTCAGGTCA
Antisense primer	GCTGTGTACGGAGAGACGAA
Plasmid	entire cluster/pcDNA3
Sense primer	ATGAGGAAGGGGCTGAGG
Antisense primer	TCAGAAGTCAGGCCAGCAAT
Plasmid	let-7e/pcDNA3
Sense primer	CTGTCTGTCTGTCGGGTCTG
Antisense primer	GCAGGGACAAGGACAGAAAA
Plasmid	miR-125a/pcDNA3
Sense primer	TGCCTATCTCCATCTCTGACC
Antisense primer	TGGTGGTCAAATGTCATGCT
Plasmid	miR-511/pcDNA3
Sense primer	AGCTGATAATGGGGGAAAGG
Antisense primer	CCCACGTCTCCTCATGTCTT
Plasmid	luc-fos/psiCHECK-2
Sense primer	GGGGGCAGGGAAGGGGAGGCA
Antisense primer	CGCATTCAACTTAAATGCTT
Plasmid	luc-NFKB1/psiCHECK-2
Sense primer	TGCTGACAATTTCCCACACC
Antisense primer	GGTCATCAATTTGCTTTTCC
Plasmid	luc-NFKBIZ/psiCHECK-2
Sense primer	ATCTGCCTGCCTTAGTCT
Antisense primer	ACCATCAGTTTTTCCAATGT
Plasmid	luc-TNFalpha/psiCHECK-2
Sense primer	TTATTACCCCCTCCTTCAGA
Antisense primer	ATTACAGACACAACTCCCCT
Plasmid	luc-TLR4/psiCHECK-2
Sense primer	ATGGAAATTGTTTATTTATGACAACAT
Antisense primer	AGGTCTAGGGTGATTGAAC
Plasmid	luc-CD14/psiCHECK-2
Sense primer	TGGATAACCTGACACTGGA
Antisense primer	ATGAAGAAAGCCTAAGTATG
Plasmid	luc-IL-6/psiCHECK-2
Sense primer	GTCAGAAACCTGTCCACT
Antisense primer	AATATGTATAAGTTAGCCAT

Plasmid	luc-CCL3/psiCHECK-2
Sense primer	CTGAGCCTTGGGAACAT
Antisense primer	AGAGCATCTTTATTATTTCC
Plasmid	luc-CXCL8/psiCHECK-2
Sense primer	CCAAGAGAATATCCGAACT
Antisense primer	CAAAGAGAATCCCAATAAGC

Table 3. Primers used for mutating/deleting miRNA seed regions

luc-mut-NFKB1/psiCHECK-2 CCGTGTAAACGTTTGCCCTA TAGGGCAAACGTTTACACGG
luc-mut-NFKBIZ/psiCHECK-2 GTTTGACCCAGTATGTCTTGTAGTTAGTTATAATCACCTTGTATCT AGATACAAGGTGATTATAACTAACTACAAGACATACTGGGTCAAAC
luc-mut-TNFalpha/miR-187/psiCHECK-2 GATGTTTCCAGACTTCCTTGAGGAGCCCAGCC GGCTGGGCTCCTCAAGGAAGTCTGGAAACATC
luc-mut-TNFalpha/miR-125a/psiCHECK-2 TCTGGAATCTGGAGACAGCCTTTGGTTCTGGC GCCAGAACCAAAGGCTGTCTCCAGATTCCAGA
luc-mut-TLR4/miR-125a/psiCHECK-2 AAGAAAAGGACAATCAGGATGTCATAAATGAAAATAAAAACCACAATGAG CTCATTGTGGTTTTTATTTTCATTTATGACATCCTGATTGTCCTTTTCTT
luc-mut-TLR4/let-7e/psiCHECK-2 CCATGACAAAGAAAGTCATTTCAACTCTTATCAAGTTGAATAA TTATTCAACTTGATAAGAGTTGAAATGACTTTCTTTGTCATGG
luc-mut-CD14/psiCHECK-2 CTGCCTTGGCTTCGAGTCCCGTCAGG CCTGACGGGACTCGAAGCCAAGGCAG
luc-mut-IL-6/psiCHECK-2 CATTTCTTGGAAAGTGTAGGCTCAAATAAATGGCTAACTT AAGTTAGCCATTTATTTGAGCCTACACTTTCCAAGAAATG
luc-mut-CCL3/miR-125a/psiCHECK-2 AAATGTGTATCGGATGCTTTTGTGGCTGTGATCGG CCGATCACAGCCACAAAAGCATCCGATACACATTT
luc-mut-CCL3/let-7e/psiCHECK-2 GTGTGACCTCCACAGCTTTCTATGGACTGGTTGT ACAACCAGTCCATAGAAAGCTGTGGAGGTCACAC
luc-mut-CXCL8/miR-125a/psiCHECK-2 GATGTTTTATTAGATAAATTTCGGGTTTTTAGATTAAAC GTTTAATCTAAAAACCCGAAATTTATCTAATAAAACATC
luc-mut-CXCL8/let-7e/psiCHECK-2 AAGTATTAGCCACCATCTCACAGTGATGTTGTGAGG CCTCACAACATCACTGTGAGATGGTGGCTAATACTT

Results

LPS induces up-regulation of several microRNAs in human monocytes and neutrophils

To identify miRNAs potentially involved in the responses of peripheral human PMN and monocytes to stimuli of bacterial origin, the miRNA pattern of expression was investigated in PMN and monocytes stimulated for 8 h with 100 ng/ml LPS using a TaqMan-based Low Density Array. As shown in Figure 10A and 10B, LPS induced an up-regulation of 12 miRNAs in PMN and/or monocytes respectively, whereas no miRNA was significantly down-regulated. LPS-induced miRNAs identified in the array were evaluated in a time-course analysis by qRT-PCR (Figure 10C).

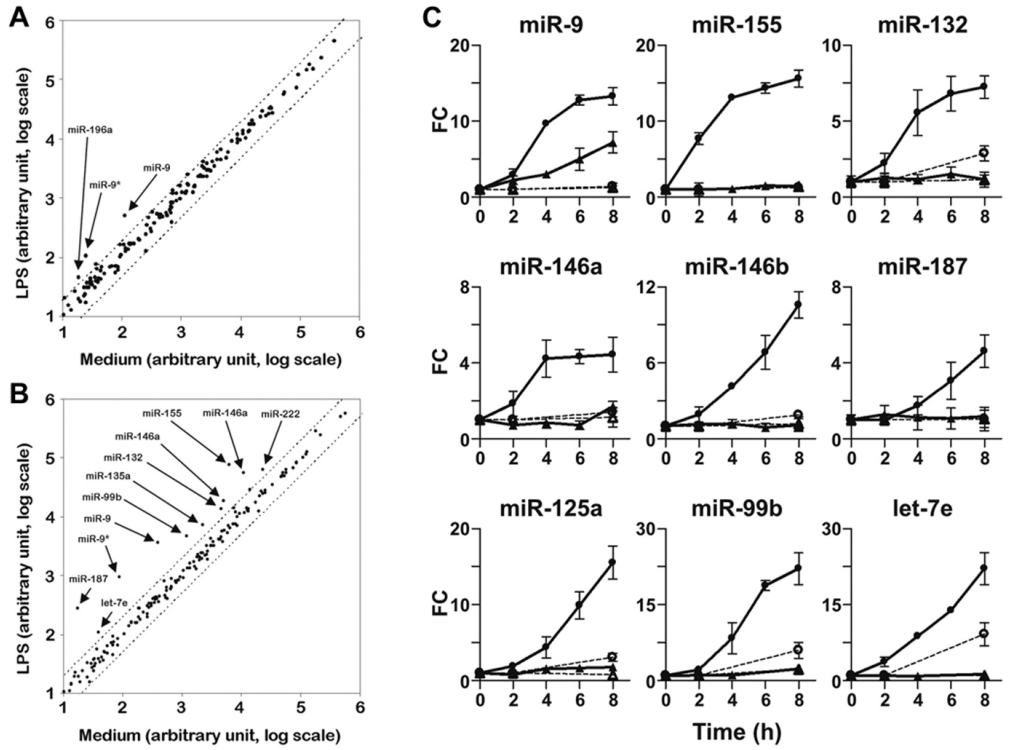

Figure 10. **miRNAs induced by LPS in PMN and monocytes.** PMN (A) and monocytes (B) were cultured for 8 h in medium alone or in the presence of 100 ng/ml LPS. The miRNA fraction was purified and changes in miRNA expression levels were determined using a micro fluidic card as described in *Materials and Methods*. Results are expressed as arbitrary units on a log scale using RNU44 as reference control. The mean values of 2 individual experiments performed are shown. Dotted lines represent the 2 and 0.5 boundary values for fold induction. In panel C, the time-course analysis of the single miR is shown. PMN and monocytes were cultured for the indicated times in medium alone (- -△- - PMN, - -o- - monocytes) or in the presence of 100 ng/ml LPS (—▲— PMN, —•— monocytes). miRNA fraction was purified and miR-9, miR-155, miR-132, miR-146a, miR-146b, miR-187, miR-125a, miR-99b, and let-7e expression was determined by RT-qPCR and normalized to the let-7a levels, as described in *Materials and Methods*. The results are expressed as fold change and are representative of 3 individual experiments.

Consistent with the array data, the expression of miR-155, miR-132, miR-146a, miR-146b, miR-187, miR-125a, miR-99b, and let-7e rapidly increased in LPS-treated monocytes but not in PMN (Figure 10C). Interestingly, both 3'-end (miR-9) and 5'-end (miR-9*) forms of miR-9 were the only miRNAs consistently induced by LPS in both PMN and monocytes, being already detectable after 2 h and steadily increasing over the time period assessed (Figure 10C). Conversely, the induction of miR-222 and miR-196a observed in the array analysis was not confirmed by RT-qPCR analysis (not shown). miR-9 was then chosen for a more detailed analysis, given that it is the only miRNA up-regulated in response to LPS in both cell types and that it has not been previously reported to be involved in the inflammatory response.

miR-9 expression is modulated in both monocytes and PMNs: biogenesis and regulation

LPS triggers different patterns of responses in PMN and monocytes, partly because of the selective activation of the different MyD88- and TRIF-dependent signaling pathways downstream of the pattern-recognition receptor TLR4 (Akira et al., 2006). To investigate the requirement of MyD88 and/or TRIF adaptors in the induction of miR-9 expression by LPS and to evaluate miR-9 regulation by other TLRs, PMN and monocytes were stimulated with Pam_3CSK_4 (100 ng/ml), a synthetic lipoprotein agonist at TLR2 that selectively requires TIRAP/MyD88; Resiquimod (R848, 10 mM), a TRL7/8 ligand signaling through MyD88 only; or polyinosinic:polycytidylic acid (poly(I:C)) (50 μg/ml), a synthetic mimetic of viral double-stranded RNA (dsRNA) that interacts with endosomal TLR3 and utilizes TRIF-mediated signaling (Akira et al., 2006). As shown in Figure 11A, activation of TLR2 and TLR7/8 resulted in up-regulation of miR-9 expression in both cell types, while that of TLR3 was ineffective. Conversely, poly(I:C) readily induced miR-155 in monocytes (Figure 11B), as previously reported in other cell types (O'Connell et al., 2007), demonstrating that the lack of miR-9 induction was not due to a general failure of monocytes to activate the TRIF-dependent pathway downstream TLR3. In agreement with the lack of TLR3 expression in human PMN (Tamassia et al., 2008), poly(I:C) had no effect on miR-9 expression in this cell type (Figure 11A). Taken

together, these data suggest that in human phagocytes, activation of the MyD88-dependent signaling pathway is necessary and sufficient to increase miR-9 expression in response to LPS and that no additional TRIF-dependent signals are required.

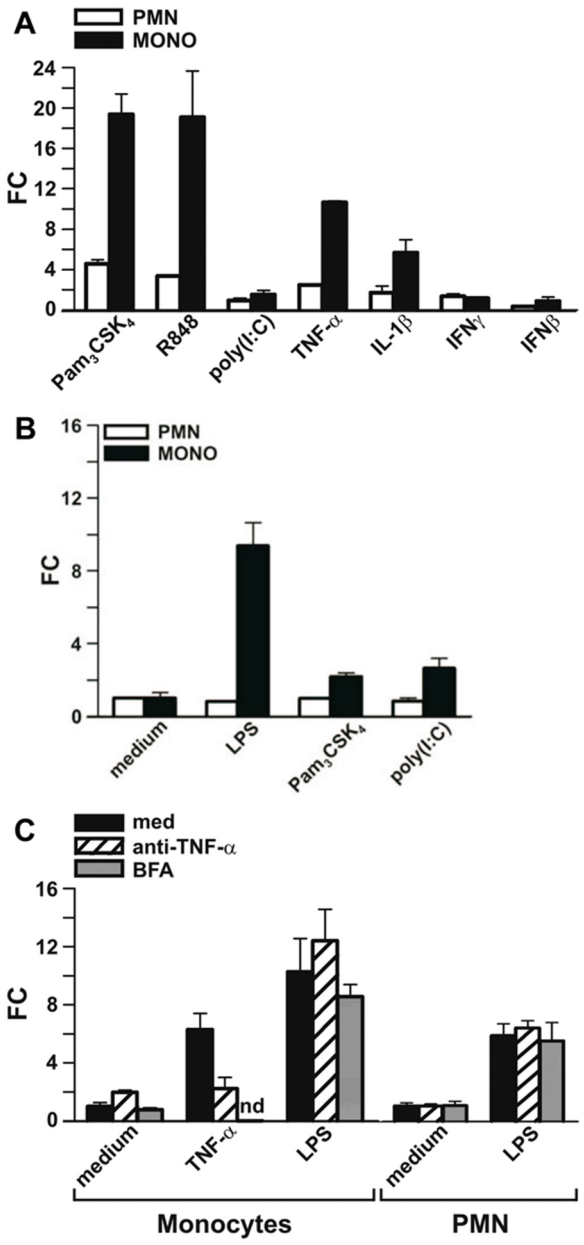

Figure 11. miR-9 is induced by MyD88-activating pathways. miR-155 is evaluated as a control. (A) PMN and monocytes were cultured for 8 h with 100 ng/ml LPS, 100 ng/ml Pam_3CSK_4, 10 μM R848 or 50 μg/ml poly(I:C), TNFα, 20 ng/ml IL-1β, 1000 U/ml IFNγ, or 1000 U/ml IFNβ. miRNA fraction was extracted and analyzed for miR-9 expression by RT-qPCR. (B) PMN and monocytes were cultured for 8 h with 100 ng/ml LPS, 100 ng/ml Pam_3CSK_4, or 50 μg/ml poly(I:C). miRNA fraction was extracted and analyzed for miR-155 expression by RT-qPCR. miRNA expression is depicted as fold change units after let-7a normalization. Data show 1 experiment representative of 3. (C) Monocytes and PMN were pretreated for 30 min with medium (black bars), 10 μg/ml anti-TNFα MoAbs (hatched bars), or 5 μg/ml brefeldin A (gray bars) before stimulation with TNFα or LPS. miRNA fraction was ex-tracted after 8 h and analyzed for miR-9 expression by RT-qPCR. miRNA expression is depicted as fold change units after let-7a normalization. Data show one experiment representative of 3. nd: not determined.

To test whether cytokines involved in the response to bacterial and/or viral infection are also effective at inducing miR-9 expression, PMN and monocytes were stimulated with TNFα (5 ng/ml), IL-1β (20 ng/ml), IFNγ (1000 U/ml), or IFNβ (1000 U/ml). The proinflammatory cytokines TNFα and IL-1β increased miR-9 levels in both PMN and monocytes, while IFNγ and IFNβ were ineffective (Figure 11A). Since miR-9 is up-regulated by both LPS and TNFα, we tested whether TLR4

induction of miR-9 required a TNFα autocrine signaling as previously reported for miR-155 (O'Connell et al., 2007). Anti TNFα MoAbs completely blocked miR-9 induction by TNFα, but were ineffective when LPS was used (Figure 11C), indicating that TNFα is not involved in the induction of miR-9 by LPS. In addition, up-regulation of miR-9 expression was not modified by treatment with Brefeldin A before LPS stimulation, ruling out the possible involvement of soluble mediators released in response to LPS for miR-9 up-regulation (Figure 11C). Taken together, these data candidate miR-9 as a novel miRNA involved in the responses of human phagocytes to selected stimuli of bacterial origin or proinflammatory cytokines.

In both mouse and human genomes, miR-9 can be generated by processing of 3 different miR-9 primary transcripts encoded by distinct genes (C1orf61 for pri-miR-9–1, BC036480 for pri-miR-9–2, and CR612213 for pri-miR-9–3, respectively). LPS induced a time-dependent increase in pri-miR-9–1 levels and had no effect on the other 2 miR-9 primary transcripts, both in PMN and monocytes (Figure 12A). The miR-9–1 primary transcript derives from the C1orf61 locus which encodes for CROC-4 protein, a transcriptional activator for the c-fos proto-oncogene (Jeffrey et al., 2000). An EST database analysis revealed the existence of an internal product of the C1orf61 locus. Both transcriptional units (here called CROC-4a and CROC-4b: Figure 12B) generate the miR-9–1 precursor and are activated by LPS in PMN and monocytes (Figure 12C). Analysis of the C1orf61 locus with the transcription start sites predictor SwitchGear software (available at http://genome.ucsc.edu) supports the existence of an internal transcriptional unit. Inspection of the genomic sequence located 2 kb upstream of the predicted start sites of the 2 transcripts identified putative promoter regions with consensus binding sites for known LPS-sensitive transcription factors, including NF-κB (Figure 12B). The observation that miR-9 induction by LPS depends on the activation of the MyD88 pathway and the identification of NF-κB consensus binding sites within the 2 putative pri-miR-9–1 promoters suggested that the miR-9 induction by LPS may result from the transcriptional activity of NF-κB. This hypothesis was confirmed by the suppressive effect on LPS-dependent miR-9 induction of NF-κB inhibitors (MG- 132, BAY-117082 and PDTC). In contrast, inhibitors of p38 (SB-203580) and JNK (SP-600125) were ineffective (Figure 12D). Collectively, these data demonstrate that in

PMN and monocytes inflammatory stimuli sustain the NF-κB-dependent transactivation of C1orf61 locus and consequent production of pri-miR-9 –1.

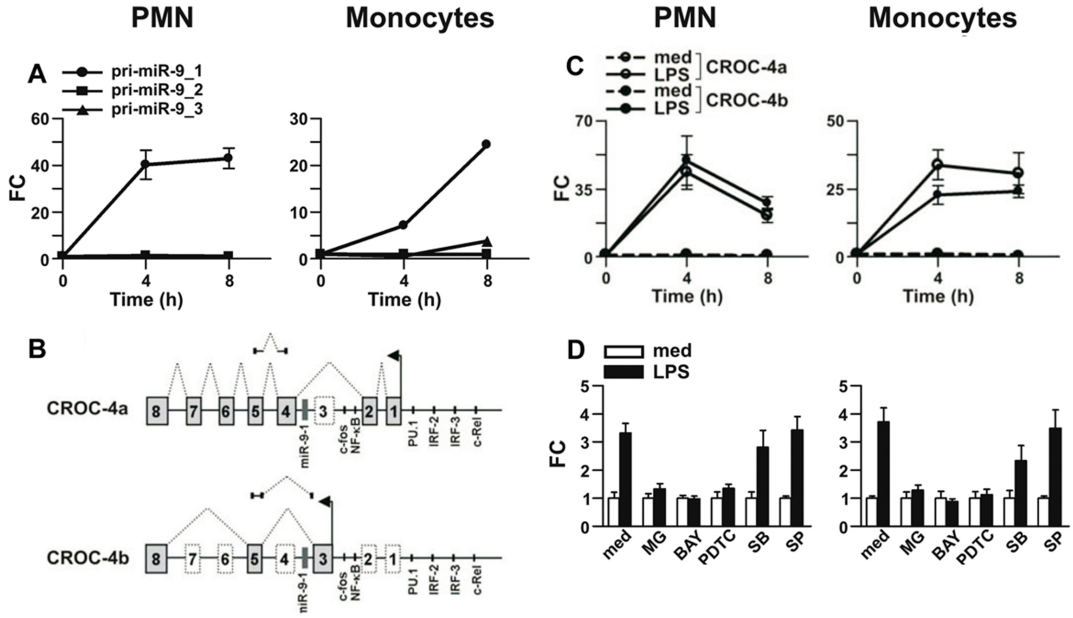

Figure 12. LPS up-regulates pri-miR-9–1 in a NF-κB-dependent manner. (A) PMN and monocytes were cultured in the presence or absence of LPS for the indicated time; total RNA was extracted and pri-miR-9-1 (—•—), pri-miR-9-2 (—■—), and pri-miR-9-3(—▲—) were analyzed by RT-qPCR and normalized to the 18S RNA as described in *Materials and Methods*. Results show that only pri-miR-9-1, but not pri-miR-9-2 or pri-miR-9-3, was induced by LPS. (B) Schematic representation of premiR-9-1 genomic locus on intron 3 of the CROC-4-encoding gene. The CROC-4a and CROC-4b transcriptional units, the putative transcription factor binding sites, and the PCR products identifying the products of the 2 transcriptional units (oligonucleotides are shown as thick and amplicons as thin lines) are shown. (C) PMN (Left) and monocytes (Right) were cultured in the presence or absence of 100 ng/ml LPS for indicated time, total RNA was extracted, and CROC-4a (—O—) and CROC-4b (—•—) were analyzed by RT-qPCR in triplicate samples and normalized to the 18S RNA as described in Materials and Methods. Results show that both CROC-4a and CROC-4b were induced by LPS in both cell types. Both transcripts did not change expression levels in the absence of LPS (dotted lines). (D) PMN and monocytes were pretreated for 30 min with medium, 10 μM MG-132, 10 μM BAY-117082, 300 μM PDTC, 20 μM SP-600125, or 10 μM SB-203580 and subsequently cultured for 8 h with or without LPS. miR-9 expression levels were determined by RT-qPCR and expressed as fold change after let-7a normalization. Data show 1 experiment representative of at least 2 for each panel.

miR-9 affects NFKB1 production during inflammatory response

To gain insight on the biological relevance of miR-9 induction under inflammatory conditions, we searched for predicted miR-9 targets, focusing our attention on regulators of transcription, which have been frequently shown to be preferential miRNA targets (Asirvatham et al., 2008). In agreement with this, the

public database of animal miRNA miRGen (available at http://www.diana.pcbi.upenn.edu/miRGen/v3/miRGen.html), which integrates analysis from PicTar (Krek et al., 2005), MiRanda (John et al., 2004), and TargetScan (Lewis et al., 2003), predicted among high score miR-9 targets the transcriptional regulators Onecut2 and PRDM1/Blimp-1, which have been previously validated as miR-9 targets but are not expressed in PMN and monocytes (data not shown). A miR-9 seed was also predicted in one of few highly conserved regions present in the 3'-UTR of the NFKB1 gene. Interestingly, the potential regulatory loop between NF-κB and miR-9 was also confirmed by the miPromotor software (http://wiki.binf.ku.dk/MiTools). Thus, to test whether miR-9 post-transcriptionally affects NFKB1, reporter construct containing the renilla luciferase gene fused to the NFKB1 3'-UTR (luc-NFKB1) was transiently transfected in HEK-293 cells together with expression plasmids encoding miR-9. As shown in Figure 13, miR-9 significantly reduced luc-NFKB1 luciferase activity, and the introduction of point mutations in the miR-9 seed in the 3'-UTR of NFKB1 (luc-mut-NFKB1) reverted the inhibitory activity of miR-9, demonstrating that the NFKB1 3'-UTR contains an active seed of miR-9. The specificity of miR-9 was also demonstrated by the lack of effect on a c-fos 3'-UTR reporter construct (luc-fos), which does not contain a miR-9 seed. Conversely, the c-fos 3'-UTR presents a miR-155 seed and was significantly inhibited by miR-155, in agreement with previous reports (Gottwein et al., 2007) (Figure 13).

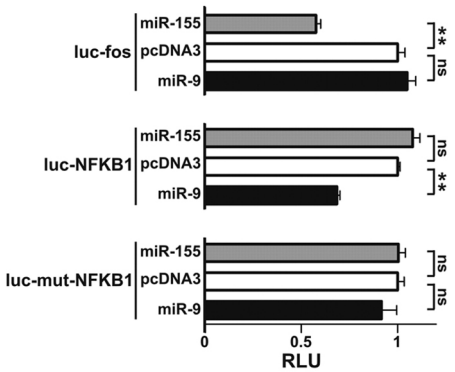

Figure 13. The NFKB1 gene is a molecular target of miR-9. The indicated luciferase constructs (luc vectors) were cotransfected with expression vectors encoding miR-155 (gray columns), miR-9 (black columns), or the pcDNA3 empty vector (white columns). Results are expressed as mean (± SD, n = 3) of the ratio between renilla luciferase and firefly control luciferase activities (RLU), adjusted to 1. **: $P < 0.01$; ns: $P > 0.05$.

Finally, we analyzed the expression profile of NFKB1 in monocytes stimulated with LPS at the transcript and protein levels (Figure 14A and 14B). Interestingly, LPS rapidly induced a consistent increase in NFKB1 transcripts (Figure 14A), but the transcript up-regulation was not paralleled by a comparable increase of the corresponding protein, which showed constant expression levels

(Figure 14B). These results indicate that NFKB1 is subjected to a LPS-dependent regulation at the transcriptional level and suggest that a second regulatory mechanism, acting at the post-transcriptional level, is also operative. In agreement with this finding, monocytes over-expressing miR-9 showed a reduced expression of the endogenous NFKB1/p105 protein (Figure 14C). Conversely, transfection of monocytes with either the pcDNA3 empty vector or with the miR-155-encoding vector did not alter the levels of expression of the endogenous NFKB1/p105 protein (Figure 14C), confirming the specificity of the effect of miR-9.

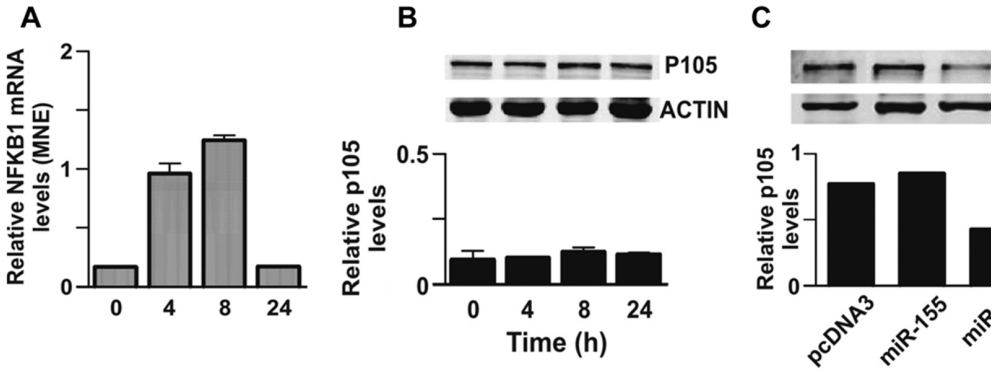

Figure 14. Analysis of the endogenous miR-9 target: NFKB1 mRNA and protein expression. (A) monocytes were cultured in the presence of LPS for the indicated times. Total RNA was purified and used to assay NFKB1 mRNA expression by RT-qPCR, as described in Materials and Methods. Relative NFKB1 gene expression is depicted as MNE units after GAPDH normalization. Data reported are representative of 3 independent experiments. (B) Whole-cell extracts (20 μg) were usually loaded on gels and immunoblots were performed by simultaneously using Abs specific for NFKB1 and Abs specific for actin, followed by incubation with Alexa Fluor-680 goat anti-rabbit Abs. One experiment representative of 3 is shown. The relative NFKB1/p105 levels (±SD, n = 3), quantified as described in *Materials and Methods*, are reported below each panel. (C) 6×10^6 monocytes were transfected with 5 μg of pcDNA3 empty vector, miR-155-encoding vector, or miR-9-encoding vector as described in *Material and Methods*. 48 hours posttransfection, 20 μg of were usually loaded on gels and NFKB1/p105 protein was detected as described above. The relative NFKB1/p105 levels, normalized for the total actin, are reported below the Western blot. One experiment representative of 2 is shown in panel C.

LPS-dependent expression induction of certain miRNAs is sustained by IL-10 autocrine action on monocytes

Recently, McCoy CE and colleagues demonstrated that TLR4-dependent miR-155 induction in murine macrophages is inhibited by IL-10 stimulation (McCoy et al., 2010). Therefore, we decided to perform such analysis in human peripheral blood monocytes on the complete panel of microRNAs previously described. As shown in Figure 15, IL-10 affected the expression of all the quantified microRNAs in

different way: the LPS-mediated over-expression of the well defined "pro-inflammatory" microRNAs (miR-155, miR-146a and miR-9) was reduced by IL-10 presence; in contrast, IL-10 induced miR-146b miR-187, miR-125a, let-7e and miR-99b expression while it strongly potentiated LPS-dependent miR-187, miR-125a, let-7e and miR-99b induction. Taken together, these data suggest that IL-10 can modify microRNA expression profile in monocytes after LPS exposure and point out miR-187 and miR-99b~7e~125a cluster as LPS-induced miRNAs strongly up-regulated by IL-10.

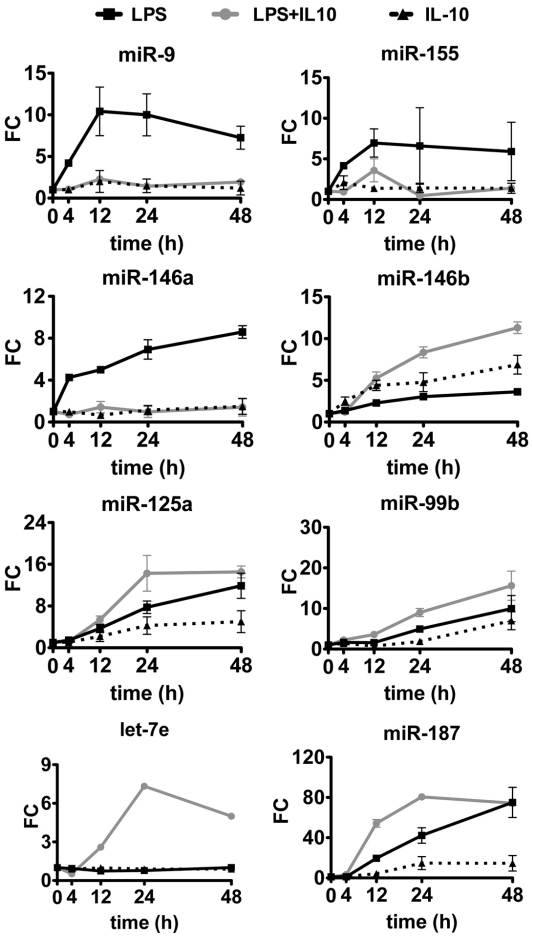

Figure 15. miRNAs induced by LPS/IL-10 in monocytes. A time-course analysis of miRNAs considered are shown. Freshly isolated monocytes were cultured for the indicated times in medium alone or in the presence of 100 ng/ml LPS (−■−), 30 ng/ml IL10 (- -▲- -) or the combination of both stimuli (−•− in grey). Total RNA was extracted and miR-9, miR-155, miR-146a, miR-146b, miR-125a, miR-99b, let-7e and miR-187 expression was determined by RT-qPCR and normalized to the snRNU6 levels, as described in *Materials and Methods*. The results are expressed as fold change respect to the medium condition and are representative of at least 2 individual experiments.

In human monocytes LPS stimulates IL-10 production, which in turn acts on monocytes in an autocrine manner (Saraiva and O'Garra, 2010). In order to investigate whether endogenously produced IL-10 plays a role in the expression of miR-187 and miR-99b~7e~125a cluster induced by LPS, monoclonal anti-IL-10R antibody (or isotype-specific control IgG) was added to monocytes, and the expression levels of miRs were analyzed 24 h after LPS stimulation (Figure 16). Blocking endogenous IL-10 action reduced the induction of miR-187, miR-125a, let-7e or miR-99b by LPS and significantly increased LPS-induced miR-155 expression (Figure 16).

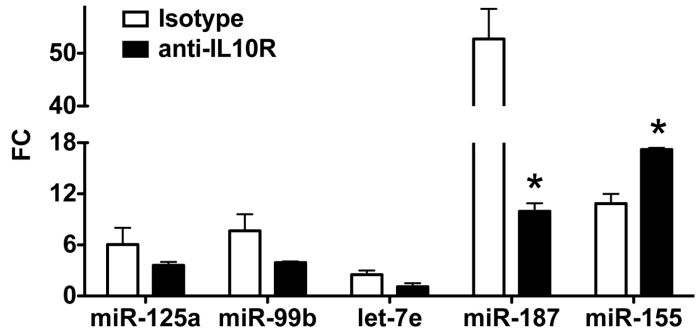

Figure 16. Modulation of IL-10 inuced miRNAs expression by endogenous IL-10. Monocytes were pretreated for 30 min with 1 μg/ml anti-IL-10R MoAbs (black bars) or 1 μg/ml Isotype control (white bars) before stimulation with 100 ng/ml LPS. Total RNA was extracted after 24 h and analyzed for indicated miRNA expression by RT-qPCR. miRNA expression is depicted as fold change respect to unstimulated control units after snRNU6 normalization. The results are representative of 2 individual experiments. *: $P < 0.05$

Mature miR-187 is generated by processing of a precursor (pre-miR-187), transcribed from an intronic region located on chromosome 18 (Figure 17A) Mature miR-125a, let-7e and miR-99b come from a single transcript located on chromosome 19 upstream a long non coding RNA transcript (UNQ2487, NR_024330.1) (Figure 17B). Expression of mature miR-99b~7e~125a cluster and UNQ2487 are induced at the same time in our experimental conditions (Figure 17C), suggesting co-transcription of these RNAs.

Taken together, these data support a role for endogenous IL-10 in LPS-induced miRNAs expression, and, above all, call attention to miR-187 and miR-99b~7e~125a cluster as unique miRNAs induced by LPS in an IL-10 dependent manner.

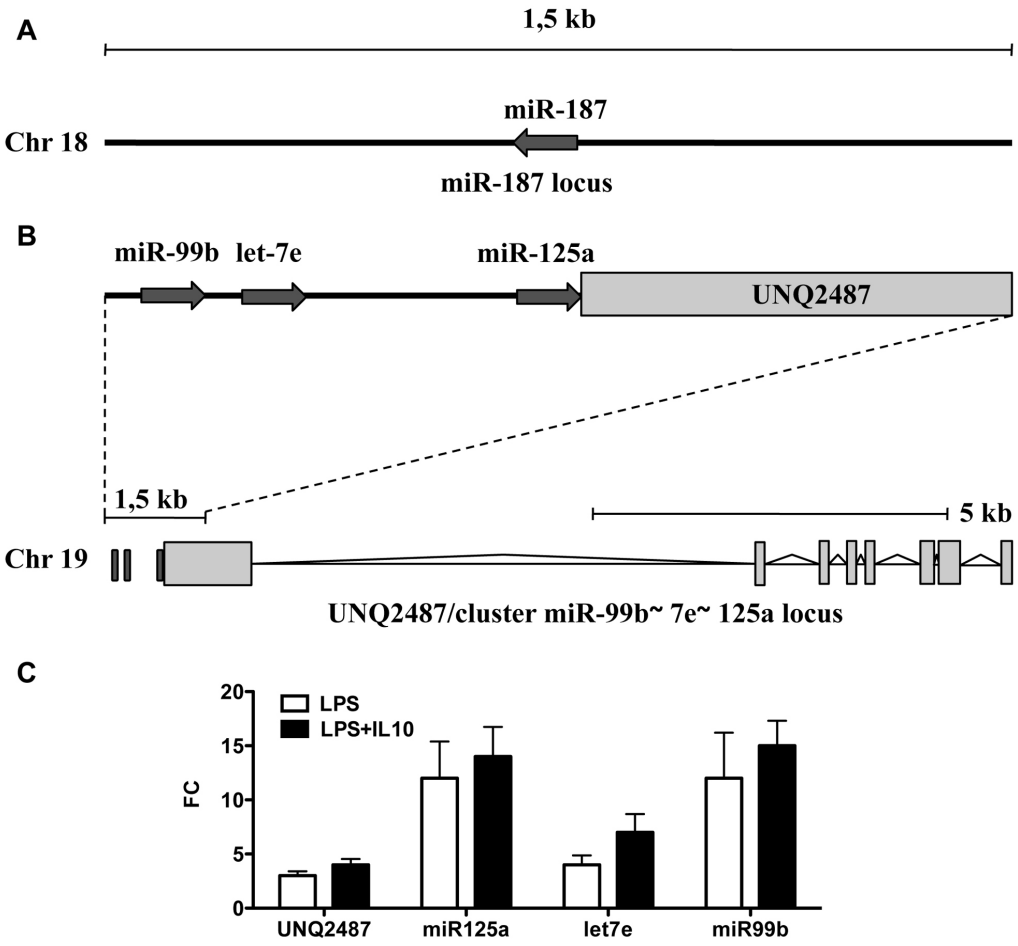

Figure 17: miR-99b, let-7e and miR-125a are a product of a unique transcript. Schematic representation of pre-miR-187 genomic locus (A) or UNQ2487/cluster miR99b~7e~125a genomic locus (B). Grey bars represent exon, triangles are introns, whereas black lines are not-annotated genomic regions. Chromosome in which the regions are located are indicated on the left. (C) Monocytes were cultured for 24 h in the presence of 100 ng/ml LPS alone (white bars) or in combination with 30 ng/ml IL-10 (black bars). Results are showh as fold change respect to untreated cells after normalization to the snRNU6 levels. Datas are representative of 2 experiments.

Over-expression of IL-10-induced microRNAs in a human monocytic cell line impairs cell response to LPS

MicroRNAs are key regulators of gene expression. Despite great advances, the miRNA world remains largely not explored and discovering the function of a single microRNA is challenging. First, each miRNA has numerous putative targets that have disparate functions (Lewis et al., 2003), with no means to decide a priori which one is most meaningful. Second, the degree of target down-regulation imposed by miRNAs often tends to be quantitatively modest: measured at the protein

level, an over-expressed miRNA usually inhibits most of its endogenous targets by less than 50% (Thomas et al., 2010).

In a recently published review, Inui Masafumi and colleagues showed that signal transduction pathways are prime candidates for miRNA-mediated regulation in animal cells. They assessed that signaling cascade are highly dynamic and non-stoichiometric molecular ensembles, which translate into well established dose-dependent responses. As such, they are the ideal targets for the degree of quantitative fluctuations imposed by miRNAs. This might enable the multi-gene regulatory capacity of miRNAs to remodel the signaling landscape, both in a positive and in a negative way (Inui et al., 2010).

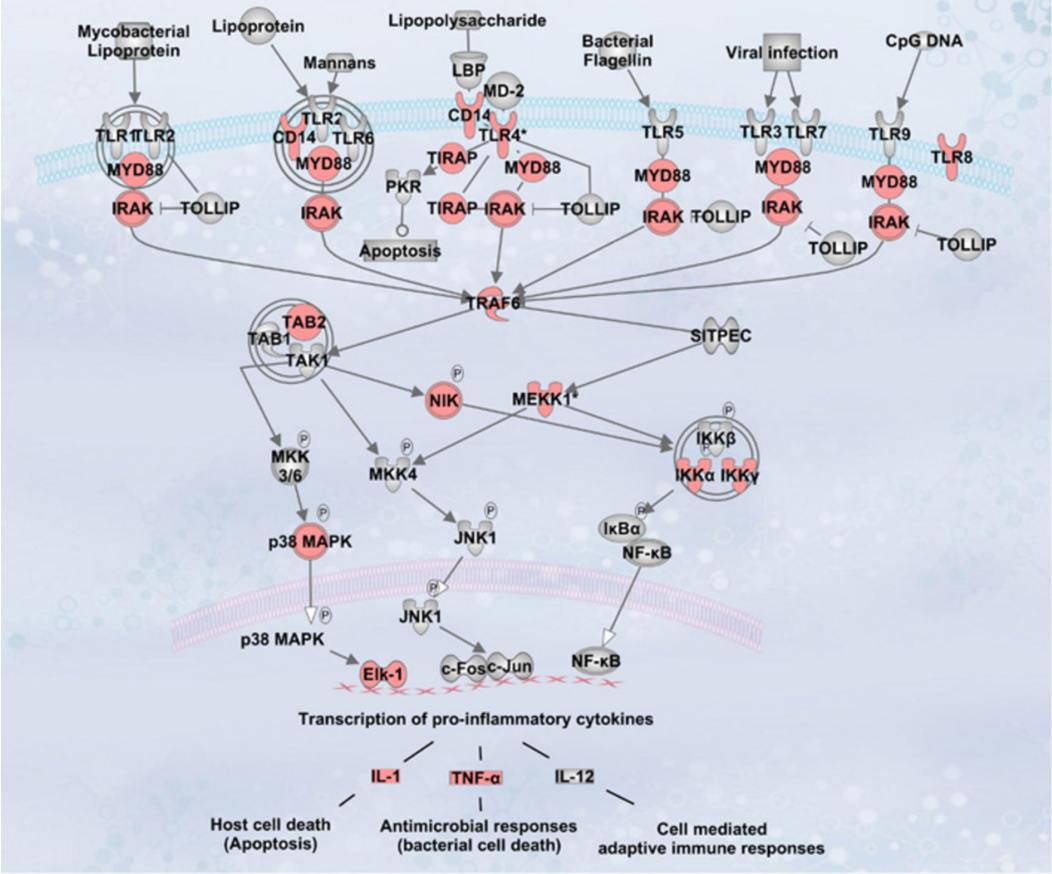

Figure 18: TLR signaling molecules affected by miR-187 and entire cluster. A list of molecules recognized by TargetScan software as targets of miR-187, miR-99b, let-7e and miR-125a was integrated with IPA "canonical pathway" informations. A schematic representation of Toll-like Receptor Signaling pathway is reported. The direct putative targets of analyzed microRNAs are highlighted here in red.

To visually underline the impact of LPS-dependent IL-10-potentiated expression of miR-187 and miR-99b~7e~125a cluster in LPS-driven signaling

cascade, we took advantage of two prediction software: TargetScan, a widely used on-line software predicting microRNA's target mRNAs, and Ingenuity Pathway software (IPA), that models conventional signaling pathway starting from a list of apparently random molecules. Integrating the list of all miR-187, miR-99b, let-7e and miR-125a targets generated by TargetScan with pathway information produced by the IPA software, we observed that TLR signaling pathway is one of the most affected by the concomitant expression of these microRNAs (Figure 18).

To confirm the *in silico* prediction, an *in vitro* cellular model was produced over-expressing microRNAs of interest (miR-187 and miR-99b~7e~125a cluster) or a scramble sequence as a control in THP-1 cells, a human monocytic cell line,

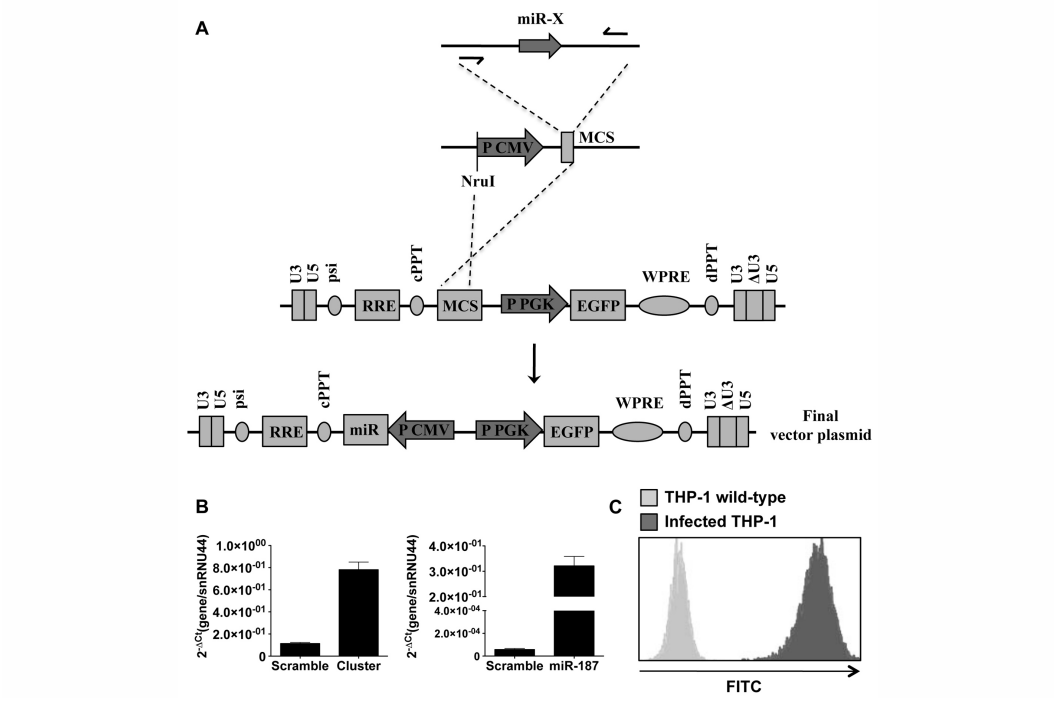

Figure 19: THP-1 infection with lentiviral particles induce a strong up-regulation in miR cloned in plasmid constructs together with EGFP protein expression. (A) A schematic representation of lentiviral plasmid construction is here reported. Briefly, 300-600 bp-long region including pre-miR sequence was cloned in pcDNA3 empty vector. miR-expressing region was then subcloned in lentiviral vector plasmid together with CMV promoter (coming from pcDNA3 plasmid) in tail-to-tail orientation respect to EGFP ORF. (B) Total RNA was extracted from THP-1 infected with lentiviral particle carrying entire cluster region cloned vector, miR-187 region cloned vector or scramble region cloned vector (as indicated in the bar graph). RNAs were retrotranscribed and analyzed for miR-125a (left panel) or miR-187 (right panel) expression by RT-qPCR. miRNA expression is reported as $2^{-\Delta Ct}$ normalized to snRNU44 levels. (C) A FACS analysis of infected THP-1 versus not-infected THP-1 (THP-1 wild-type) is here reported.

through lentiviral infection. The genomic sequences in which miR-187 or miR-99b~7e~125a cluster are located were cloned (459 base-pairs or 842 base-paires, respectively) in pcDNA3, a vector with a multiple cloning site downstream CMV eukaryotic constitutive promoter. This promoter region together with pre-miR sequences were then subcloned in the lentiviral vector pRRLSIN.cPPT.PGK-GFP.WPRE (Addgene) that, per se, induces constitutive expression of EGFP protein gene, labelling infected cells with green fluorescent protein (Figure 19A). Lentiviral particles produced with these vectors (as indicated in "material and methods" issue) drives integration of pre-miRs sequences downstream the CMV promoter in THP-1 genome, leading to microRNA (or control sequence) constitutive over-expression together with EGFP production (Figure 19B).

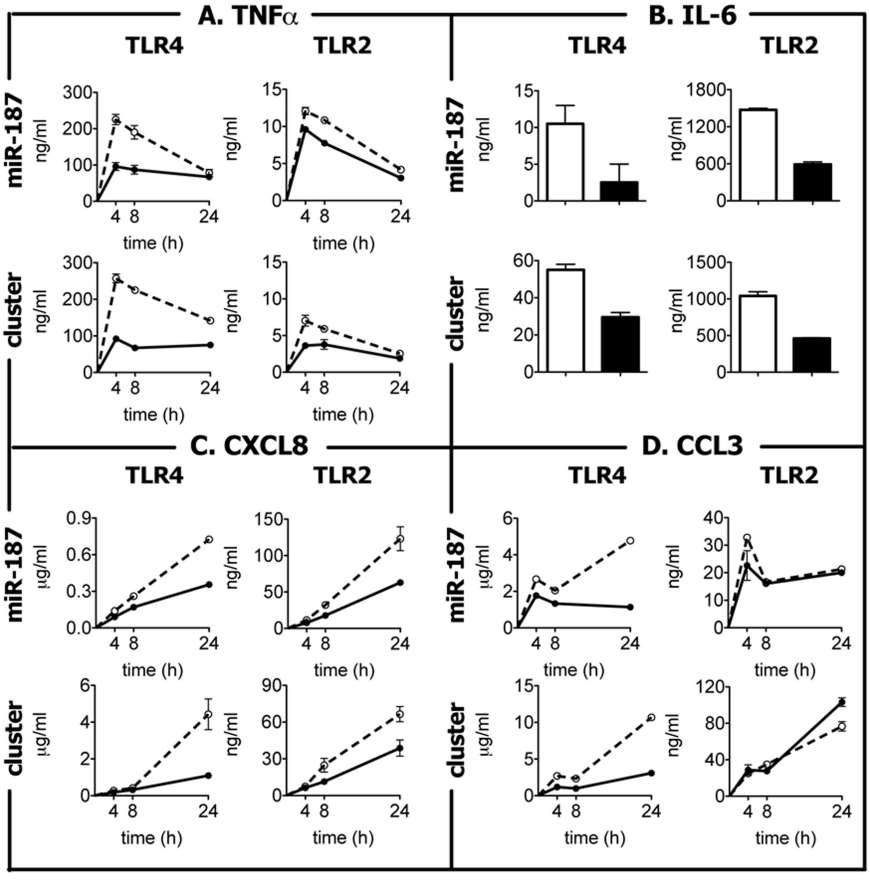

Figure 20: Global impairment in pro-inflammatory cytokines and chemokines production in THP-1 infected cell line. THP-1 overexpressing miR-187, entire cluster or a scramble sequence were seeded and stimulated with 1 µg/µL LPS (TLR4 agonist) or 1 µg/µL Pam_3CSK_4 (TLR2 agonist). Supernatants were collected and analyzed by ELISA assay at indicated time points except for IL-6 measurements (panel B) that was carried only after 24 h. Dotted lines or withe bars indicate scramble-THP-1 production of indicated cytokine/chemokine, whereas black lines or bars indicate miR-187 or cluster-THP-1 (as indicated on the left) production. Every experiment was carryed in duplicated. Datas show one of at least 2 individual experiments.

Over-expressing cell lines were therefore exposed to different stimuli to assess their ability in inducing a proinflammatory signaling cascade. Keeping in mind the *in silico* predicted impact of these microRNAs on TLRs pathway, we performed THP-1 stimulation with LPS and Pam_3CSK_4, specific TLR4 and TLR2 agonists respectively. Several pro-inflammatory mediators were measured at different time points till 24 h except for IL-6, whose production remains undetectable at early time points. As shown in Figure 20, a general impairment in producing cytokines and chemokines were observed both in THP-1-187 and in THP-1-cluster compared with controls. In particular, TLR4-dependent pathway appeared to be more regulated than TLR2-dependent cascade. Indeed, TLR4 lost almost 50% of its capacity in inducing pro-inflammatory molecules production, whereas both miR-187 and cluster over-expressing cells showed less impairment in TLR2 agonist-induced cytokines and chemokines production.

Among all these microRNAs, ability of let-7e in reducing TLR4 receptor expression and therefore signaling cascade activation has already been described in murine macrophages (Androulidaki et al., 2009). Moreover, *in silico* analysis of microRNA-99b targets alone revealed very few molecules related to inflammation (data not shown). We therefore decided to use THP-1 cells over-expressing miR-187, miR-125a or let-7e alone to better characterize their modality in inducing TLRs desensitization. To this aim, we took advantage of THP-1 ability in producing CXCL10 in response to several stimuli. Stimulations with TLR4 or TLR2 agonists and IFNγ were performed and CXCL10 production levels in cell free supernatants were measured by ELISA. As shown in Figure 21, TLR4 and TLR2-dependent CXCL10 production were severely impaired in THP-1 over-expressing miR-187 and miR-125a compared to the control, whereas let-7e affected only TLR4-dependent response, confirming the ability of let-7e in modulating TLR4 receptor expression previously observed by Androulidaki A and colleagues (Androulidaki et al., 2009). IFNγ exposure led to the same produced CXCL10 amount in all cell types analyzed.

Therefore, miR-187 and miR-99b~7e~125a cluster activity were analyzed independently.

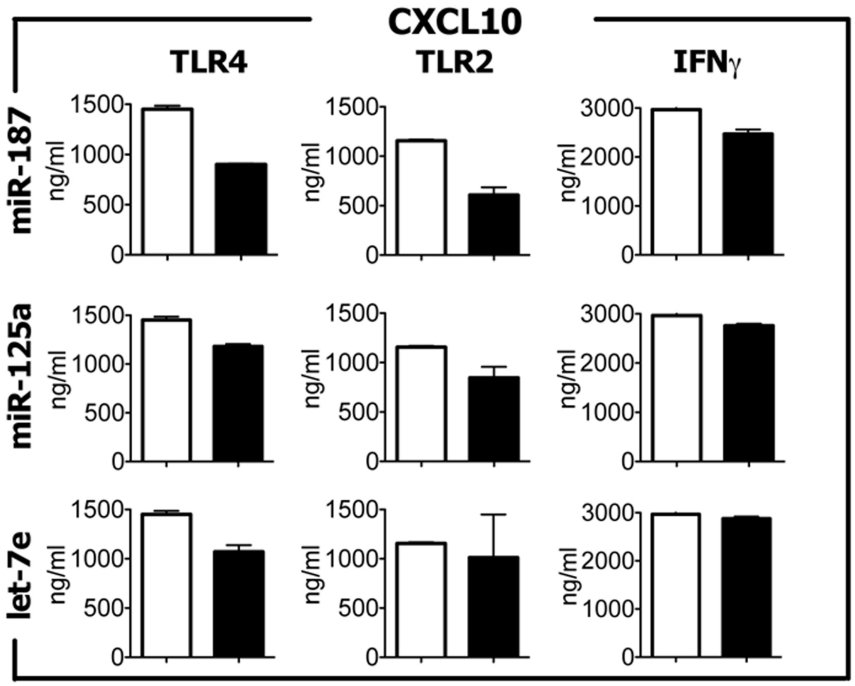

Figure 21: miR-187 or cluster-dependent inhibition of TLR4 or TLR2 pathway. THP-1 overexpressing miR-187, entire cluster or a scramble sequence were seeded and stimulated with 1 μg/μL LPS (TLR4), 1 μg/μL Pam_3CSK_4 (TLR2) or 100 ng/μL IFNγ. CXCL10 production level of THP-1 overexpressing scramble sequence (white bars) or indicated miRs (black bars) are here reported. Every experiment was carryed in duplicated. Datas show one of at least 2 individual experiments.

miR-187 inhibits IkBζ expression

Among the potential targets predicted by TargetScan used to performed the *in silico* analysis schematically represented in Figure 18, we focused our attention on inflammation-related transcription factor-encoding genes, as identified by GO-terms association, that have been frequently shown to be preferential miRNA targets (Asirvatham et al., 2008). This analysis indicated a potentially favourable interaction between the miR-187 and one 7mer site in the 3'UTR region of the transcriptional activator NFKBIZ transcript. Additional data indirectly supported the prediction of NFKBIZ as one of miR-187 target genes. In fact, the kinetic of expression of the NFKBIZ gene product, the IkBζ protein, inversely correlates with the expression of miR-187 in monocytes stimulated with LPS (Figure 22). This inverse correlation is even stronger in monocytes stimulated with LPS plus IL-10, in which miR-187 expression is potentiated, whereas the expression of IkBζ is further reduced, particularly at 12 and 24 h when this effect is the most pronounced. Luciferase assay

was utilized to confirm the predicted interaction of miR-187 with the 3'UTR of NFKBIZ transcript (Figure 23A). Luciferase activity is reduced by ~ 30% in HEK293 cells transfected with a luciferase reporter construct expressing the NFKBIZ 3'UTR together with mIR-187 mimic but not with a miR-scramble (miR-scr) used as control. Point mutation of the miR-187 seed region in the NFKBIZ 3'UTR abolished miR-187-mediated inhibition of luciferase activity (Figure 23A), demonstrating the specificity of miR-187-NFKBIZ 3'UTR interaction.

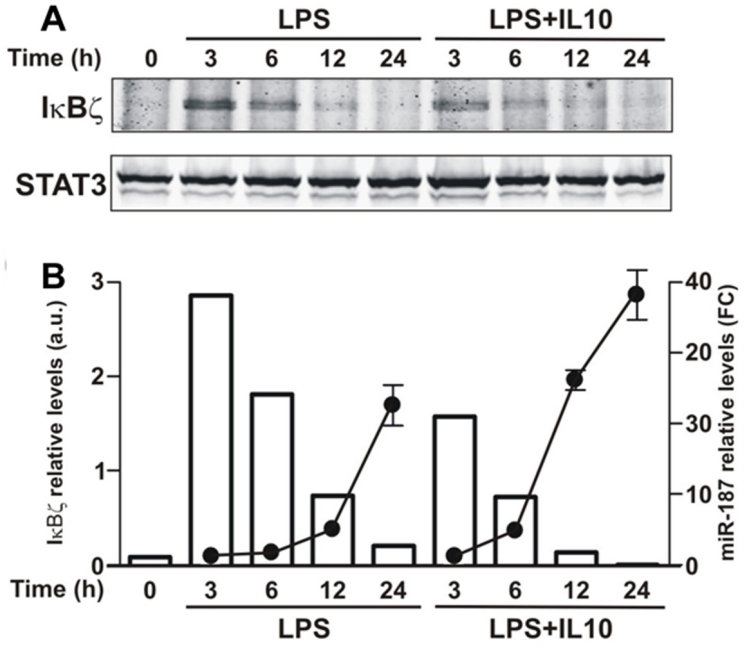

Figure 22: IκBζ protein levels down-modulation correlates with miR-187 up-regulation in stimulated human monocytes. Monocytes were cultured for the indicated times with LPS alone or in combination with IL-10. (A) Whole-cell extracts and small RNA fractions were purified in parallel as described in *Materials and Methods*. 100 μg proteins were loaded on gels and immunoblots were performed by using specific anti-IκBζ and anti-STAT3 Abs. (B) Quantitative analysis of the expression levels of IκBζ and miR-187. The relative IκBζ protein levels, quantified by the Odyssey software and normalized for the total STAT3, are reported as white bars, whereas black dots show the expression of miR-187 determined by RT-qPCR on small RNA fractions purified from the same samples. One experiment representative of three is shown.

In order to provide direct evidence for NFKBIZ as a physiologic target of miR-187, we tested whether miR-187 can directly influence the levels of the endogenous IkBζ protein expression (Figure 23B-E). A miR-187 mimic or a scramble negative control were transiently transfected in monocytes and the level of IkBζ protein expression following LPS stimulation was assessed (Figure 23B). Three hours after LPS stimulation, a reduction of the IkBζ protein levels was observed in

monocytes over-expressing the miR-187 mimic as compared to monocytes transfected with the miR-scr control (Figure 23B and C). In a complementary approach, we functionally inhibited miR-187 by transiently transfecting monocytes with a specific miR-187 single strand DNA-LNA knockdown probe (as-miR-187) or with a scramble miRNA knock-down probe (as-miR-scr) as a control. Reduction of miR-187 expression increases IkBζ protein levels in monocytes stimulated for 3 h with LPS and LPS plus IL-10, respectively (Figure 23D and 23E).

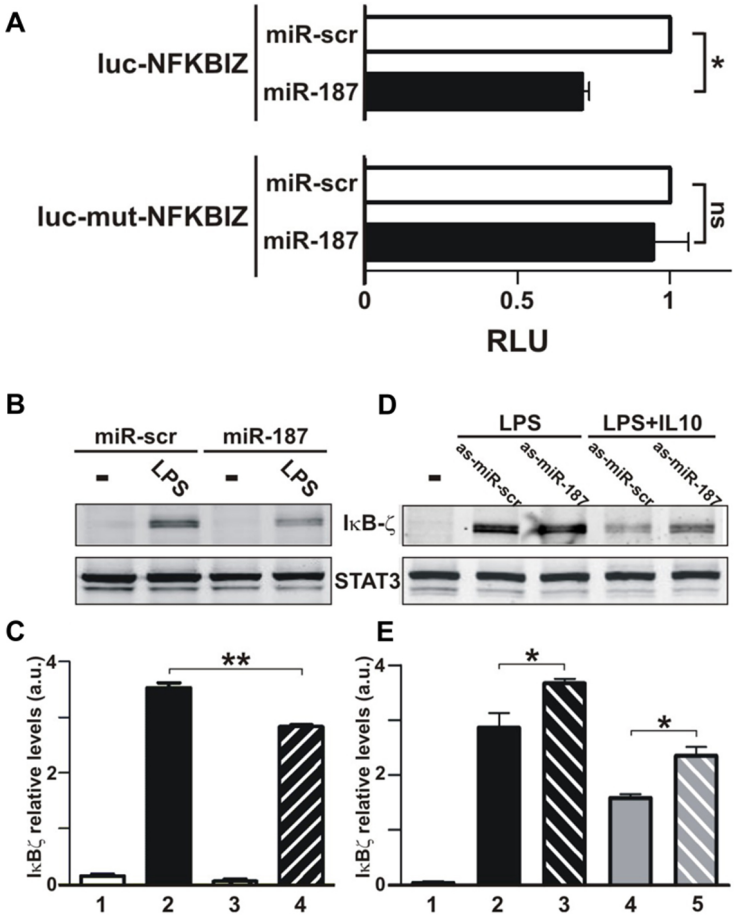

Figure 23: NFKBIZ is a predicted miR-187 target gene. (A) HEK 293T cells were cotransfected with the indicated luciferase constructs and 100 nM miR-187 mimics or scramble control. Results are expressed as mean (±SD, n = 3) of the ratio between renilla luciferase and firefly control luciferase activities (RLU), adjusted to 1. *: $P < 0.05$; ns: $P > 0.05$. (B) and (D) Total cell extracts (100 µg) were usually loaded on gels and immunoblots were performed by simultaneously using Abs specific for IκBζ and STAT3, followed by incubation with Alexa Fluor 680 goat anti-rabbit and IRDyeTM 800 goat anti-mouse Abs. One experiment representative of three is shown. (C) and (E) The blots were scanned on the Odyssey Infrared Imaging System at 700 nm and 800 nm wavelenght. The relative IκBζ levels (±SEM; n = 3), as quantified by the Odyssey software and normalized for the total STAT3 are reported (**: $P < 0.01$; *: $P < 0.05$).

Collectively, these data identify NFKBIZ as an endogenous miR-187 target and indicate the induction of miR-187 as one of the mechanisms utilized by IL-10 to suppress LPS-induced IkBζ expression.

IL-10-dependent IL-6 down-modulation occurs also through miR-187 activity on IκBζ

In mouse macrophages the nuclear protein IkBζ, also known as MAIL (Kitamura et al., 2000), is induced upon TLR/IL-1R stimulation and is required for the LPS-dependent induction of IL-6 (Kitamura et al., 2000; Seshadri et al., 2009; Yamamoto et al., 2004). We confirmed that human monocytes exposed to LPS up-regulate IkBζ expression which in turn triggers IL-6 production (Figure 24). A dose-dependent IkBζ knockdown by specific IkBζ-silencing siRNA (si-IkBζ) leads to a decrease in LPS-induced IL-6 production. Conversely, LPS-induced TNFα production in si-IkBζ- and si-ctrl-transfected monocytes are comparable (Figure 24D).

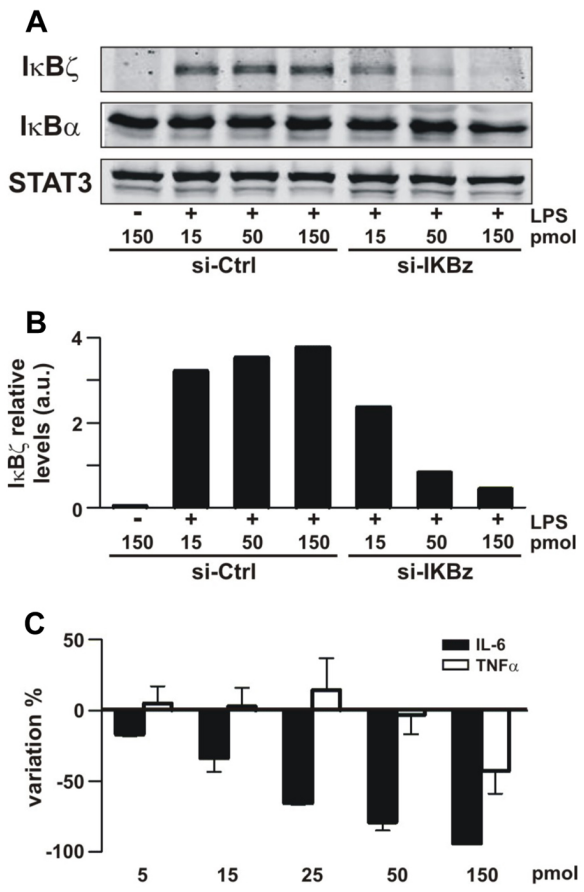

Figure 24: Knockdown of IκBζ suppresses the LPS-induced IL-6 production. Freshly purified monocytes were transfected with increasing amounts of si-IκBζ siRNA or with the same amount of siRNA control. The day after, monocytes were stimulated with LPS for 3 h, supernatants were collected and cells lysed in order to extract total RNA and proteins in parallel. 100 µg of whole cell extracts were analyzed by immunoblotting and the levels of IκBζ, IκBα and STAT3 were detected at the Odyssey Infrared Imaging System (A). Panel (B) show the levels of IκBζ normalized versus total STAT3, as detected in one experiment representative of three performed. IL-6 and TNFα were detected on cell-free supernatants by ELISA and the variation in cytokine release observed in monocytes transfected with si-IκBζ versus si-Ctrl were plottd in panel (C) (mean percentage ± SEM, n = 3)

To investigate whether the negative regulation of IkBζ by miR-187 was functionally involved in the production of IL-6 by LPS and in its regulation by IL-10, human monocytes were transfected with miR-187 mimic or with si-IkBζ along with their relative controls, and the expression of IL-6 was analyzed after 3 h of LPS activation (Figure 25). The reduction of IkBζ expression mediated by either miR-187 or si-IkBζ causes a significant and comparable reduction of the LPS-induced IL-6 transcription (Figure 25), thus demonstrating that miR-187 reduces IL-6 expression by knocking down the expression of its transcriptional activator IkBζ.

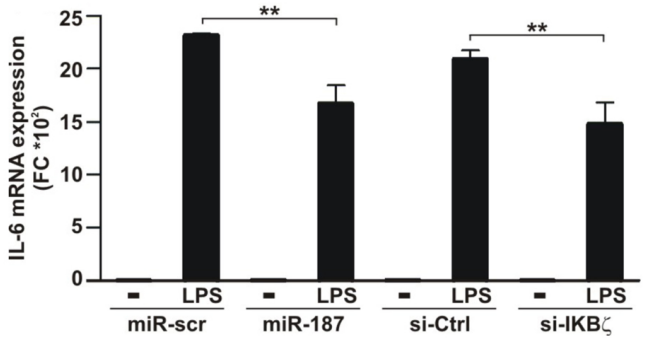

Figure 25: LPS-induced IL-6 gene expression is reduced by miR-187-mediated IκBζ knockdown. Monocytes were transfected with 150 pmol miR-scr or miR-187 mimic or 15 pmol si-Ctrl or si-IκBζ siRNA, let reover for 18 h, stimulated with LPS for 3 h, and then processed for IL-6 expression analysis. Total RNA was purified and the expression of IL-6 mRNA was analyzed by RT-qPCR as described in *Materials and Methods*. IL-6 expression is expressed as fold change after normalization to the GAPDH housekeeping. Means ± SEM are reported (n = 5, **: $P < 0.01$)

We then measured LPS-induced IL-6 production in supernatants of monocytes in which the IkBζ level was either knocked down by miR-187 over-expression or increased by as-miR-187 (Figure 26A-D). LPS-induced IL-6 production is reduced in monocytes over-expressing miR-187 as compared to monocytes transfected with miR-scr (Figure 26A), whereas miR-187 knockdown by as-miR-187 results in increased IL-6 production (Figure 26B). Finally, secretion of IL-6 by monocytes stimulated with LPS plus IL-10 is also affected by miR-187-dependent modulation of IkBζ protein level (Figure 26C). LPS-induced IL-10 production was not modified by any conditions (Fig. 26E-G), thus excluding a role for IL-10 in the inhibition and/or increase in LPS-induced IL-6. Collectively, these data demonstrate that IL-10, through the miR-187-mediated reduction ok IkBζ, indirectly inhibits LPS-induced IL-6 transcription and production.

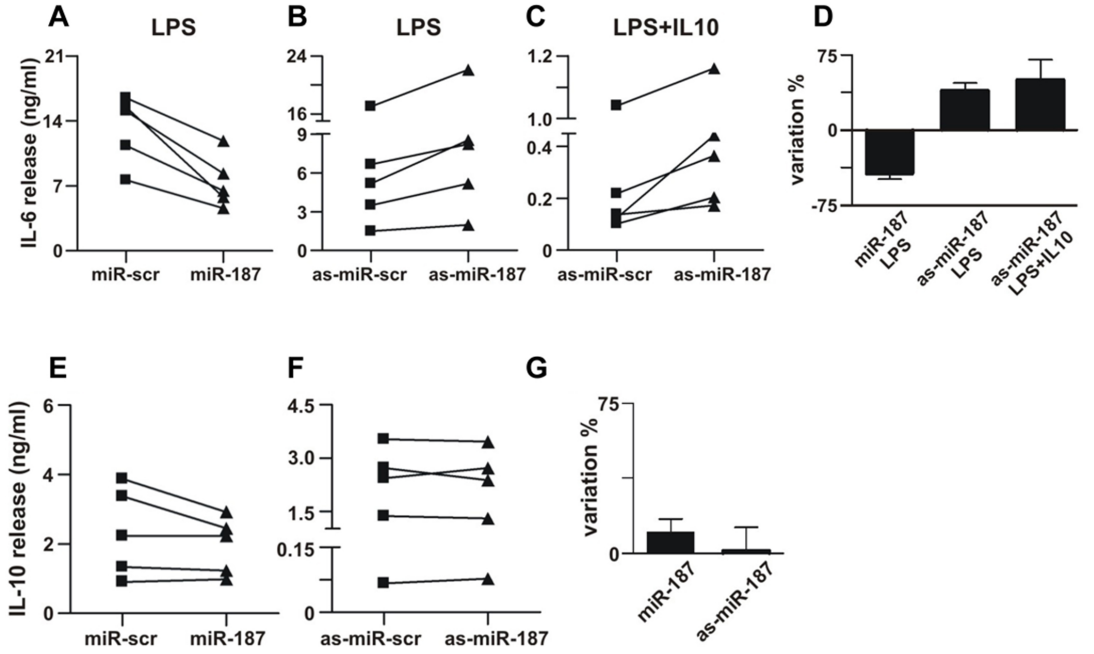

Figure 26: IL-6 production, but not IL-10 production, is affected by overexpression and/or silencing of miR-187. Monocytes were transfected with miR-187 mimic or as-miR-187, along with their respective miR-scr and as-miR-scr controls, as indicated. IL-6 levels were determined by ELISA in cell-free supernatants collected 24 h after stimulation with LPS alone (A, B) or with LPS plus IL-10 (C). In parallel, IL-10 levels were quantified only after stimulation with LPS alone (E, F). Changes in the levels of IL-6 (D) or IL-10 (G) release by miR-187-transfected versus mIR-scr-transfected monocytes and as-miR-187 transfected versus as-mIR-scr-transfected monocytes are shown (mean % variation ± SEM; n = 5).

miR-187 dampens inflammation through direct interaction with TNFα 3'UTR

Consistent with observation in macrophages from IkBζ knockout mice (Kitamura et al., 2000), the production of LPS-induced TNFα was not significantly modified in si-IkBζ-transfected monocytes (Figure 24D). Conversely, a marked reduction in TNFα production was observed in response to LPS in human monocytes over-expressing the miR-187 mimic, as compared to the miR-scr (Figure 27A and 27C). To exclude an off-target effect of miR-187 over-expression, we investigated whether miR-187 knockdown was affecting TNFα production as well. Monocytes transfected with as-miR-187 or with as-miR-scr were stimulated with LPS or LPS plus IL-10, and TNFα release was determined in cell free supernatant at 24 h. LPS-induced TNFα production was only slightly, but not significantly, affected by miR-187 knockdown (not shown). On the contrary, blocking miR-187 expression give rise to a significantly higher TNFα release in response to LPS plus IL-10 (Figure 27B and 27C). Taken together, these data point to TNFα as an additional miR-187

target. Indeed, a miR-187 seed was predicted in the 3'UTR of the TNFα transcript by both Miranda and TargetScan algorithms. To confirm TNFα mRNA as a direct target of miR-187, a reporter construct containing the Renilla luciferase gene fused to the TNFα 3'UTR (luc-TNFα) was transiently transfected into HEK293 cells together with miR-187 mimic or miR-scr. As shown in Figure 27D, miR-187 significantly reduced luc-TNFα luciferase activity, and the introduction of point mutations in the miR-187 seed in the TNFα 3'UTR (luc-mut-TNFα) reverted the inhibitory activity of miR-187, demonstrating that TNFα 3'UTR contains an active seed of miR-187. Collectively, these data demonstrate that miR-187 is part of the IL-10 dependent post-transcriptional control of TNFα.

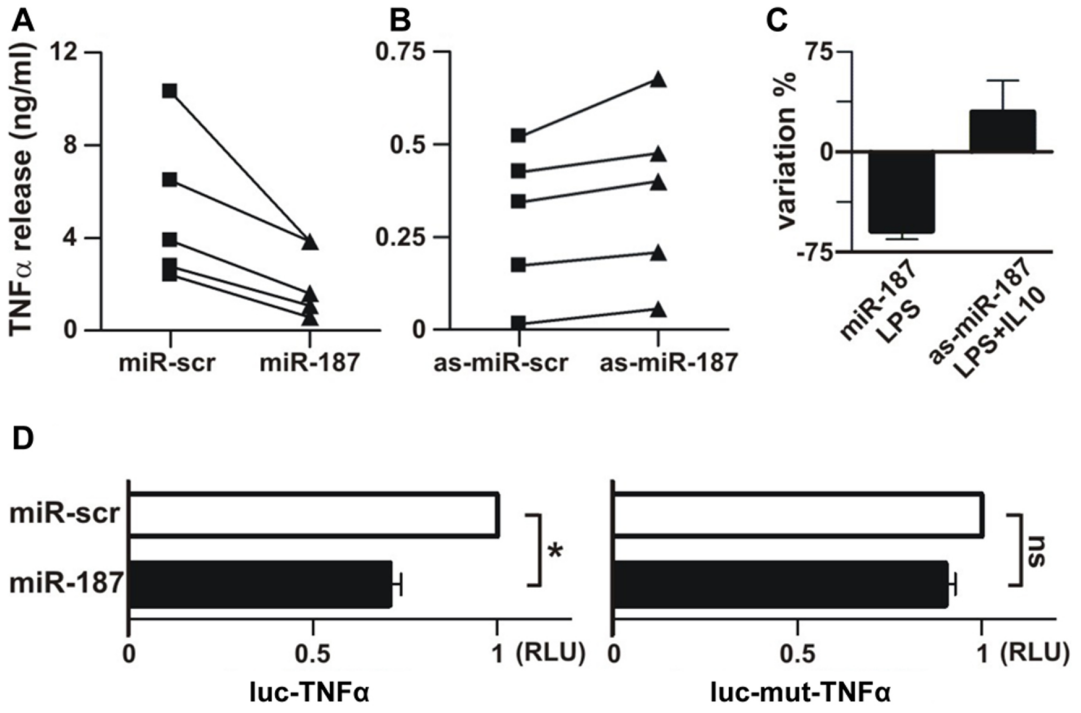

Figure 27: TNFα is a direct target of miR-187. The amount of TNFα was quantified by ELISA in cell-free supernatants collected after 24 h from monocytes transfected with miR-187 mimics or miR-scr and stimulated with LPS (A) or transfected with as-miR-187 or as-miRscr and stimulated with LPS plus IL10 (B). (C) Percent variation of TNFα released by monocytes transfected with miR-187 mimic or as-miR-187 versus their respective controls is shown (mean ± SEM, n = 5). (D) HEK 293T cells were cotransfected with the indicated luciferase constructs and 100 nM miR-187 mimics or scramble control. Results are expressed as mean (±SD, n = 3) of the ratio between renilla luciferase and firefly control luciferase activities (RLU), adjusted to 1. *: $P < 0.05$; ns: $P > 0.05$.

miR-99b~7e~125a cluster modulate cytokines and chemokines release in response to LPS

In parallel, we analyzed the activity of miR-99b~7e~125a cluster on TLR signaling, among receptor proteins or effector molecules. Analyzing the 3'UTR of TLR4, TLR2 or their co-receptors, we identified several seed regions recognized by cluster members in both TLR4 and CD14, a TLR4 signaling partner protein involved in recognizing pathogen-derived molecules that has been demonstrated as fundamental in TLR4-dependent signaling transduction (Ostuni et al., 2010).

Luciferase assays were performed to assess the activity of miR-125a and let-7e on TLR4 3'UTR, cotrasfecting miR-125a mimic, let-7e mimic or the scramble

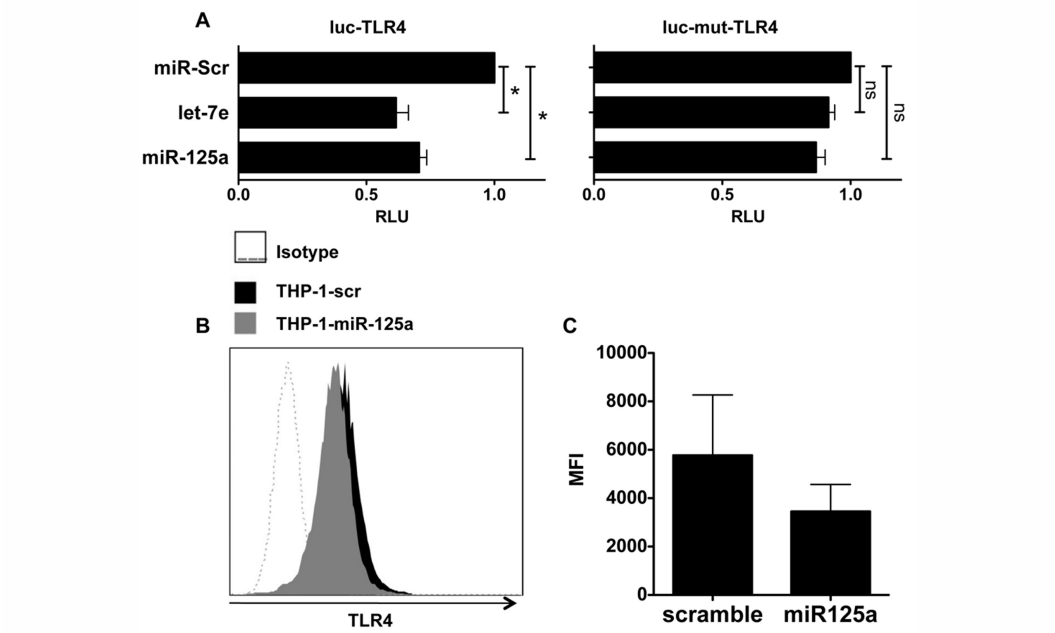

Figure 28: TLR4 recepor is a direct target of miR-125a. (A) HEK 293T cells were cotransfected with the indicated luciferase constructs and 100 nM miR-125a or let-7e mimics or scramble control. Results are expressed as mean (±SD, n = 3) of the ratio between renilla luciferase and firefly control luciferase activities (RLU), adjusted to 1. *: $P < 0.05$; ns: $P > 0.05$. (B) FACS analysis indicative of three indipendent experiments of THP-1 overexpressing miR-125a or a scramble sequence after staining with APC-conjugated anti-hTLR4 Abs is here reported. (C) Mean of the fluorescence intensity subctracted of the isotypic control obtained in three experiments is shown in the bar graph. *: $P < 0.05$; ns: $P > 0.05$.

control together with a reporter construct expressing *Renilla* luciferase coding sequence fused with TLR4 3'UTR in HEK293 cell line. Over-expression of miR-125a or let-7e inhibits luciferase expression as compared to the control. This inhibition was restored by mutating miR-125a or let-7e seed region respectively,

demonstrating the specificity of the observed down-modulation. These results confirm what has been already shown about let-7e ability in TLR4 regulation ((Androulidaki et al., 2009) and Figure 28C). To go deep in demonstrating the *in silico*-predicted and with luciferase assay validated miR-125a activity, TLR4 protein expression on miR-125a over-expressing THP-1 cells surface were investigated. As shown in Figure 28B and C, TLR4 translation was inhibited in miR-125a over-expressing THP-1 as compared with the control.

The same experiments were performed to assess the CD14 post-translational inhibition operated by miR-125a. As shown in Figure 29A, luciferase assay sustained the hypothesis of miR-125a activity on both TLR4 and its co-receptor CD14 3'UTR, inhibiting the *Renilla* luciferase – wild-type 3'UTR CD14 fused construct expression but not its mutated counterpart when cotrasfected together with miR-125a mimic. The same reduction amount was observed also at protein level, reducing CD14 receptor detection through FACS analysis on membrane of THP-1 over-expressing miR-125a as compared to control cells (Figure 29B).

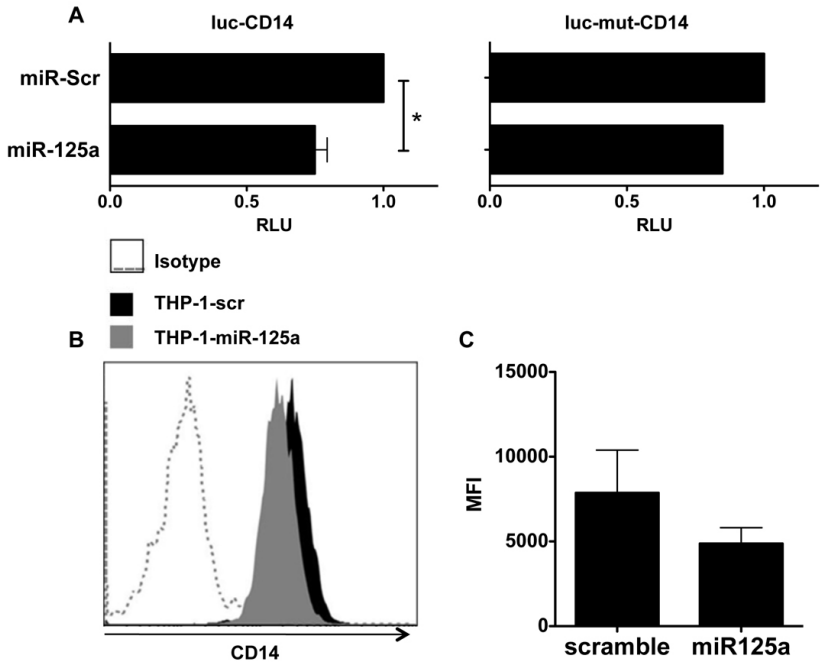

Figure 29: CD14 co-receptor protein is a miR-125a target gene. (A) HEK 293T cells were cotransfected with the indicated luciferase constructs and 100 nM miR-125a mimics or scramble control. Results are expressed as mean (±SD, n = 3) of the ratio between renilla luciferase and firefly control luciferase activities (RLU), adjusted to 1. *: $P < 0.05$; ns: $P > 0.05$. (B) FACS analysis indicative of three indipendent experiments of THP-1 overexpressing miR-125a or a scramble sequence after staining with APC-conjugated anti-hTLR4 Abs is here reported. (C) Mean of the fluorescence intensity subctracted of the isotypic control obtained in three experiments is shown in the bar graph. *: $P < 0.05$; ns: $P > 0.05$.

These results are consistent with the global impairment in TLR4 response observed in Figure 20 and 21 and suggest that miR-125a exerts post-transcriptional inhibition on both TLR4 receptor and its coreceptor CD14 by direct targeting their 3'UTR, leading to down-modulation of protein expression on monocytes surface and negative regulation of cell response to pathogens. In contrary, these data can not explain the impairment observed in TLR2-dependent cytokines and chemokines production.

To this aim, an *in silico* analysis of 3'UTR of all the cytokines and chemokine tested by ELISA (Figure 20 and 21) were conducted. Surprisingly, we found at least one seed region of cluster's microRNAs in every pro-inflammatory mediator tested, apart from CXCL10. We therefore decide to clone their 3'-untranslated regions and fuse each of them with *Renilla* luciferase gene in order to test miR-125a and let-7e ability in reducing luciferase expression and activity. As

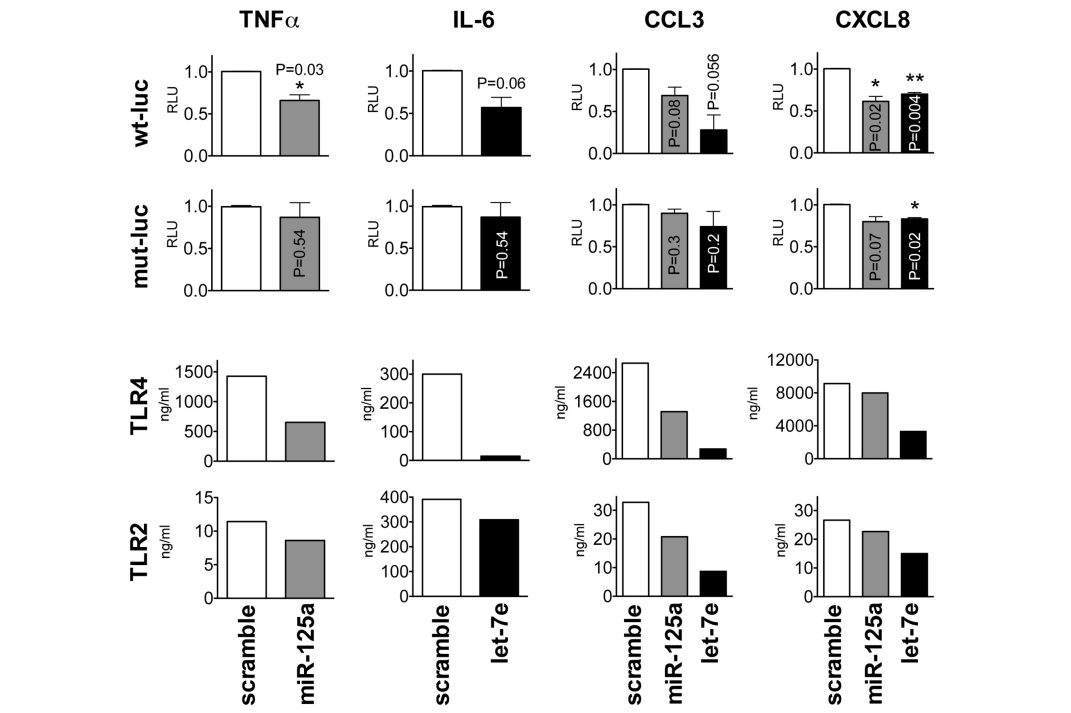

Figure 30: miR-125a and let-7e show a unique capacity in downmodulating several cytokines and chemokines mRNA. In panel (A) different combinations of target-miR luciferase reporter assays are shown. Indicated construct were cotransfected with 100 nM of indicated miR mimics or scramble control. Results are expressed as mean (±SD, n = 3) of the ratio between renilla luciferase and firefly control luciferase activities (RLU), adjusted to 1. *: $P < 0.05$; ns: $P > 0.05$. (B) THP-1 over-expressing indicated miR or scramble sequence were seeded and stimulated for 24 h with 1 µg/µL LPS (TLR4) or with 1 µg/µL Pam_3CSK_4 (TLR2). Cell-free supernatants were then collected and indicated cyokines and chemokines were quantified by ELISA. Datas from one single experiment are here shown.

shown in Figure 30A, luciferase assays confirmed miR-125a and let-7e inhibitory effects on CCL3 and CXCL8 3'UTRs, whereas TNFα 3'UTR was affected only by miR-125a and IL-6 by let-7e. The specificity of miR:3'UTR base-pairing was demonstrated by mutating the respective seed regions in every fusion gene construct. In order to confirm these and previous observations (Figure 20), the impairment in cytokines and chemokines production by THP-1 over-expressing single miR in comparison with control was investigated. As shown in Figure 30B, both miR-125a and let-7e over-expression in THP-1 led to a general TLR4 and TLR2 unresponsiveness as compared to the control, suggesting a multi-targeting activity on entire pro-inflammatory signaling cascade, from ligand engagement (TLR4 inhibition), to signaling transmission (not yet investigated) till pro-inflammatory mediators translation and production (Figure 30).

Glucocorticoids up-regulate miR-511, a product of MRC1 gene locus

miRNA genes are scattered in all chromosomes in humans except for the Y chromosome. Approximately 50% of known miRNAs are found in clusters (Lagos-Quintana et al., 2001; Lau et al., 2001; Mourelatos et al., 2002) and they are transcribed as polycistronic primary transcripts (Lee et al., 2002). Several analyses of miRNA gene locations showed that the majority (~70%) of mammalian miRNA genes are located in defined transcription units (TUs). It has been demonstrated that many miRNA genes were found in the introns in the sense orientation (Rodriguez et al., 2004). The major part of these intronic miRNAs are in the introns of protein-coding genes, whereas few miRNAs are in the introns of noncoding RNAs (ncRNAs). In some cases, miRNAs are present in either an exon or an intron depending on the alternative splicing pattern. So, miRNA genes can be categorized based on their genomic locations: intronic miRNA in protein coding TU, intronic miRNA in noncoding TU and exonic miRNA in noncoding TU (Kim and Nam, 2006).

Recently, several microarray analysis of LPS or alternative stimuli-induced gene expression in human monocytes, macrophages or neutrophils have been published by our group (Martinez et al., 2006; Recalcati et al., 2010; Tamassia et al., 2007). A collection of all these data was generated and a list of genes modulated in

response to pro- or anti-inflammatory mediators came out from this analysis. Taking advantage from Genome Browser database (http://genome.ucsc.edu/), we identified intronic miRNAs located in annotated genes in both protein coding and noncoding TU, confirming what have been already observed about miR-155 (O'Connell et al., 2007) and miR-146a (Taganov et al., 2006). In Figure 31A, a part of the entire list is reported.

Between all miRNAs listed, miRNA-511 leap at us as it is an intronic miRNA located in 5th intron of mannose receptor (known as MRC1 or CD206) (Figure 31B), a protein known to be expressed by monocytes and macrophages exposed to anti-inflammatory mediators (Fraser et al., 1998; Stein et al., 1992). It has also been shown to be up-regulated in tumour associated macrophages, as they acquire alternative polarization in tumour micro-environment (Allavena et al., 2010).

A

miR	Affycode	expression
hsa-miR-24-2	1555847_a_at	0,017328324
hsa-miR-215	217900_at	-1,787213867
hsa-miR-194-1	217900_at	-1,787213867
hsa-miR-553	203594_at	-1,456273723
hsa-miR-146a	232504_at	1,73605241
hsa-miR-155	229437_at	3,232527064
hsa-miR-9-1	205103_at	0,36848719

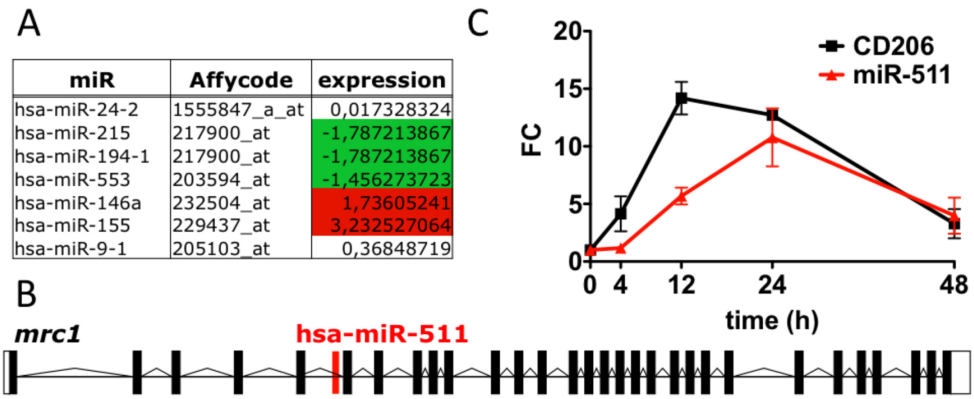

Figure 31: miR-511 is located in mannose receptor 5th intron and both locus products are expressed in monocytes in a glucocorticoid-induced manner. (A) The table here reported correlate a selection of 7 microRNAs and the affycode associated to their locus with expression variation in monocytes in response to LPS. Datas come from our pubblished observations. (B) A schematic representation of CD206/MRC1 gene locus is here reported. Squares represent exon (untranslated regions in white, coding sequence in black), triangles represent introns, red line represents miR-511 as indicated. (C) Freshly isolated monocytes were stimulated with 10^{-5} M dexamethasone and collected at indicated time points. Total RNA was extracted and miR-511 and CD206 (MRC1) expression was analyzed by RT-qPCR as indicated in *Materials and Methods*. Results are reported as fold change respect to unstimulated cells after normalization on snRNU6 levels.

Among several anti-inflammatory cytokines responsible of mannose receptor gene induction (Martinez et al., 2009), glucocorticoids have also been reported to induce MRC1 gene expression *in vitro* in human peripheral blood monocytes after 16-48 h exposure (Hogger et al., 1998; van der Goes et al., 2000). Therefore, expression of both MRC1 gene and miR-511 were tested in RNA extracted from

human peripheral blood monocytes treated with glucocorticoids (Dexamethasone/DEXA) at different time points. As shown in Figure 31C, both microRNA and its host gene were significantly up-regulated already after 12 h of stimulation.

In silico analysis predicted and functional assays confirmed TGFβ signaling cascade affection by miR-511

The most peculiar feature of microRNAs is that a single miR interacts and consequently modulates large panel of targets. An analysis similar to that applied on LPS-dependent IL-10-potentiated microRNAs putative targets genes with the Ingenuity Pathway software (IPA) was performed on the miR-511 targets list generated by TargetScan. We identified a list of signaling pathways "affected" by miR-511. In this list, the most represented pathway was the TGFβ signaling pathway, with 25 different predicted target molecules (highlighted in red in Figure 32).

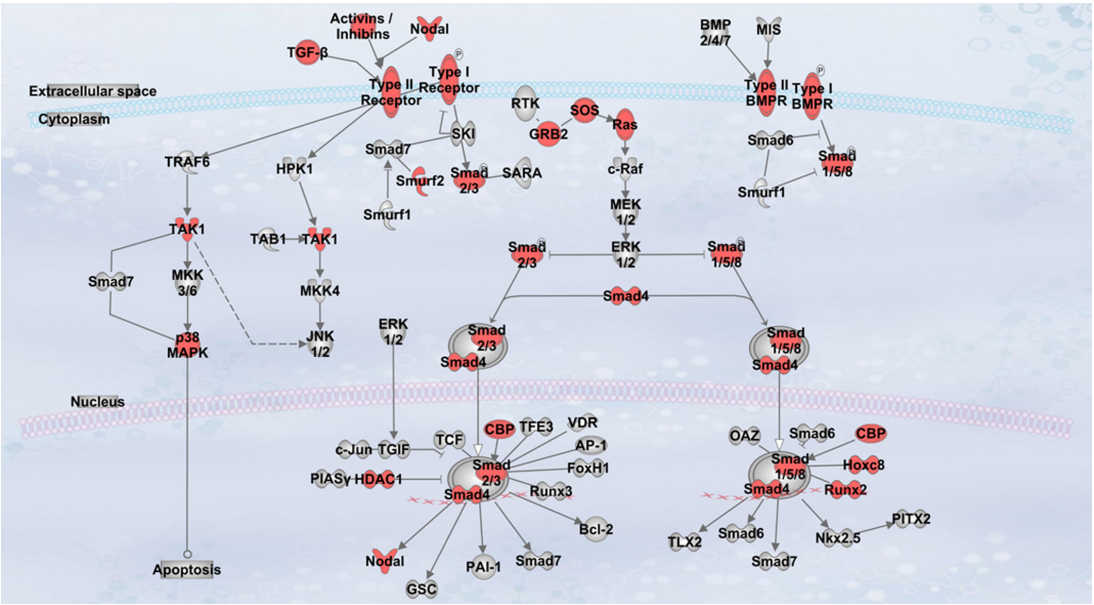

Figure 32: miR-511 targeting of TGFβ signaling pathway. A list of putative target molecules of miR-511 generated by TargetScan software was integrated with IPA "canonical pathways" informations. A schematic representation of TGFβ signaling pathway is reported. Putative targets of miR-511 are highlighted here in red.

In order to verify *in silico* analysis prediction, we generated a cellular model by infecting the human monocytic cell line THP-1 with lentiviral particles sustaining constitutive expression of miR-511 or a scramble sequence as a control together with EGFP (Figure 19A). The constitutive expression of EGFP protein and microRNA-

511 was tested by FACS analysis (not shown) and qRT-PCR (TaqMan microRNA Assay, Figure 33A), respectively. We then performed some cell-based assays to test the ability of infected THP-1 to respond to TGFβ, such as apoptosis assay (Figure 33B) and gene expression analysis (Figure 33C). In the former, THP-1 cells were exposed to two different apoptosis inducing stimuli, such as starvation (RPMI without FBS) or 10 ng/ml TGFβ. THP-1 that constitutively produced microRNA-511

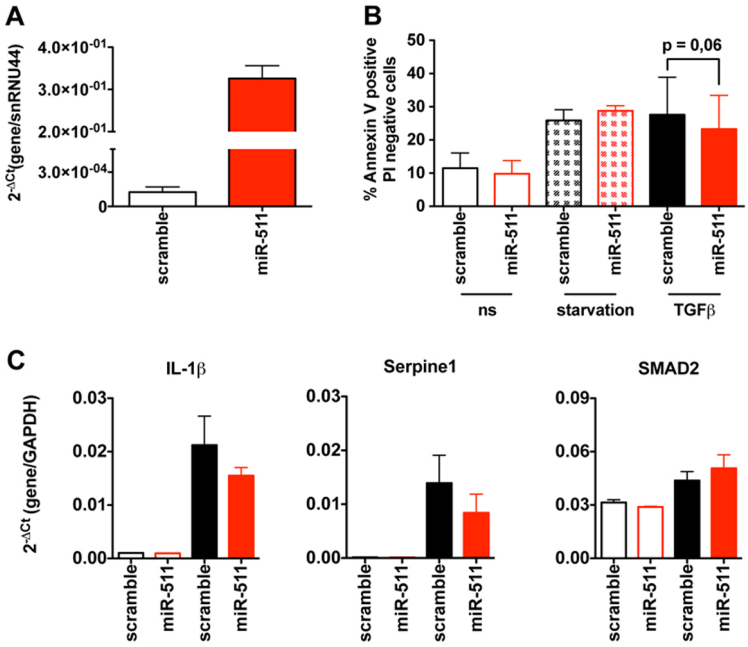

Figure 33: miR-511 dampens TGFβ cellular response in THP-1 over-expressing cells. (A) THP-1 infected with lentiviral particle carrying miR-511 genomic region or scramble sequence were lysed with TRIZOL reagent and total RNA was extract. miR-511 expression levels was analyzed by RT-qPCR as indicated in *Materials and Methods*. Datas were normalized with $2^{-\Delta Ct}$ method on snRNU6 levels as housekeeping. (B, C) THP-1 over-expressing miR-511 or a scramble sequence were starved and then seeded in normal medium, in medium without FBS or with 10 ng/µL TGFβ for 48 h. (B) Apoptosis grade were analyzed with Annexin-V staining. FACS analysis was performed. Results are shown here as percentage (mean ± SEM, n = 3) of annexin-V positive and 7-AAC negative cells (early apoptotic cells). (C) Total RNA was extracted and genes expression was analyzed as described in *Materials and Methods*. Datas were normalized with $2^{-\Delta Ct}$ method on GAPDH levels as housekeeping. Empty bars represents unstimulated cells, fill bars cells stimulated with 10 ng/ml TGFβ.

showed a lower amount of apoptotic cells (Annexin V positive and PI negative cells) only if stimulated with TGFβ, whereas starvation induced similar apoptosis grade in both infected cell types. In the latter experiment, IL-1β and Serpine1 mRNA were measured as indicators of TGFβ induced gene expression, whereas SMAD2 mRNA was considered an internal control, as its expression does not change after TGFβ exposure. As shown in Figure 33C, THP-1 that constitutively express miR-511

displayed a gene expression impairment if compared to the control. Taken together, these data suggest that THP-1 over-expressing miR-511 showed a diminished TGFβ-induced cellular responses.

After TGFβ receptor activation, SMADs protein are phosphorylated and eterodimerize with SMAD4 to translocate in the nucleus and start gene transcription (Ten Dijke et al., 2002). TGFβ receptor cross-phosphorylation also causes engagement of TGFβ activated kinase 1 (TAK1) that works as a bridge between classical TGFβ signaling pathway and MAPKs cascade (Sakurai et al., 2002). We therefore performed phosphorylation assays by western blot analysis on control THP-1 versus miR-511 over-expressing THP-1 to clarify which harm of TGFβ signaling pathway is most affected by the presence of miR-511 (Figure 34). As highlighted in Figure 34A and 34B, miR-511 over-expression in THP-1 monocytic cells completely abolished MAPKs activation, whereas slightly reduces SMAD2 phosphorylation (Figure 34C). SMAD3 activation were not affected by miR-511 presence in the cells. Taken together, all these data suggests that miR-511 over-expression in the monocytic cell line THP-1 attenuates TGFβ signaling and its biological activity, operating a strong inhibition of SMAD-independent signaling pathway.

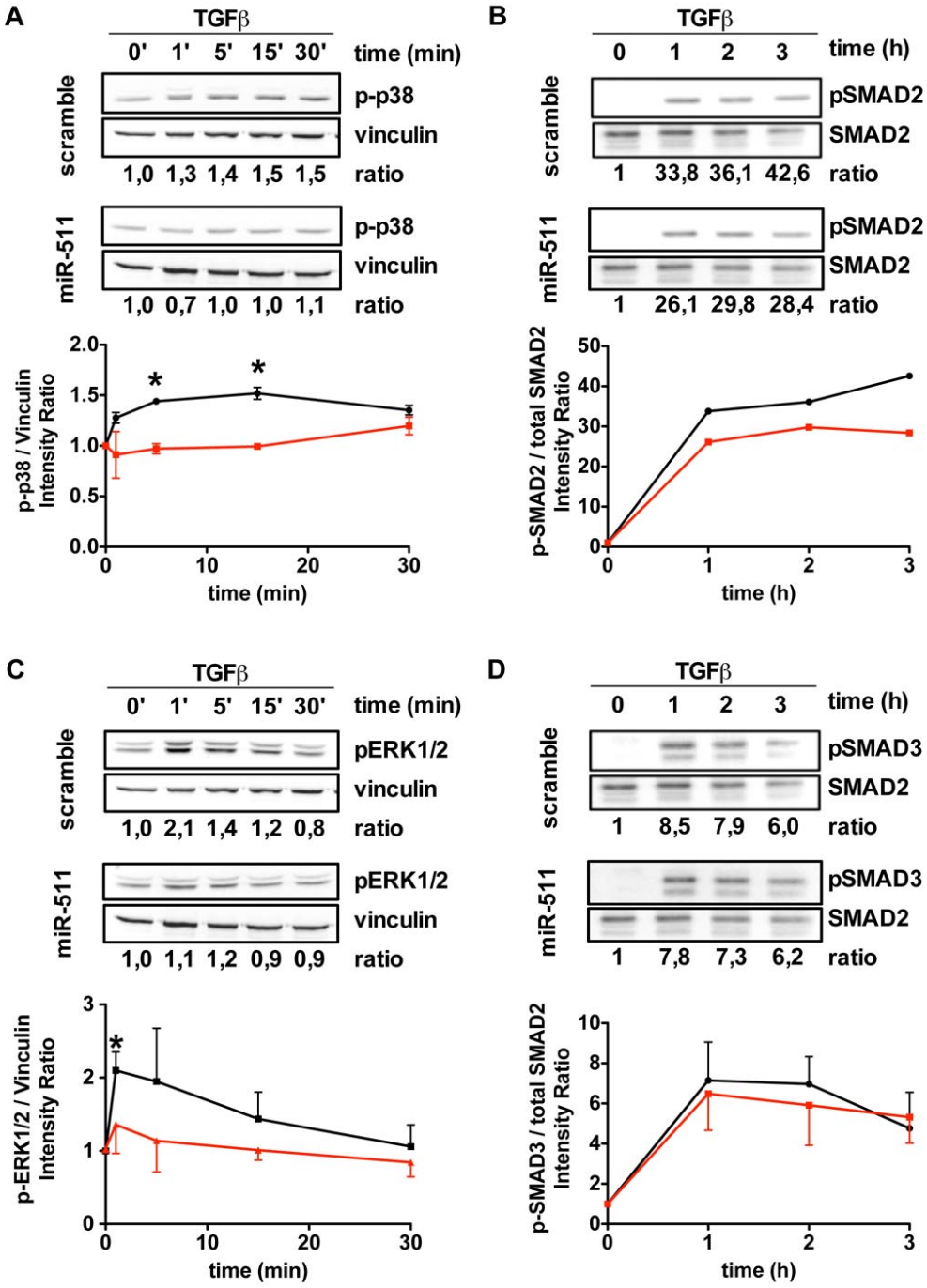

Figure 34: **miR-511 inhibits Erk and p38 MAPK activation but not Smad2 or Smad3 phosphorylation.** THP-1 over-expressing miR-511 or a scrable sequence were stimulated at indicated time points with 10 ng/µL TGFβ. 35 µg of whole-cell extracts were then loaded on a SDS-PAGE gel and membrane immuoblotted with anti-phospho-p38 Abs (A), anti-phospho-ERK1/2 (C) and anti-vinculin Abs (A, C), anti-phospho-Smad2 (B), anti-phospho-Smad3 (D) and anti-total Smad2 (B, D). ECL was then added and proteins detected by chemioluminescence. ImageJ software was then used to quantify the chemioluminescence intensity. Datas are presented as ratio between indicated protein and housekeeping. Intensity Ratio was calculated as mean ± SEM of three independent experiments (A, C), of one single experiment (B) or two independent experiments (D).

Discussion and future perspectives

As already discussed here and as this work demonstrated in several ways, microRNAs can be considered as master regulators of innate immune cells function. Starting from the seminal work of Taganov and colleagues (Taganov et al., 2006), it was clear to all scientific community that microRNAs play an important role in modulating and tuning every step in inflammatory reaction.

In this work we firstly described TLR4-dependent modulation of microRNA profile in two primary innate immunity cell types, represented by monocytes and neutrophils. A low-density TaqMan-based array was performed on monocytes and neutrophils stimulated for 8 h with LPS and microRNAs expressed by stimulated cells were compared to unstimulated controls. This experiment confirmed miR-155, miR-146a and miR-132 expression induction in human peripheral blood monocytes, previously observed by Taganov et al. in the THP-1 human monocytic cell line (Taganov et al., 2006), but also identified miRNAs never reported before in this context, including miR-187, the miR-99b~7e~125a cluster, miR-9/9*, miR-135a and miR-222 in monocytes, and miR-196a and miR-9/9* in neutrophils. No down-regulated miRs were observed. In order to confirm our analysis and to better characterize miRs induction, cells were stimulated and miR expression singularly analysed at different time points. All up-regulated miRs were confirmed, except for miR-135a and miR-222 in monocytes and miR-196a in neutrophils, and all of them showed a relatively slow kinetic, with the remarkable exception of miR-155, which appear as the only miR greatly induced at very short (2 h) time-point after LPS stimulation.

This experiment identified miR-9/9* as the only LPS-induced microRNA in both monocytes and neutrophils. miR-9 was firstly observed as a fundamental regulator in neurons development, where it is involved in a double negative feedback loop with the transcription factors REST and CREB, the first responsible of inhibition of neurogenesis and direct target of miR-9 and the latter implicated in inducing miR-9 and, thus, neural development (Smirnova et al., 2005; Wu and Xie, 2006). The fundamental role of miR-9 in the nervous system development was then confirmed by the phenotype of a miR-9 knockout mouse (Shibata et al., 2011). A role for miR-9 in immune system was also already reported, as it was found to down-regulate the transcription factor PRDM1/Blimp-1 in Hodgkin/Reed-Sternberg cells,

thus interfering with normal B cell terminal differentiation and contributing to the pathogenesis of Hodgkin lymphoma (Nie et al., 2008). Finally, miR-9 misregulation was observed in several tumours. Nevertheless, our identification of miR-9 as an LPS-responsive miRNA in human PMN and monocytes represents the first evidence linking miR-9 to the innate immune response.

To further investigate miR-9 biology in the context of innate immunity, its regulation was then investigated. In human monocytes and neutrophils, TRL4 engagement triggers different signaling cascades depending on the MAL/MyD88 or TRAM/TRIF adaptor molecules, the first being responsible of activation of NF-κB and MAPK signaling cascade, the latter recognized as type I IFN expression activator. Different TLRs can activate MyD88 with (TLR2) or without (TLR7/8) MAL scaffolding property or can selectively signal through TRAM/TRIF adaptors (TLR3). We observed that agonists inducing TLR2 or TLR7/8 triggering are also able to up-regulate miR-9 expression, whereas TLR3 agonists are not. In line with previous observations, TLR3 induced a signaling pathway responsible for miR-155 expression only in monocytes, as neutrophils do not express TLR3 receptor. miR-9 expression was also activated stimulating monocytes and neutrophils with TNFα and IL-1β. These observation, together with the late kinetic in miR-9 expression, argued against the possibility of a TNFα-dependent autocrine loop acting on monocytes and neutrophils, as previously reported for miR-155 (O'Connell et al., 2007), and this hypothesis was definitively put aside when incubation of monocytes with anti-TNFα antibody completely blocked TNFα-induced but not LPS-induced miR-9 expression. TNFα and TLR engagement are both responsible of NF-κB and MAPK activation, though through different signaling pathways. Our data suggested the possibility that miR-9 expression was NF-κB and/or MAPK activation-dependent. Consistent witht this, pre-incubation of monocytes and neutrophils with three different NF-κB inhibitors (MG-132, BAY-117082 and PDTC) completely blocked miR-9 induction, while inhibition of p38 (SB-203580) or JNK (SP-600125) were ineffective, demonstrating that NF-κB-activation is required for miR-9 up-regulation.

In the human genome, three different pri-miR-9 precursors exist, encoded as intronic miR by three different genes. We investigated all three potential precursors and demonstrated that LPS exclusively induced transcription of the C1orf61 gene,

using two different transcriptional units called CROC-4a and CROC-4b equally contributing to miR-9 induction in response to LPS. Thus, LPS induce production of miR-9-1 by two transcripts derived from the C1orf61 gene. Of note, the role of miR-9 in the development of nervous system has recently been ascribed to miR-9-2 and miR-9-3, while miR-9-1 was found not to be expressed in the CNS. Thus, the three miR-9 encoded by the human genome have evolved distinct functions in different tissues and biological processes.

microRNAs mechanism of action is now well characterized. Several studies analysed microRNA-induced transcriptome and proteome variations (Selbach et al., 2008), concluding that a single miR can modulate hundreds of targets in the cell. This result is due not only to the degenerated nature of miRNA-mRNA target recognition, as only 6-8 nucleotides are sufficient to guarantee miRNA-dependent inhibition, but also to the evolutionarily dictated ability of miRNAs to preferentially target transcription factors (Asirvatham et al., 2008). These observations, together with the already demonstrated ability of miR-9 to inhibit transcription factors involved in neurogenesis (Wu and Xie, 2006), drove us to concentrate our attention on transcription factors predicted to be targets of miR-9. Among these, the NF-κB1 molecule, a Rel family member involved in NF-κB transcription activity. In particular, NFKB1 mRNA translates for two different isoforms, a full-length p105 isoform and a smaller protein of 50 kDa (p50). As p105, NF-κB1 has inhibitory function, acting like an IκB protein. Once processed, p50 can heterodimerize, usually with p65, to induce gene expression. In the cell, the ratio between p105 and p50 designs its function. A seed region of miR-9 is present in the 3'untranslated region of NFKB1 mRNA and its activity was tested in a luciferase assay on both wild-type and mutated form of the 3'UTR, confirming strong miR-9 activity and binding specificity.

After LPS exposure, human monocytes induce a wide transcription activation, in part due to NF-κB activation. We demonstrated that also NFKB1 mRNA levels are enhanced as a part of this transcriptional program, but unexpectedly the NF-κB1/p105 protein amount did not change. This expression pattern completely fits with microRNA-induced gene expression regulation, as they are known to be responsible in maintenance of cellular homeostasis avoiding

undesired protein over-expression. miR-9-induced inhibition of p105/p50 protein translation was therefore tested transfecting miR-9 expressing plasmid or its control in freshly purified human monocytes, establishing its negative control on NF-κB1 protein translation.

In summary, TLRs engagement induces a wide change in gene transcription, particularly due to NF-κB family members activation. As NF-κB1 can have different functional effects based on its relative expression in the cell, its levels must be finely regulated. p105 itself was observed working as IκB family proteins. p50 homodimers, lacking a transciption activation domain, function as repressors, whereas heterodimers act as transcription activators. On the basis of our observation, miR-9 expression is one of the mechanisms that both monocytes and neutrophils may use to manage p105 production and therefore p105/p50 ratio in the cells, thus allowing the proinflammatory phase of the LPS response to correctly proceed.

IL-10 is a key molecule in the control of an inflammatory response and it is among those genes overexpressed in monocytes exposed to pro-inflammatory stimuli. Its anti-inflammatory properties, together with its prompt release after TLR triggering, make it one of the most important autocrine negative feedback mechanism in resolving acute inflammation. Recently, an IL-10-dependent miR-155 expression inhibition was observed in murine macrophages (McCoy et al., 2010). IL-10-induced transcription variation of LPS-induced miRNAs was therefore tested. Our data support a complex modulation of LPS-induced microRNA exerted by IL-10. miR-155 expression inhibition was confirmed also in freshly isolated human monocytes, together with the down-modulation of miR-146a and miR-9. By contrast, miR-146b, miR-99b~7e~125a cluster and miR-187 are strongly induced by costimulation of LPS and IL-10, and miR-146a is induced also by IL-10 itself.

To understand whether LPS-induced miR-187 and miR-99b~7e~125a cluster induction was due to the autocrine effect of IL-10 on monocytes, an inhibition of the IL-10 receptor was obtained by pre-treating monocytes with an IL-10R-blocking antibody before LPS stimulation. Avoiding IL-10 triggering of its receptor, miR-187 strong induction was completely blocked and a similar effect on miR-99b~7e~125a cluster was observed. Conversely, LPS effect on miR-155 was potentiated,

confirming the inhibitory role of IL-10 on this miR. While miR-187 is generated by processing of a precursor located in an intergenic region on chromosome 18, the miR-99b~7e~125a cluster comes from a common precursor located upstream a long non-coding RNA (UNQ2487). Expression analysis of these microRNAs and their neighbour ncRNA point to their co-expression, but further experiments, such as expression kinetic, are required.

In 2010, in a brilliant review on miRNA regulation of transcription, Inui and colleagues suggested that rather than looking for miRNA-target pairs to predict miRNA biological finctions, researchers should ask which biological processes might be prime candidates for miRNA-mediated regulation. In this perspective, signaling complexes are highly dynamic and are able to translate stimuli into well-established dose-dependent responses. Hence, signal transduction pathways result as prime candidates for miRNA-mediated regulation in animal cells, as the multi-targeting capacity of miRNAs make them able to remodel the signaling landscape, facilitating or opposing the transmission of information to downstream effectors in a desirable manner. Taking advantage of the on-line software TargetScan to predict miRNA targets and of the Ingenuity Pathway (IPA) software to allocate genes in well-established signaling pathways, a list of signaling cascades affected by miR-187 and miR-99b~7e~125a cluster was generated. Among several signaling pathways, the TLR signaling pathway emerged for the multi-targeting activity of these miRs. Subsequent experiments confirmed the capacity of both miR-187 and the cluster to impact on the acute inflammatory response, as the THP-1 monocytic cell line transduced with miR-187 or the cluster showed inhibition of TLR4 and TLR2-dependent pro-inflammatory cytokines (TNFα and IL-6) and chemokines (CCL3 and CXCL8). Similar results were observed stimulating miR-99b~7e~125a cluster overexpressing THP-1 with TLR4 and TLR2 agonists and IFNγ using CXCL10 production as a read-out. CXCL10 was previously shown to be a potent effector of IFN-antiviral response (Qi et al., 2009) and therefore produced by THP-1 cells also in response to both TLR agonists and type II interferon. Confirming its role as negative regulator of reaction against invading pathogens, miR-187 over-expression inhibited TLR4 and TLR2-dependent but not IFNγ-induced CXCL10 production. In parallel, over-expression of miR-125a and let-7e exerted different function in

response to these stimulations. Indeed, miR-125a show the same capacity of the entire cluster overexpression in impairing TLR2 and TLR4-induced response, whereas let-7e over-expression influenced only TLR4 signaling pathway, as already published (Androulidaki et al., 2009), demonstrating to be ineffective in inhibiting TLR2-dependent CXCL10 production. As for miR-187, IFNγ stimulation was not affected by miRs overexpression. Further experiments are needed to verify miR-99b contribution in miR cluster global impairment of inflammatory stimulations.

As for miR-9, the list of miR-187 putative targets was screened in search of transcription factors known to be involved in the inflammatory process. Among these, the atypical member of NF-κB inhibitors IκBs family IκBζ attracted our attention. IκBζ has been shown to be required for LPS-dependent IL-6 transcription and production (Kitamura et al., 2000; Seshadri et al., 2009; Yamamoto et al., 2004), and its prompt induction after LPS triggering is negatively modulated by IL-10. Several lines of evidence suggested miR-187 as a regulator of IκBζ expression and function. First, we observed an inverse correlation of miR-187 and NFKBIZ mRNA expression. Second, miR-187 overexpression inhibited translation of the IκBζ 3'UTR reporter construct, demonstrating binding specificity as deletion of miR-187 seed region completely restored the expression of the reporter gene. Third, forced expression of miR-187 in freshly purified human monocytes significantly inhibits LPS-dependent IκBζ up-regulation. Fourth, in a complementary approach, inhibition of the LPS- or LPS plus IL-10-dependent miR-187 expression induces IκBζ up-regulation. Fifth, transfection of miR-187 mimic effects on IL-6 transcription and production overlap with knock down of IκBζ with siRNA technology, while it has no effect on IL-10 production, demonstrating the specific activity of miR-187 on IκBζ protein.

The miR-187-driven inhibition of IκBζ, however, is not sufficient to explain the global impairment in TLR response observed in miR-187 overexpressing THP-1 cells. This phenotype can be partially explained by the proven capacity of miR-187 to directly target TNFα mRNA and therefore inhibit its production, here uncovered by using luciferase reporter assay and transfecting freshly purified monocytes both with miR mimic and with anti-sense miR. TNFα has been already shown to induce a great number of pro-inflammatory events, including activation of key transcription

factors such as NF-κB, AP-1 and p38 activation, all fundamental for transcription activation and production of inflammatory cytokines and chemokines (Aggarwal, 2003). Therefore, an inhibition in TNFα release can easily result in abrogation of a fundamental positive feedback loop in the production of inflammatory mediators.

Our data suggesting a significant role of the miR-99b~7e~125a cluster in acute inflammatory response prompted us to further investigate the role of these molecules in the immune system. As far as let-7 family members, 13 genomic loci, some of which clustered together have been identified, but as some of these loci encode identical mature miRNAs the number of distinct mature let-7 miRNAs stands at ten: let-7a through let-7i plus the related miR-98 and miR-202. The mature let-7a is identical across animal species from C. elegans to humans, while the other family members share the seed sequence but differ in the remaining nucleotides to varying extents. These miRs are expressed in various different adult tissues. In contrast to their expression in differentiated tissues, mature let-7 family members are absent in human and mouse embryonic stem cells or pluripotent cell populations, and increasing expression upon differentiation seems to be a common theme (Bussing et al., 2008). Recent publications sustain a role for let-7 members in the immune system, as some members of these family are regulated in several inflammatory contests (Androulidaki et al., 2009; Chen et al., 2007b; Satoh et al., 2011; Sharbati et al., 2011; Zhang et al., 2011), some members were shown to directly regulate TLR4 receptor expression, thus modulating macrophage and dendritic cell responses to LPS (Androulidaki et al., 2009; Chen et al., 2007b; Satoh et al., 2011), and let-7b and let-7i were shown to modulate cytokine production by direct targeting IFNβ and SOCS1, respectively (Sharbati et al., 2011; Zhang et al., 2011). Conversely, a poor literature is presently available on the miR-99 family members, miR-99a and miR-99b, which seem to be involved in positive or negative regulation of tumour development, depending on cancer progression and types (Witwer et al., 2010). Also the miR-125a was first identified as an oncosuppressor miRNA, as several study reported its capacity to induce apoptosis or inhibit metastatic potential of cancer cells (Guo et al., 2009; Li et al., 2009); (Jiang et al., 2011; Wang et al., 2009), and a polimorphism located in the eighth nucleotide of the mature miR form was associated with breast cancer risk in different countries (Peterlongo et al., 2011).

Very recently, miR-125a was also linked to macrophage biology, as it is up-regulated in response to different pathogens (Monk et al., 2010; Schnitger et al., 2011) and has been shown to play a non-redundant role in hematopoiesis (Gerrits et al., 2011; Guo et al., 2010). Notably, Zhao and colleagues observed an increased production of CCL5 in the contest of systemic lupus erythematosus and linked this phenomenon to the up-regulation of KLF13, a transcription factor responsible of CCL5 transcription, as a consequence of miR-125a down-modulation (Zhao et al., 2010). Also the other miR-125 family member, miR-125b, is well known to be involved in cancer and immunity biology. First identified as involved in cancer regression (Ozen et al., 2008; Visone et al., 2007), growing evidence indicate miR-125b as a master regulator of both transcription factors (Bak1, Stat3, IRF4; (Chaudhuri et al., 2011; Surdziel et al., 2011) and immune mediators (TNFα; (Tili et al., 2007). Curiously, miR-125b is down-modulated by LPS, opposite to our observation of miR-125a being induced (Androulidaki et al., 2009; Tili et al., 2007). As the cluster product with the highest predicted amount of targets involved in the immune response, our attention was concentrated on miR-125a. A seed region for miR-125a was detected both in TLR4 and in its co-receptor CD14 3'UTRs and luciferase reporter assays demonstrated activity and binding specificity to these two regions. FACS analysis of miR-125a overexpressing THP-1 monocytic cells confirmed the negative regulation exerted by this microRNA on the two receptor molecules. TLR4 inhibition, however, is not sufficient to explain the miR-125a-induced impairment in both TLR2 and TLR4 cellular response. In 2007, Tili and colleagues demonstrated in murine macrophages how miR-125b and miR-155 collaborate in negatively and positively regulating TNFα mRNA translation, respectively. Our experiments confirmed the ability of miR-125a in inhibiting TNFα translation, acting both on receptor that induce its production (TLR4 and CD14) and on the cytokine itself. As hypothesized for miR-187, TNFα inhibition is sufficient to explain the spread miR-125a-dependent inhibition of cytokine and chemokine production. Moreover, we predicted and experimentally validated at least one seed region of miR-125a or let-7e in several proinflammatory cytokines and chemokines, with the exception of CXCL10. Hence, the miR-99b~7e~125a cluster show the unique feature in being direct inhibitor of both TLR4 receptor and several pro-inflammatory effectors.

In summary, our results highlight a novel set of microRNA induced by LPS and potentiated by IL-10 that operate a wide inhibition of the TLR4 function, from its expression to the ability to induce pro-inflammatory mediators transcription and production. These microRNAs qualify themselves as new anti-inflammatory weapons used by IL-10 to extinguish inflammation.

In the last part of this thesis project, experiments have been focused on microRNAs regulation by glucocorticoids. In this respect, we identified miR-511 as a novel microRNA located in the fifth intron of the mannose receptor MRC1. This gene encodes for a protein known to be expressed by monocytes, up-regulated in their differentiation to macrophages and dendritic cells and over-expressed in response to anti-inflammatory stimuli (Martinez et al., 2009). Among anti-inflammatory stimuli, glucocorticoids can induce MRC1 expression in human monocytes and macrophages. Concomitant up-regulation of miR-511 with its host gene was therefore tested and confirmed at different time points.

Little is known about miR-511, except for a recently published paper describing miR-511 as a regulator of TLR4 protein expression (Tserel et al., 2011). To go deep in understanding its biological function, an analysis of its predicted target genes was conducted using the IPA software as described for miR-187 and cluster. Analysis revealed the TGFβ signaling pathway as one of the most affected among canonical pathways taken into account by IPA. Gene expression regulation, apoptosis and Smads/MAPK cascade activation are all know effects of TGFβ signaling. Results suggested a strong inhibition of TGFβ-dependent activation of both the Extracellular Signal-Regulated Kinases (ERK1/2) and p38 activation module by miR-511. These two modules are activated with different signaling mechanisms. The first is activated by Raf-Ras-MEK pathway, usually activated directly by tyrosin kinase receptor; the latter is activated by Smad-TAK1 activation together with JNK module. Preliminary results suggest inhibition of the three MAP kinases signaling cascades, but further experiment must be conducted to validate JNK1 and 3 activation. These results underline miR-511 as a new molecular mechanism used by glucocorticoids to control pro-inflammatory mediators release by inhibiting the MAPK cascade. Furthermore, in 2003 Peltier and colleagues

demonstrated that TGFβ, via upregulation of both Smad and MAPK signaling cascade, is also able to increase macrophages sensitivity to glucocorticoids. In this contest, our results could be considered as a negative feedback exerted by glucocorticoids to avoid excessive response of monocytes to their action.

In conclusion, microRNAs function in every step of acute inflammation. LPS sensing induces upregulation of a set of microRNA, including miR-9, miR-155, miR-146a and miR-132 that, in different ways, control pro-inflammatory mediators. At the same time, in a highly regulated and fine-tuned manner, anti-inflammatory effectors are produced. Acting in concert with the previously activated signaling pathways, these mediators induce a second set of microRNAs, including miR-187, miR-125a, let-7e and miR-99b, that by a multi-targeting strategy stop inflammation acting in particular on the NF-κB signaling pathway and on the final effectors themself. At the end, the systemic action of glucocorticoids allow the extinguishing of inflammation, turning off pro-inflammatory transcription factors and in turn activating their own transcription program that consist, among other genes, in at least one microRNA (miR-511) which inhibit TGFβ activity and prevent MAPK cascade further activation (Figure 35).

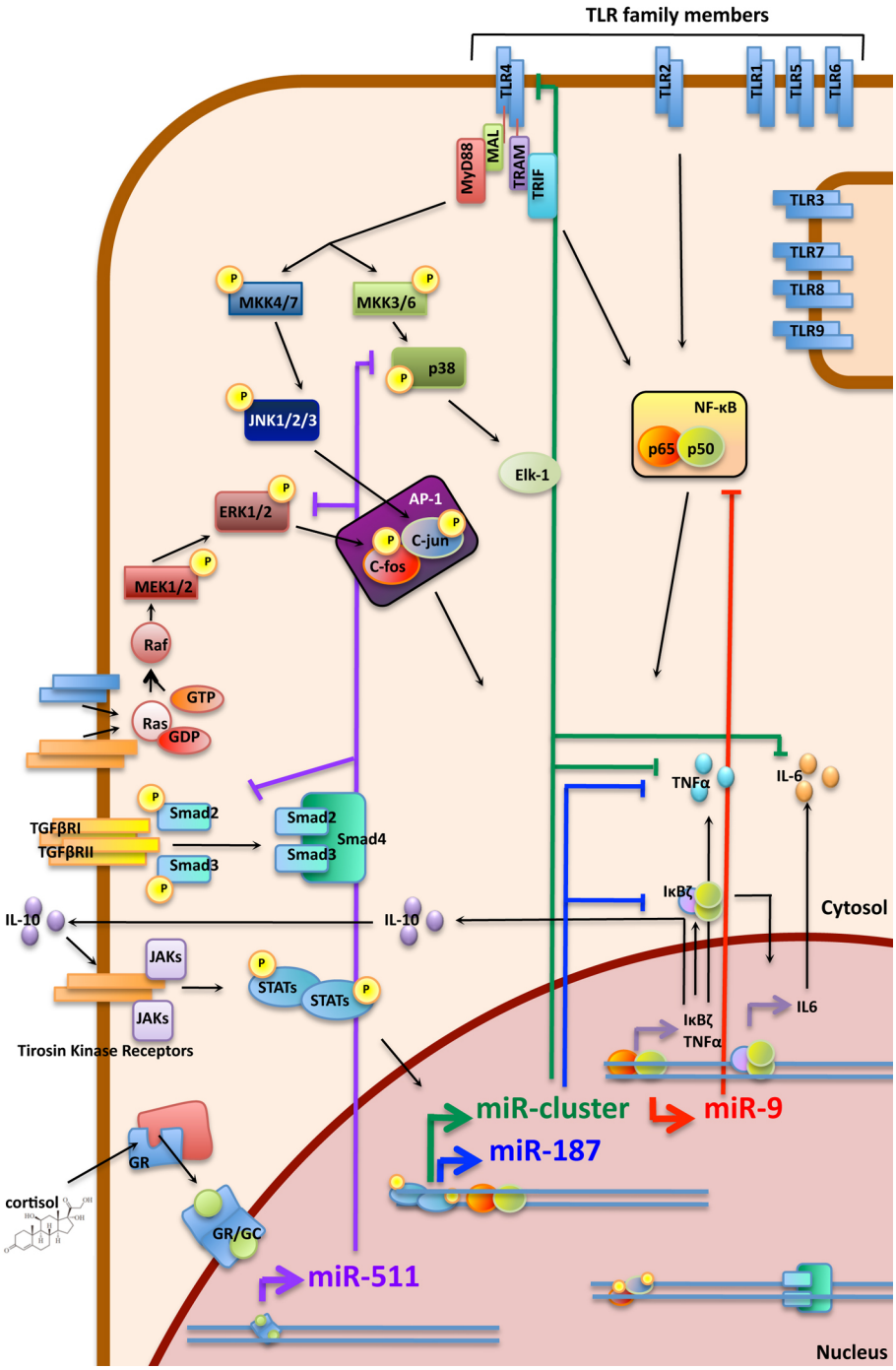

Figure 35. Schematic model of pro- and anti-inflammatory microRNAs impact on inflammation-driven signaling pathways uncovered with this work. TLR4 engagement triggers a complicate signaling cascade that induces miRs transcription (the uncovered action of miR-9 is here reported). TLR-dependent IL-10 release exherts a feedback activity up-regulating miR-187 and miR cluster that, in turn, inhibit excessive pro-inflammatory mediators release. Finally, systemic production of cortisol induces miR-511 that negatively modulates SMAD and MAPK signaling pathways.

References

Abraham, S.M., Lawrence, T., Kleiman, A., Warden, P., Medghalchi, M., Tuckermann, J., Saklatvala, J., and Clark, A.R. (2006). Antiinflammatory effects of dexamethasone are partly dependent on induction of dual specificity phosphatase 1. J Exp Med *203*, 1883-1889.

Aggarwal, B.B. (2003). Signaling pathways of the TNF superfamily: a double-edged sword. Nat Rev Immunol *3*, 745-756.

Agrawal, S., Agrawal, A., Doughty, B., Gerwitz, A., Blenis, J., Van Dyke, T., and Pulendran, B. (2003). Cutting edge: different Toll-like receptor agonists instruct dendritic cells to induce distinct Th responses via differential modulation of extracellular signal-regulated kinase-mitogen-activated protein kinase and c-Fos. J Immunol *171*, 4984-4989.

Akbari, O., Panjwani, N., Garcia, S., Tascon, R., Lowrie, D., and Stockinger, B. (1999). DNA vaccination: transfection and activation of dendritic cells as key events for immunity. J Exp Med *189*, 169-178.

Akira, S., and Takeda, K. (2004). Toll-like receptor signaling. Nat Rev Immunol *4*, 499-511.

Akira, S., Uematsu, S., and Takeuchi, O. (2006). Pathogen recognition and innate immunity. Cell *124*, 783-801.

Allavena, P., Chieppa, M., Bianchi, G., Solinas, G., Fabbri, M., Laskarin, G., and Mantovani, A. (2010). Engagement of the mannose receptor by tumoral mucins activates an immune suppressive phenotype in human tumor-associated macrophages. Clin Dev Immunol *2010*, 547179.

Anderson, P. (2008). Post-transcriptional control of cytokine production. Nat Immunol *9*, 353-359.

Androulidaki, A., Iliopoulos, D., Arranz, A., Doxaki, C., Schworer, S., Zacharioudaki, V., Margioris, A.N., Tsichlis, P.N., and Tsatsanis, C. (2009). The kinase Akt1 controls macrophage response to lipopolysaccharide by regulating microRNAs. Immunity *31*, 220-231.

Arbibe, L., Mira, J.P., Teusch, N., Kline, L., Guha, M., Mackman, N., Godowski, P.J., Ulevitch, R.J., and Knaus, U.G. (2000). Toll-like receptor 2-mediated NF-kappa B activation requires a Rac1-dependent pathway. Nat Immunol *1*, 533-540.

Ashcroft, G.S. (1999). Bidirectional regulation of macrophage function by TGF-beta. Microbes Infect *1*, 1275-1282.

Asirvatham, A.J., Gregorie, C.J., Hu, Z., Magner, W.J., and Tomasi, T.B. (2008). MicroRNA targets in immune genes and the Dicer/Argonaute and ARE machinery components. Mol Immunol *45*, 1995-2006.

Auffray, C., Fogg, D., Garfa, M., Elain, G., Join-Lambert, O., Kayal, S., Sarnacki, S., Cumano, A., Lauvau, G., and Geissmann, F. (2007). Monitoring of blood vessels and tissues by a population of monocytes with patrolling behavior. Science *317*, 666-670.

Auffray, C., Sieweke, M.H., and Geissmann, F. (2009). Blood monocytes: development, heterogeneity, and relationship with dendritic cells. Annu Rev Immunol *27*, 669-692.

Bakheet, T., Williams, B.R., and Khabar, K.S. (2006). ARED 3.0: the large and diverse AU-rich transcriptome. Nucleic Acids Res *34*, D111-114.

Banchereau, J., and Steinman, R.M. (1998). Dendritic cells and the control of immunity. Nature *392*, 245-252.

Bartel, D.P. (2004). MicroRNAs: genomics, biogenesis, mechanism, and function. Cell *116*, 281-297.

Batuman, O.A., Ferrero, A., Cupp, C., Jimenez, S.A., and Khalili, K. (1995). Differential regulation of transforming growth factor beta-1 gene expression by glucocorticoids in human T and glial cells. J Immunol *155*, 4397-4405.

Beeson, P.B. (1947). Tolerance to Bacterial Pyrogens : I. Factors Influencing Its Development. J Exp Med *86*, 29-38.

Beutler, B., Milsark, I.W., and Cerami, A.C. (1985). Passive immunization against cachectin/tumor necrosis factor protects mice from lethal effect of endotoxin. Science *229*, 869-871.

Bhattacharyya, S., Balakathiresan, N.S., Dalgard, C., Gutti, U., Armistead, D., Jozwik, C., Srivastava, M., Pollard, H.B., and Biswas, R. (2011). Elevated miR-155 promotes inflammation in cystic fibrosis by driving hyperexpression of interleukin-8. J Biol Chem *286*, 11604-11615.

Blobe, G.C., Schiemann, W.P., and Lodish, H.F. (2000). Role of transforming growth factor beta in human disease. N Engl J Med *342*, 1350-1358.

Bogdan, C., Paik, J., Vodovotz, Y., and Nathan, C. (1992). Contrasting mechanisms for suppression of macrophage cytokine release by transforming growth factor-beta and interleukin-10. J Biol Chem *267*, 23301-23308.

Boldin, M.P., Taganov, K.D., Rao, D.S., Yang, L., Zhao, J.L., Kalwani, M., Garcia-Flores, Y., Luong, M., Devrekanli, A., Xu, J.*, et al.* (2011). miR-146a is a significant brake on autoimmunity, myeloproliferation, and cancer in mice. J Exp Med *208*, 1189-1201.

Boonstra, A., Rajsbaum, R., Holman, M., Marques, R., Asselin-Paturel, C., Pereira, J.P., Bates, E.E., Akira, S., Vieira, P., Liu, Y.J.*, et al.* (2006). Macrophages and myeloid dendritic cells, but not plasmacytoid dendritic cells, produce IL-10 in response to MyD88- and TRIF-dependent TLR signals, and TLR-independent signals. J Immunol *177*, 7551-7558.

Borregaard, N. (2010). Neutrophils, from marrow to microbes. Immunity *33*, 657-670.

Brinkmann, V., Reichard, U., Goosmann, C., Fauler, B., Uhlemann, Y., Weiss, D.S., Weinrauch, Y., and Zychlinsky, A. (2004). Neutrophil extracellular traps kill bacteria. Science *303*, 1532-1535.

Bussing, I., Slack, F.J., and Grosshans, H. (2008). let-7 microRNAs in development, stem cells and cancer. Trends Mol Med *14*, 400-409.

Cargnello, M., and Roux, P.P. (2011). Activation and function of the MAPKs and their substrates, the MAPK-activated protein kinases. Microbiol Mol Biol Rev *75*, 50-83.

Carmody, R.J., Ruan, Q., Palmer, S., Hilliard, B., and Chen, Y.H. (2007). Negative regulation of toll-like receptor signaling by NF-kappaB p50 ubiquitination blockade. Science *317*, 675-678.

Carty, M., Goodbody, R., Schroder, M., Stack, J., Moynagh, P.N., and Bowie, A.G. (2006). The human adaptor SARM negatively regulates adaptor protein TRIF-dependent Toll-like receptor signaling. Nat Immunol *7*, 1074-1081.

Cassatella, M.A. (1999). Neutrophil-derived proteins: selling cytokines by the pound. Adv Immunol *73*, 369-509.

Cavaillon, J.M., and Adib-Conquy, M. (2006). Bench-to-bedside review: endotoxin tolerance as a model of leukocyte reprogramming in sepsis. Crit Care *10*, 233.

Cavaillon, J.M., Adrie, C., Fitting, C., and Adib-Conquy, M. (2005). Reprogramming of circulatory cells in sepsis and SIRS. J Endotoxin Res *11*, 311-320.

Cella, M., Salio, M., Sakakibara, Y., Langen, H., Julkunen, I., and Lanzavecchia, A. (1999). Maturation, activation, and protection of dendritic cells induced by double-stranded RNA. J Exp Med *189*, 821-829.

Ceppi, M., Pereira, P.M., Dunand-Sauthier, I., Barras, E., Reith, W., Santos, M.A., and Pierre, P. (2009). MicroRNA-155 modulates the interleukin-1 signaling pathway in activated human monocyte-derived dendritic cells. Proc Natl Acad Sci U S A *106*, 2735-2740.

Cesana, M., Cacchiarelli, D., Legnini, I., Santini, T., Sthandier, O., Chinappi, M., Tramontano, A., and Bozzoni, I. (2011). A long noncoding RNA controls muscle differentiation by functioning as a competing endogenous RNA. Cell *147*, 358-369.

Chang, H., Brown, C.W., and Matzuk, M.M. (2002). Genetic analysis of the mammalian transforming growth factor-beta superfamily. Endocr Rev *23*, 787-823.

Chatzikyriakidou, A., Voulgari, P.V., Georgiou, I., and Drosos, A.A. (2010a). A polymorphism in the 3'-UTR of interleukin-1 receptor-associated kinase (IRAK1), a target gene of miR-146a, is associated with rheumatoid arthritis susceptibility. Joint Bone Spine *77*, 411-413.

Chatzikyriakidou, A., Voulgari, P.V., Georgiou, I., and Drosos, A.A. (2010b). The role of microRNA-146a (miR-146a) and its target IL-1R-associated kinase (IRAK1) in psoriatic arthritis susceptibility. Scand J Immunol *71*, 382-385.

Chaudhuri, A.A., So, A.Y., Sinha, N., Gibson, W.S., Taganov, K.D., O'Connell, R.M., and Baltimore, D. (2011). MicroRNA-125b Potentiates Macrophage Activation. J Immunol *187*, 5062-5068.

Chen, C.Y., and Shyu, A.B. (1995). AU-rich elements: characterization and importance in mRNA degradation. Trends Biochem Sci *20*, 465-470.

Chen, C.Z., Li, L., Lodish, H.F., and Bartel, D.P. (2004). MicroRNAs modulate hematopoietic lineage differentiation. Science *303*, 83-86.

Chen, G., Zhuchenko, O., and Kuspa, A. (2007a). Immune-like phagocyte activity in the social amoeba. Science *317*, 678-681.

Chen, X.M., Splinter, P.L., O'Hara, S.P., and LaRusso, N.F. (2007b). A cellular micro-RNA, let-7i, regulates Toll-like receptor 4 expression and contributes to cholangiocyte immune responses against Cryptosporidium parvum infection. J Biol Chem *282*, 28929-28938.

Chen, Z.J. (2005). Ubiquitin signaling in the NF-kappaB pathway. Nat Cell Biol *7*, 758-765.

Cobb, M.H., and Goldsmith, E.J. (1995). How MAP kinases are regulated. J Biol Chem *270*, 14843-14846.

Colotta, F., Re, F., Polentarutti, N., Sozzani, S., and Mantovani, A. (1992). Modulation of granulocyte survival and programmed cell death by cytokines and bacterial products. Blood *80*, 2012-2020.

Cusson-Hermance, N., Khurana, S., Lee, T.H., Fitzgerald, K.A., and Kelliher, M.A. (2005). Rip1 mediates the Trif-dependent toll-like receptor 3- and 4-induced NF-{kappa}B activation but does not contribute to interferon regulatory factor 3 activation. J Biol Chem *280*, 36560-36566.

del Fresno, C., Garcia-Rio, F., Gomez-Pina, V., Soares-Schanoski, A., Fernandez-Ruiz, I., Jurado, T., Kajiji, T., Shu, C., Marin, E., Gutierrez del Arroyo, A.*, et al.* (2009). Potent phagocytic activity with impaired antigen presentation identifying lipopolysaccharide-tolerant human monocytes: demonstration in isolated monocytes from cystic fibrosis patients. J Immunol *182*, 6494-6507.

Deng, L., Wang, C., Spencer, E., Yang, L., Braun, A., You, J., Slaughter, C., Pickart, C., and Chen, Z.J. (2000). Activation of the IkappaB kinase complex by TRAF6 requires a dimeric ubiquitin-conjugating enzyme complex and a unique polyubiquitin chain. Cell *103*, 351-361.

Derynck, R., and Zhang, Y.E. (2003). Smad-dependent and Smad-independent pathways in TGF-beta family signaling. Nature *425*, 577-584.

Dillon, S., Agrawal, A., Van Dyke, T., Landreth, G., McCauley, L., Koh, A., Maliszewski, C., Akira, S., and Pulendran, B. (2004). A Toll-like receptor 2 ligand stimulates Th2 responses in vivo, via induction of extracellular signal-regulated kinase mitogen-activated protein kinase and c-Fos in dendritic cells. J Immunol *172*, 4733-4743.

Dobrovolskaia, M.A., and Vogel, S.N. (2002). Toll receptors, CD14, and macrophage activation and deactivation by LPS. Microbes Infect *4*, 903-914.

Du, X., Poltorak, A., Silva, M., and Beutler, B. (1999). Analysis of Tlr4-mediated LPS signal transduction in macrophages by mutational modification of the receptor. Blood Cells Mol Dis *25*, 328-338.

El Gazzar, M., Church, A., Liu, T., and McCall, C.E. (2011). MicroRNA-146a regulates both transcription silencing and translation disruption of TNF-alpha during TLR4-induced gene reprogramming. J Leukoc Biol *90*, 509-519.

El Gazzar, M., and McCall, C.E. (2011). MicroRNAs regulatory networks in myeloid lineage development and differentiation: regulators of the regulators. Immunol Cell Biol.

Elenkov, I.J., Papanicolaou, D.A., Wilder, R.L., and Chrousos, G.P. (1996). Modulatory effects of glucocorticoids and catecholamines on human interleukin-12 and interleukin-10 production: clinical implications. Proc Assoc Am Physicians *108*, 374-381.

Engel, M.E., McDonnell, M.A., Law, B.K., and Moses, H.L. (1999). Interdependent SMAD and JNK signaling in transforming growth factor-beta-mediated transcription. J Biol Chem *274*, 37413-37420.

Fabian, M.R., Sonenberg, N., and Filipowicz, W. (2010). Regulation of mRNA translation and stability by microRNAs. Annu Rev Biochem *79*, 351-379.

Farazi, T.A., Juranek, S.A., and Tuschl, T. (2008). The growing catalog of small RNAs and their association with distinct Argonaute/Piwi family members. Development *135*, 1201-1214.

Feng, G.J., Goodridge, H.S., Harnett, M.M., Wei, X.Q., Nikolaev, A.V., Higson, A.P., and Liew, F.Y. (1999). Extracellular signal-related kinase (ERK) and p38 mitogen-activated protein (MAP) kinases differentially regulate the lipopolysaccharide-mediated induction of inducible nitric oxide synthase and IL-12 in macrophages: Leishmania phosphoglycans subvert macrophage IL-12 production by targeting ERK MAP kinase. J Immunol *163*, 6403-6412.

Fiorentino, D.F., Bond, M.W., and Mosmann, T.R. (1989). Two types of mouse T helper cell. IV. Th2 clones secrete a factor that inhibits cytokine production by Th1 clones. J Exp Med *170*, 2081-2095.

Fogg, D.K., Sibon, C., Miled, C., Jung, S., Aucouturier, P., Littman, D.R., Cumano, A., and Geissmann, F. (2006). A clonogenic bone marrow progenitor specific for macrophages and dendritic cells. Science *311*, 83-87.

Foster, S.L., Hargreaves, D.C., and Medzhitov, R. (2007). Gene-specific control of inflammation by TLR-induced chromatin modifications. Nature *447*, 972-978.

Foster, S.L., and Medzhitov, R. (2009). Gene-specific control of the TLR-induced inflammatory response. Clin Immunol *130*, 7-15.

Franchimont, D. (2004). Overview of the actions of glucocorticoids on the immune response: a good model to characterize new pathways of immunosuppression for new treatment strategies. Ann N Y Acad Sci *1024*, 124-137.

Fraser, I.P., Koziel, H., and Ezekowitz, R.A. (1998). The serum mannose-binding protein and the macrophage mannose receptor are pattern recognition molecules that link innate and adaptive immunity. Semin Immunol *10*, 363-372.

Fridlender, Z.G., Sun, J., Kim, S., Kapoor, V., Cheng, G., Ling, L., Worthen, G.S., and Albelda, S.M. (2009). Polarization of tumor-associated neutrophil phenotype by TGF-beta: "N1" versus "N2" TAN. Cancer Cell *16*, 183-194.

Galanos, C., and Freudenberg, M.A. (1993). Mechanisms of endotoxin shock and endotoxin hypersensitivity. Immunobiology *187*, 346-356.

Gallucci, S., and Matzinger, P. (2001). Danger signals: SOS to the immune system. Curr Opin Immunol *13*, 114-119.

Garlanda, C., Bottazzi, B., Bastone, A., and Mantovani, A. (2005). Pentraxins at the crossroads between innate immunity, inflammation, matrix deposition, and female fertility. Annu Rev Immunol *23*, 337-366.

Gasque, P. (2004). Complement: a unique innate immune sensor for danger signals. Mol Immunol *41*, 1089-1098.

Geissmann, F., Auffray, C., Palframan, R., Wirrig, C., Ciocca, A., Campisi, L., Narni-Mancinelli, E., and Lauvau, G. (2008). Blood monocytes: distinct subsets, how they relate to dendritic cells, and their possible roles in the regulation of T-cell responses. Immunol Cell Biol *86*, 398-408.

Geissmann, F., Prost, C., Monnet, J.P., Dy, M., Brousse, N., and Hermine, O. (1998). Transforming growth factor beta1, in the presence of granulocyte/macrophage colony-stimulating factor and interleukin 4, induces differentiation of human peripheral blood monocytes into dendritic Langerhans cells. J Exp Med *187*, 961-966.

Gerrits, A., Walasek, M.A., Olthof, S., Weersing, E., Ritsema, M., Zwart, E., van Os, R., Bystrykh, L.V., and de Haan, G. (2011). Genetic screen identifies microRNA cluster 99b/let-7e/125a as a regulator of primitive hematopoietic cells. Blood.

Ghosh, S., May, M.J., and Kopp, E.B. (1998). NF-kappa B and Rel proteins: evolutionarily conserved mediators of immune responses. Annu Rev Immunol *16*, 225-260.

Gordon, S. (1998). The role of the macrophage in immune regulation. Res Immunol *149*, 685-688.

Gordon, S. (2002). Pattern recognition receptors: doubling up for the innate immune response. Cell *111*, 927-930.

Gottwein, E., Mukherjee, N., Sachse, C., Frenzel, C., Majoros, W.H., Chi, J.T., Braich, R., Manoharan, M., Soutschek, J., Ohler, U*., et al.* (2007). A viral microRNA functions as an orthologue of cellular miR-155. Nature *450*, 1096-1099.

Govinden, R., and Bhoola, K.D. (2003). Genealogy, expression, and cellular function of transforming growth factor-beta. Pharmacol Ther *98*, 257-265.

Granowitz, E.V., Santos, A.A., Poutsiaka, D.D., Cannon, J.G., Wilmore, D.W., Wolff, S.M., and Dinarello, C.A. (1991). Production of interleukin-1-receptor antagonist during experimental endotoxaemia. Lancet *338*, 1423-1424.

Guido, E.C., Delorme, E.O., Clemm, D.L., Stein, R.B., Rosen, J., and Miner, J.N. (1996). Determinants of promoter-specific activity by glucocorticoid receptor. Mol Endocrinol *10*, 1178-1190.

Guo, S., Lu, J., Schlanger, R., Zhang, H., Wang, J.Y., Fox, M.C., Purton, L.E., Fleming, H.H., Cobb, B., Merkenschlager, M*., et al.* (2010). MicroRNA miR-125a controls hematopoietic stem cell number. Proc Natl Acad Sci U S A *107*, 14229-14234.

Guo, X., Wu, Y., and Hartley, R.S. (2009). MicroRNA-125a represses cell growth by targeting HuR in breast cancer. RNA Biol *6*, 575-583.

Hacker, H., and Karin, M. (2006). Regulation and function of IKK and IKK-related kinases. Sci STKE *2006*, re13.

Hacker, H., Mischak, H., Hacker, G., Eser, S., Prenzel, N., Ullrich, A., and Wagner, H. (1999). Cell type-specific activation of mitogen-activated protein kinases by CpG-DNA controls interleukin-12 release from antigen-presenting cells. EMBO J *18*, 6973-6982.

Hadji-Azimi, I., Coosemans, V., and Canicatti, C. (1987). Atlas of adult Xenopus laevis laevis hematology. Dev Comp Immunol *11*, 807-874.

Halees, A.S., El-Badrawi, R., and Khabar, K.S. (2008). ARED Organism: expansion of ARED reveals AU-rich element cluster variations between human and mouse. Nucleic Acids Res *36*, D137-140.

Hao, S., and Baltimore, D. (2009). The stability of mRNA influences the temporal order of the induction of genes encoding inflammatory molecules. Nat Immunol *10*, 281-288.

Hayashi, F., Means, T.K., and Luster, A.D. (2003). Toll-like receptors stimulate human neutrophil function. Blood *102*, 2660-2669.

Hayden, M.S., and Ghosh, S. (2004). Signaling to NF-kappaB. Genes Dev *18*, 2195-2224.

Hayden, M.S., and Ghosh, S. (2008). Shared principles in NF-kappaB signaling. Cell *132*, 344-362.

He, L., and Hannon, G.J. (2004). MicroRNAs: small RNAs with a big role in gene regulation. Nat Rev Genet *5*, 522-531.

Hemmi, H., Takeuchi, O., Kawai, T., Kaisho, T., Sato, S., Sanjo, H., Matsumoto, M., Hoshino, K., Wagner, H., Takeda, K.*, et al.* (2000). A Toll-like receptor recognizes bacterial DNA. Nature *408*, 740-745.

Herbomel, P., Thisse, B., and Thisse, C. (1999). Ontogeny and behaviour of early macrophages in the zebrafish embryo. Development *126*, 3735-3745.

Hoffmann, A., Levchenko, A., Scott, M.L., and Baltimore, D. (2002). The IkappaB-NF-kappaB signaling module: temporal control and selective gene activation. Science *298*, 1241-1245.

Hoffmann, J.A., Kafatos, F.C., Janeway, C.A., and Ezekowitz, R.A. (1999). Phylogenetic perspectives in innate immunity. Science *284*, 1313-1318.

Hofmann, T.G., and Schmitz, M.L. (2002). The promoter context determines mutual repression or synergism between NF-kappaB and the glucocorticoid receptor. Biol Chem *383*, 1947-1951.

Hogger, P., Dreier, J., Droste, A., Buck, F., and Sorg, C. (1998). Identification of the integral membrane protein RM3/1 on human monocytes as a glucocorticoid-inducible member of the scavenger receptor cysteine-rich family (CD163). J Immunol *161*, 1883-1890.

Hoshino, K., Takeuchi, O., Kawai, T., Sanjo, H., Ogawa, T., Takeda, Y., Takeda, K., and Akira, S. (1999). Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are

hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product. J Immunol *162*, 3749-3752.

Imai, K., Takeshita, A., and Hanazawa, S. (2000). Transforming growth factor-beta inhibits lipopolysaccharide-stimulated expression of inflammatory cytokines in mouse macrophages through downregulation of activation protein 1 and CD14 receptor expression. Infect Immun *68*, 2418-2423.

Ing, N.H. (2005). Steroid hormones regulate gene expression posttranscriptionally by altering the stabilities of messenger RNAs. Biol Reprod *72*, 1290-1296.

Inui, M., Martello, G., and Piccolo, S. (2010). MicroRNA control of signal transduction. Nat Rev Mol Cell Biol *11*, 252-263.

Ismaili, N., and Garabedian, M.J. (2004). Modulation of glucocorticoid receptor function via phosphorylation. Ann N Y Acad Sci *1024*, 86-101.

Janeway, C.A., Jr. (1989). Approaching the asymptote? Evolution and revolution in immunology. Cold Spring Harb Symp Quant Biol *54 Pt 1*, 1-13.

Janeway, C.A., Jr., and Medzhitov, R. (2002). Innate immune recognition. Annu Rev Immunol *20*, 197-216.

Jeffrey, P.L., Capes-Davis, A., Dunn, J.M., Tolhurst, O., Seeto, G., Hannan, A.J., and Lin, S.L. (2000). CROC-4: a novel brain specific transcriptional activator of c-fos expressed from proliferation through to maturation of multiple neuronal cell types. Mol Cell Neurosci *16*, 185-196.

Jenkins, K.A., and Mansell, A. (2010). TIR-containing adaptors in Toll-like receptor signaling. Cytokine *49*, 237-244.

Jiang, L., Huang, Q., Chang, J., Wang, E., and Qiu, X. (2011). MicroRNA HSA-miR-125a-5p induces apoptosis by activating p53 in lung cancer cells. Exp Lung Res *37*, 387-398.

John, B., Enright, A.J., Aravin, A., Tuschl, T., Sander, C., and Marks, D.S. (2004). Human MicroRNA targets. PLoS Biol *2*, e363.

Kagan, J.C., Su, T., Horng, T., Chow, A., Akira, S., and Medzhitov, R. (2008). TRAM couples endocytosis of Toll-like receptor 4 to the induction of interferon-beta. Nat Immunol *9*, 361-368.

Kang, Y., Chen, C.R., and Massague, J. (2003). A self-enabling TGFbeta response coupled to stress signaling: Smad engages stress response factor ATF3 for Id1 repression in epithelial cells. Mol Cell *11*, 915-926.

Karin, M., and Ben-Neriah, Y. (2000). Phosphorylation meets ubiquitination: the control of NF-[kappa]B activity. Annu Rev Immunol *18*, 621-663.

Karreth, F.A., Tay, Y., Perna, D., Ala, U., Tan, S.M., Rust, A.G., DeNicola, G., Webster, K.A., Weiss, D., Perez-Mancera, P.A*., et al.* (2011). In vivo identification of tumor- suppressive PTEN ceRNAs in an oncogenic BRAF-induced mouse model of melanoma. Cell *147*, 382-395.

Kawai, T., and Akira, S. (2007). Signaling to NF-kappaB by Toll-like receptors. Trends Mol Med *13*, 460-469.

Kehrl, J.H., Wakefield, L.M., Roberts, A.B., Jakowlew, S., Alvarez-Mon, M., Derynck, R., Sporn, M.B., and Fauci, A.S. (1986). Production of transforming growth factor beta by human T lymphocytes and its potential role in the regulation of T cell growth. J Exp Med *163*, 1037-1050.

Kelly, L.M., Englmeier, U., Lafon, I., Sieweke, M.H., and Graf, T. (2000). MafB is an inducer of monocytic differentiation. EMBO J *19*, 1987-1997.

Kenny, E.F., and O'Neill, L.A. (2008). Signaling adaptors used by Toll-like receptors: an update. Cytokine *43*, 342-349.

Kile, B.T., Nicola, N.A., and Alexander, W.S. (2001). Negative regulators of cytokine signaling. Int J Hematol *73*, 292-298.

Kim, V.N., and Nam, J.W. (2006). Genomics of microRNA. Trends Genet *22*, 165-173.

Kimbrell, D.A., and Beutler, B. (2001). The evolution and genetics of innate immunity. Nat Rev Genet *2*, 256-267.

Kitamura, H., Kanehira, K., Okita, K., Morimatsu, M., and Saito, M. (2000). MAIL, a novel nuclear I kappa B protein that potentiates LPS-induced IL-6 production. FEBS Lett *485*, 53-56.

Krek, A., Grun, D., Poy, M.N., Wolf, R., Rosenberg, L., Epstein, E.J., MacMenamin, P., da Piedade, I., Gunsalus, K.C., Stoffel, M.*, et al.* (2005). Combinatorial microRNA target predictions. Nat Genet *37*, 495-500.

Kuwata, H., Watanabe, Y., Miyoshi, H., Yamamoto, M., Kaisho, T., Takeda, K., and Akira, S. (2003). IL-10-inducible Bcl-3 negatively regulates LPS-induced TNF-alpha production in macrophages. Blood *102*, 4123-4129.

Kyriakis, J.M., Banerjee, P., Nikolakaki, E., Dai, T., Rubie, E.A., Ahmad, M.F., Avruch, J., and Woodgett, J.R. (1994). The stress-activated protein kinase subfamily of c-Jun kinases. Nature *369*, 156-160.

Lagos-Quintana, M., Rauhut, R., Lendeckel, W., and Tuschl, T. (2001). Identification of novel genes coding for small expressed RNAs. Science *294*, 853-858.

Lau, N.C., Lim, L.P., Weinstein, E.G., and Bartel, D.P. (2001). An abundant class of tiny RNAs with probable regulatory roles in Caenorhabditis elegans. Science *294*, 858-862.

Lechner, J., Welte, T., and Doppler, W. (1997). Mechanism of interaction between the glucocorticoid receptor and Stat5: role of DNA-binding. Immunobiology *198*, 112-123.

Lee, T.H., Shank, J., Cusson, N., and Kelliher, M.A. (2004). The kinase activity of Rip1 is not required for tumor necrosis factor-alpha-induced IkappaB kinase or p38 MAP kinase activation or for the ubiquitination of Rip1 by Traf2. J Biol Chem *279*, 33185-33191.

Lee, Y., Jeon, K., Lee, J.T., Kim, S., and Kim, V.N. (2002). MicroRNA maturation: stepwise processing and subcellular localization. EMBO J *21*, 4663-4670.

Leonard, W.J., and O'Shea, J.J. (1998). Jaks and STATs: biological implications. Annu Rev Immunol *16*, 293-322.

Levashina, E.A., Langley, E., Green, C., Gubb, D., Ashburner, M., Hoffmann, J.A., and Reichhart, J.M. (1999). Constitutive activation of toll-mediated antifungal defense in serpin-deficient Drosophila. Science *285*, 1917-1919.

Lewis, B.P., Shih, I.H., Jones-Rhoades, M.W., Bartel, D.P., and Burge, C.B. (2003). Prediction of mammalian microRNA targets. Cell *115*, 787-798.

Li, W., Duan, R., Kooy, F., Sherman, S.L., Zhou, W., and Jin, P. (2009). Germline mutation of microRNA-125a is associated with breast cancer. J Med Genet *46*, 358-360.

Lindsay, M.A. (2008). microRNAs and the immune response. Trends Immunol *29*, 343-351.

Liu, Y., Cousin, J.M., Hughes, J., Van Damme, J., Seckl, J.R., Haslett, C., Dransfield, I., Savill, J., and Rossi, A.G. (1999). Glucocorticoids promote nonphlogistic phagocytosis of apoptotic leukocytes. J Immunol *162*, 3639-3646.

Louafi, F., Martinez-Nunez, R.T., and Sanchez-Elsner, T. (2010). MicroRNA-155 targets SMAD2 and modulates the response of macrophages to transforming growth factor-{beta}. J Biol Chem *285*, 41328-41336.

Lu, L.F., Thai, T.H., Calado, D.P., Chaudhry, A., Kubo, M., Tanaka, K., Loeb, G.B., Lee, H., Yoshimura, A., Rajewsky, K.*, et al.* (2009). Foxp3-dependent microRNA155 confers competitive fitness to regulatory T cells by targeting SOCS1 protein. Immunity *30*, 80-91.

Lu, N.Z., and Cidlowski, J.A. (2004). The origin and functions of multiple human glucocorticoid receptor isoforms. Ann N Y Acad Sci *1024*, 102-123.

Lu, N.Z., and Cidlowski, J.A. (2005). Translational regulatory mechanisms generate N-terminal glucocorticoid receptor isoforms with unique transcriptional target genes. Mol Cell *18*, 331-342.

Manning, G., Whyte, D.B., Martinez, R., Hunter, T., and Sudarsanam, S. (2002). The protein kinase complement of the human genome. Science *298*, 1912-1934.

Martich, G.D., Danner, R.L., Ceska, M., and Suffredini, A.F. (1991). Detection of interleukin 8 and tumor necrosis factor in normal humans after intravenous endotoxin: the effect of antiinflammatory agents. J Exp Med *173*, 1021-1024.

Martinez, F.O., Gordon, S., Locati, M., and Mantovani, A. (2006). Transcriptional profiling of the human monocyte-to-macrophage differentiation and polarization: new molecules and patterns of gene expression. J Immunol *177*, 7303-7311.

Martinez, F.O., Helming, L., and Gordon, S. (2009). Alternative activation of macrophages: an immunologic functional perspective. Annu Rev Immunol *27*, 451-483.

Martinez, F.O., Sica, A., Mantovani, A., and Locati, M. (2008). Macrophage activation and polarization. Front Biosci *13*, 453-461.

Martinon, F., and Tschopp, J. (2005). NLRs join TLRs as innate sensors of pathogens. Trends Immunol *26*, 447-454.

Massague, J. (2000). How cells read TGF-beta signals. Nat Rev Mol Cell Biol *1*, 169-178.

McCall, C.E., and Yoza, B.K. (2007). Gene silencing in severe systemic inflammation. Am J Respir Crit Care Med *175*, 763-767.

McCarthy, J.V., Ni, J., and Dixit, V.M. (1998). RIP2 is a novel NF-kappaB-activating and cell death-inducing kinase. J Biol Chem *273*, 16968-16975.

McCoy, C.E., Sheedy, F.J., Qualls, J.E., Doyle, S.L., Quinn, S.R., Murray, P.J., and O'Neill, L.A. (2010). IL-10 inhibits miR-155 induction by toll-like receptors. J Biol Chem *285*, 20492-20498.

McCusker, K., and Hoidal, J. (1989). Characterization of scavenger receptor activity in resident human lung macrophages. Exp Lung Res *15*, 651-661.

Medzhitov, R. (2010). Inflammation 2010: new adventures of an old flame. Cell *140*, 771-776.

Mege, J.L., Meghari, S., Honstettre, A., Capo, C., and Raoult, D. (2006). The two faces of interleukin 10 in human infectious diseases. Lancet Infect Dis *6*, 557-569.

Michie, H.R., Manogue, K.R., Spriggs, D.R., Revhaug, A., O'Dwyer, S., Dinarello, C.A., Cerami, A., Wolff, S.M., and Wilmore, D.W. (1988). Detection of circulating tumor necrosis factor after endotoxin administration. N Engl J Med *318*, 1481-1486.

Monk, C.E., Hutvagner, G., and Arthur, J.S. (2010). Regulation of miRNA transcription in macrophages in response to Candida albicans. PLoS One *5*, e13669.

Monneret, G., Venet, F., Pachot, A., and Lepape, A. (2008). Monitoring immune dysfunctions in the septic patient: a new skin for the old ceremony. Mol Med *14*, 64-78.

Moore, K.W., de Waal Malefyt, R., Coffman, R.L., and O'Garra, A. (2001). Interleukin-10 and the interleukin-10 receptor. Annu Rev Immunol *19*, 683-765.

Moore, K.W., Vieira, P., Fiorentino, D.F., Trounstine, M.L., Khan, T.A., and Mosmann, T.R. (1990). Homology of cytokine synthesis inhibitory factor (IL-10) to the Epstein-Barr virus gene BCRFI. Science *248*, 1230-1234.

Motoyama, M., Yamazaki, S., Eto-Kimura, A., Takeshige, K., and Muta, T. (2005). Positive and negative regulation of nuclear factor-kappaB-mediated transcription by IkappaB-zeta, an inducible nuclear protein. J Biol Chem *280*, 7444-7451.

Mourelatos, Z., Dostie, J., Paushkin, S., Sharma, A., Charroux, B., Abel, L., Rappsilber, J., Mann, M., and Dreyfuss, G. (2002). miRNPs: a novel class of ribonucleoproteins containing numerous microRNAs. Genes Dev *16*, 720-728.

Nahid, M.A., Satoh, M., and Chan, E.K. (2011). Mechanistic role of microRNA-146a in endotoxin-induced differential cross-regulation of TLR signaling. J Immunol *186*, 1723-1734.

Naiki, Y., Michelsen, K.S., Zhang, W., Chen, S., Doherty, T.M., and Arditi, M. (2005). Transforming growth factor-beta differentially inhibits MyD88-dependent, but not TRAM- and TRIF-dependent, lipopolysaccharide-induced TLR4 signaling. J Biol Chem *280*, 5491-5495.

Newfeld, S.J., Wisotzkey, R.G., and Kumar, S. (1999). Molecular evolution of a developmental pathway: phylogenetic analyses of transforming growth factor-beta family ligands, receptors and Smad signal transducers. Genetics *152*, 783-795.

Nie, K., Gomez, M., Landgraf, P., Garcia, J.F., Liu, Y., Tan, L.H., Chadburn, A., Tuschl, T., Knowles, D.M., and Tam, W. (2008). MicroRNA-mediated down-regulation of PRDM1/Blimp-1 in Hodgkin/Reed-Sternberg cells: a potential pathogenetic lesion in Hodgkin lymphomas. Am J Pathol *173*, 242-252.

O'Connell, R.M., Taganov, K.D., Boldin, M.P., Cheng, G., and Baltimore, D. (2007). MicroRNA-155 is induced during the macrophage inflammatory response. Proc Natl Acad Sci U S A *104*, 1604-1609.

O'Neill, L.A., and Bowie, A.G. (2007). The family of five: TIR-domain-containing adaptors in Toll-like receptor signaling. Nat Rev Immunol *7*, 353-364.

O'Neill, L.A., Bryant, C.E., and Doyle, S.L. (2009). Therapeutic targeting of Toll-like receptors for infectious and inflammatory diseases and cancer. Pharmacol Rev *61*, 177-197.

O'Neill, L.A., Sheedy, F.J., and McCoy, C.E. (2011). MicroRNAs: the fine-tuners of Toll-like receptor signaling. Nat Rev Immunol *11*, 163-175.

Ostuni, R., Zanoni, I., and Granucci, F. (2010). Deciphering the complexity of Toll-like receptor signaling. Cell Mol Life Sci *67*, 4109-4134.

Ozen, M., Creighton, C.J., Ozdemir, M., and Ittmann, M. (2008). Widespread deregulation of microRNA expression in human prostate cancer. Oncogene *27*, 1788-1793.

Parrillo, J.E. (1993). Pathogenetic mechanisms of septic shock. N Engl J Med *328*, 1471-1477.

Pecchi, E., Dallaporta, M., Jean, A., Thirion, S., and Troadec, J.D. (2009). Prostaglandins and sickness behavior: old story, new insights. Physiol Behav *97*, 279-292.

Peterlongo, P., Caleca, L., Cattaneo, E., Ravagnani, F., Bianchi, T., Galastri, L., Bernard, L., Ficarazzi, F., Dall'olio, V., Marme, F*., et al.* (2011). The rs12975333 variant in the miR-125a and breast cancer risk in Germany, Italy, Australia and Spain. J Med Genet *48*, 703-704.

Piemonti, L., Monti, P., Allavena, P., Sironi, M., Soldini, L., Leone, B.E., Socci, C., and Di Carlo, V. (1999). Glucocorticoids affect human dendritic cell differentiation and maturation. J Immunol *162*, 6473-6481.

Pratt, W.B., and Toft, D.O. (1997). Steroid receptor interactions with heat shock protein and immunophilin chaperones. Endocr Rev *18*, 306-360.

Preas, H.L., 2nd, Reda, D., Tropea, M., Vandivier, R.W., Banks, S.M., Agosti, J.M., and Suffredini, A.F. (1996). Effects of recombinant soluble type I interleukin-1 receptor on human inflammatory responses to endotoxin. Blood *88*, 2465-2472.

Pujols, L., Mullol, J., Perez, M., Roca-Ferrer, J., Juan, M., Xaubet, A., Cidlowski, J.A., and Picado, C. (2001). Expression of the human glucocorticoid receptor alpha

and beta isoforms in human respiratory epithelial cells and their regulation by dexamethasone. Am J Respir Cell Mol Biol *24*, 49-57.

Pulendran, B. (2005). Variegation of the immune response with dendritic cells and pathogen recognition receptors. J Immunol *174*, 2457-2465.

Qasimi, P., Ming-Lum, A., Ghanipour, A., Ong, C.J., Cox, M.E., Ihle, J., Cacalano, N., Yoshimura, A., and Mui, A.L. (2006). Divergent mechanisms utilized by SOCS3 to mediate interleukin-10 inhibition of tumor necrosis factor alpha and nitric oxide production by macrophages. J Biol Chem *281*, 6316-6324.

Qi, X.F., Kim, D.H., Yoon, Y.S., Jin, D., Huang, X.Z., Li, J.H., Deung, Y.K., and Lee, K.J. (2009). Essential involvement of cross-talk between IFN-gamma and TNF-alpha in CXCL10 production in human THP-1 monocytes. J Cell Physiol *220*, 690-697.

Quinn, J.M., and Gillespie, M.T. (2005). Modulation of osteoclast formation. Biochem Biophys Res Commun *328*, 739-745.

Quinn, S.R., and O'Neill, L.A. (2011). A trio of microRNAs that control Toll-like receptor signaling. Int Immunol *23*, 421-425.

Randolph, G.J., Inaba, K., Robbiani, D.F., Steinman, R.M., and Muller, W.A. (1999). Differentiation of phagocytic monocytes into lymph node dendritic cells in vivo. Immunity *11*, 753-761.

Re, F., Muzio, M., De Rossi, M., Polentarutti, N., Giri, J.G., Mantovani, A., and Colotta, F. (1994). The type II "receptor" as a decoy target for interleukin 1 in polymorphonuclear leukocytes: characterization of induction by dexamethasone and ligand binding properties of the released decoy receptor. J Exp Med *179*, 739-743.

Recalcati, S., Locati, M., Marini, A., Santambrogio, P., Zaninotto, F., De Pizzol, M., Zammataro, L., Girelli, D., and Cairo, G. (2010). Differential regulation of iron homeostasis during human macrophage polarized activation. Eur J Immunol *40*, 824-835.

Redecke, V., Hacker, H., Datta, S.K., Fermin, A., Pitha, P.M., Broide, D.H., and Raz, E. (2004). Cutting edge: activation of Toll-like receptor 2 induces a Th2 immune response and promotes experimental asthma. J Immunol *172*, 2739-2743.

Remy, I., Wilson, I.A., and Michnick, S.W. (1999). Erythropoietin receptor activation by a ligand-induced conformation change. Science *283*, 990-993.

Rescigno, M., Granucci, F., Citterio, S., Foti, M., and Ricciardi-Castagnoli, P. (1999). Coordinated events during bacteria-induced DC maturation. Immunol Today *20*, 200-203.

Rhen, T., and Cidlowski, J.A. (2005). Antiinflammatory action of glucocorticoids--new mechanisms for old drugs. N Engl J Med *353*, 1711-1723.

Rodriguez, A., Griffiths-Jones, S., Ashurst, J.L., and Bradley, A. (2004). Identification of mammalian microRNA host genes and transcription units. Genome Res *14*, 1902-1910.

Ruben, S.M., Klement, J.F., Coleman, T.A., Maher, M., Chen, C.H., and Rosen, C.A. (1992). I-Rel: a novel rel-related protein that inhibits NF-kappa B transcriptional activity. Genes Dev *6*, 745-760.

Ruby, J.G., Jan, C.H., and Bartel, D.P. (2007). Intronic microRNA precursors that bypass Drosha processing. Nature *448*, 83-86.

Ryseck, R.P., Bull, P., Takamiya, M., Bours, V., Siebenlist, U., Dobrzanski, P., and Bravo, R. (1992). RelB, a new Rel family transcription activator that can interact with p50-NF-kappa B. Mol Cell Biol *12*, 674-684.

Saccani, S., Marazzi, I., Beg, A.A., and Natoli, G. (2004). Degradation of promoter-bound p65/RelA is essential for the prompt termination of the nuclear factor kappaB response. J Exp Med *200*, 107-113.

Saccani, S., Pantano, S., and Natoli, G. (2003). Modulation of NF-kappaB activity by exchange of dimers. Mol Cell *11*, 1563-1574.

Sakurai, H., Nishi, A., Sato, N., Mizukami, J., Miyoshi, H., and Sugita, T. (2002). TAK1-TAB1 fusion protein: a novel constitutively active mitogen-activated protein kinase kinase kinase that stimulates AP-1 and NF-kappaB signaling pathways. Biochem Biophys Res Commun *297*, 1277-1281.

Saraiva, M., and O'Garra, A. (2010). The regulation of IL-10 production by immune cells. Nat Rev Immunol *10*, 170-181.

Sato, S., Sanjo, H., Takeda, K., Ninomiya-Tsuji, J., Yamamoto, M., Kawai, T., Matsumoto, K., Takeuchi, O., and Akira, S. (2005). Essential function for the kinase TAK1 in innate and adaptive immune responses. Nat Immunol *6*, 1087-1095.

Satoh, M., Tabuchi, T., Minami, Y., Takahashi, Y., Itoh, T., and Nakamura, M. (2011). Expression of let-7i is associated with Toll-like receptor 4 signal in coronary artery disease: Effect of statins on let-7i and Toll-like receptor 4 signal. Immunobiology.

Schaeffer, H.J., and Weber, M.J. (1999). Mitogen-activated protein kinases: specific messages from ubiquitous messengers. Mol Cell Biol *19*, 2435-2444.

Schindler, C., and Strehlow, I. (2000). Cytokines and STAT signaling. Adv Pharmacol *47*, 113-174.

Schnitger, A.K., Machova, A., Mueller, R.U., Androulidaki, A., Schermer, B., Pasparakis, M., Kronke, M., and Papadopoulou, N. (2011). Listeria monocytogenes Infection in Macrophages Induces Vacuolar-Dependent Host miRNA Response. PLoS One *6*, e27435.

Selbach, M., Schwanhausser, B., Thierfelder, N., Fang, Z., Khanin, R., and Rajewsky, N. (2008). Widespread changes in protein synthesis induced by microRNAs. Nature *455*, 58-63.

Sen, R., and Baltimore, D. (1986). Multiple nuclear factors interact with the immunoglobulin enhancer sequences. Cell *46*, 705-716.

Senftleben, U., Cao, Y., Xiao, G., Greten, F.R., Krahn, G., Bonizzi, G., Chen, Y., Hu, Y., Fong, A., Sun, S.C.*, et al.* (2001). Activation by IKKalpha of a second, evolutionary conserved, NF-kappa B signaling pathway. Science *293*, 1495-1499.

Seong, S.Y., and Matzinger, P. (2004). Hydrophobicity: an ancient damage-associated molecular pattern that initiates innate immune responses. Nat Rev Immunol *4*, 469-478.

Serbina, N.V., Jia, T., Hohl, T.M., and Pamer, E.G. (2008). Monocyte-mediated defense against microbial pathogens. Annu Rev Immunol *26*, 421-452.

Serbina, N.V., and Pamer, E.G. (2006). Monocyte emigration from bone marrow during bacterial infection requires signals mediated by chemokine receptor CCR2. Nat Immunol *7*, 311-317.

Seshadri, S., Kannan, Y., Mitra, S., Parker-Barnes, J., and Wewers, M.D. (2009). MAIL regulates human monocyte IL-6 production. J Immunol *183*, 5358-5368.

Sharbati, J., Lewin, A., Kutz-Lohroff, B., Kamal, E., Einspanier, R., and Sharbati, S. (2011). Integrated microRNA-mRNA-analysis of human monocyte derived macrophages upon Mycobacterium avium subsp. hominissuis infection. PLoS One *6*, e20258.

Shaw, G., and Kamen, R. (1986). A conserved AU sequence from the 3' untranslated region of GM-CSF mRNA mediates selective mRNA degradation. Cell *46*, 659-667.

Shi, Y., and Massague, J. (2003). Mechanisms of TGF-beta signaling from cell membrane to the nucleus. Cell *113*, 685-700.

Shibata, M., Nakao, H., Kiyonari, H., Abe, T., and Aizawa, S. (2011). MicroRNA-9 regulates neurogenesis in mouse telencephalon by targeting multiple transcription factors. J Neurosci *31*, 3407-3422.

Smirnova, L., Grafe, A., Seiler, A., Schumacher, S., Nitsch, R., and Wulczyn, F.G. (2005). Regulation of miRNA expression during neural cell specification. Eur J Neurosci *21*, 1469-1477.

Smythies, L.E., Sellers, M., Clements, R.H., Mosteller-Barnum, M., Meng, G., Benjamin, W.H., Orenstein, J.M., and Smith, P.D. (2005). Human intestinal macrophages display profound inflammatory anergy despite avid phagocytic and bacteriocidal activity. J Clin Invest *115*, 66-75.

Soehnlein, O., and Lindbom, L. (2010). Phagocyte partnership during the onset and resolution of inflammation. Nat Rev Immunol *10*, 427-439.

Solan, N.J., Miyoshi, H., Carmona, E.M., Bren, G.D., and Paya, C.V. (2002). RelB cellular regulation and transcriptional activity are regulated by p100. J Biol Chem *277*, 1405-1418.

Sontheimer, E.J. (2005). Assembly and function of RNA silencing complexes. Nat Rev Mol Cell Biol *6*, 127-138.

Stark, G.R., Kerr, I.M., Williams, B.R., Silverman, R.H., and Schreiber, R.D. (1998). How cells respond to interferons. Annu Rev Biochem *67*, 227-264.

Stein, M., Keshav, S., Harris, N., and Gordon, S. (1992). Interleukin 4 potently enhances murine macrophage mannose receptor activity: a marker of alternative immunologic macrophage activation. J Exp Med *176*, 287-292.

Stoecklin, G., and Anderson, P. (2006). Posttranscriptional mechanisms regulating the inflammatory response. Adv Immunol *89*, 1-37.

Suffredini, A.F., Fantuzzi, G., Badolato, R., Oppenheim, J.J., and O'Grady, N.P. (1999). New insights into the biology of the acute phase response. J Clin Immunol *19*, 203-214.

Suffredini, A.F., Reda, D., Banks, S.M., Tropea, M., Agosti, J.M., and Miller, R. (1995). Effects of recombinant dimeric TNF receptor on human inflammatory responses following intravenous endotoxin administration. J Immunol *155*, 5038-5045.

Sumazin, P., Yang, X., Chiu, H.S., Chung, W.J., Iyer, A., Llobet-Navas, D., Rajbhandari, P., Bansal, M., Guarnieri, P., Silva, J.*, et al.* (2011). An extensive microRNA-mediated network of RNA-RNA interactions regulates established oncogenic pathways in glioblastoma. Cell *147*, 370-381.

Sun, S.C., Asling, B., and Faye, I. (1991). Organization and expression of the immunoresponsive lysozyme gene in the giant silk moth, Hyalophora cecropia. J Biol Chem *266*, 6644-6649.

Surdziel, E., Cabanski, M., Dallmann, I., Lyszkiewicz, M., Krueger, A., Ganser, A., Scherr, M., and Eder, M. (2011). Enforced expression of miR-125b affects myelopoiesis by targeting multiple signaling pathways. Blood *117*, 4338-4348.

Taganov, K.D., Boldin, M.P., Chang, K.J., and Baltimore, D. (2006). NF-kappaB-dependent induction of microRNA miR-146, an inhibitor targeted to signaling proteins of innate immune responses. Proc Natl Acad Sci U S A *103*, 12481-12486.

Takeuchi, M., Alard, P., and Streilein, J.W. (1998). TGF-beta promotes immune deviation by altering accessory signals of antigen-presenting cells. J Immunol *160*, 1589-1597.

Takeuchi, O., Hoshino, K., Kawai, T., Sanjo, H., Takada, H., Ogawa, T., Takeda, K., and Akira, S. (1999). Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. Immunity *11*, 443-451.

Tamassia, N., Le Moigne, V., Calzetti, F., Donini, M., Gasperini, S., Ear, T., Cloutier, A., Martinez, F.O., Fabbri, M., Locati, M.*, et al.* (2007). The MyD88-independent pathway is not mobilized in human neutrophils stimulated via TLR4. J Immunol *178*, 7344-7356.

Tamassia, N., Le Moigne, V., Rossato, M., Donini, M., McCartney, S., Calzetti, F., Colonna, M., Bazzoni, F., and Cassatella, M.A. (2008). Activation of an immunoregulatory and antiviral gene expression program in poly(I:C)-transfected human neutrophils. J Immunol *181*, 6563-6573.

Tang, Y., Luo, X., Cui, H., Ni, X., Yuan, M., Guo, Y., Huang, X., Zhou, H., de Vries, N., Tak, P.P.*, et al.* (2009). MicroRNA-146A contributes to abnormal activation of the type I interferon pathway in human lupus by targeting the key signaling proteins. Arthritis Rheum *60*, 1065-1075.

Tanimura, N., Saitoh, S., Matsumoto, F., Akashi-Takamura, S., and Miyake, K. (2008). Roles for LPS-dependent interaction and relocation of TLR4 and TRAM in TRIF-signaling. Biochem Biophys Res Commun *368*, 94-99.

Tay, Y., Kats, L., Salmena, L., Weiss, D., Tan, S.M., Ala, U., Karreth, F., Poliseno, L., Provero, P., Di Cunto, F.*, et al.* (2011). Coding-independent regulation of the tumor suppressor PTEN by competing endogenous mRNAs. Cell *147*, 344-357.

Taylor, P.R., and Gordon, S. (2003). Monocyte heterogeneity and innate immunity. Immunity *19*, 2-4.

Ten Dijke, P., Goumans, M.J., Itoh, F., and Itoh, S. (2002). Regulation of cell proliferation by Smad proteins. J Cell Physiol *191*, 1-16.

Thomas, M., Lieberman, J., and Lal, A. (2010). Desperately seeking microRNA targets. Nat Struct Mol Biol *17*, 1169-1174.

Tili, E., Michaille, J.J., Cimino, A., Costinean, S., Dumitru, C.D., Adair, B., Fabbri, M., Alder, H., Liu, C.G., Calin, G.A.*, et al.* (2007). Modulation of miR-155 and miR-125b levels following lipopolysaccharide/TNF-alpha stimulation and their possible roles in regulating the response to endotoxin shock. J Immunol *179*, 5082-5089.

Tserel, L., Runnel, T., Kisand, K., Pihlap, M., Bakhoff, L., Kolde, R., Peterson, H., Vilo, J., Peterson, P., and Rebane, A. (2011). MicroRNA expression profiles of human blood monocyte-derived dendritic cells and macrophages reveal miR-511 as putative positive regulator of Toll-like receptor 4. J Biol Chem *286*, 26487-26495.

Turner, M., Chantry, D., and Feldmann, M. (1990). Transforming growth factor beta induces the production of interleukin 6 by human peripheral blood mononuclear cells. Cytokine *2*, 211-216.

Ulevitch, R.J., and Tobias, P.S. (1995). Receptor-dependent mechanisms of cell stimulation by bacterial endotoxin. Annu Rev Immunol *13*, 437-457.

Ulevitch, R.J., Tobias, P.S., and Mathison, J.C. (1984). Regulation of the host response to bacterial lipopolysaccharides. Fed Proc *43*, 2755-2759.

van der Goes, A., Hoekstra, K., van den Berg, T.K., and Dijkstra, C.D. (2000). Dexamethasone promotes phagocytosis and bacterial killing by human monocytes/macrophages in vitro. J Leukoc Biol *67*, 801-807.

van Furth, R., and Cohn, Z.A. (1968). The origin and kinetics of mononuclear phagocytes. J Exp Med *128*, 415-435.

Vasudevan, S., Tong, Y., and Steitz, J.A. (2007). Switching from repression to activation: microRNAs can up-regulate translation. Science *318*, 1931-1934.

Visone, R., Pallante, P., Vecchione, A., Cirombella, R., Ferracin, M., Ferraro, A., Volinia, S., Coluzzi, S., Leone, V., Borbone, E.*, et al.* (2007). Specific microRNAs are downregulated in human thyroid anaplastic carcinomas. Oncogene *26*, 7590-7595.

Wahl, S.M., Allen, J.B., Weeks, B.S., Wong, H.L., and Klotman, P.E. (1993). Transforming growth factor beta enhances integrin expression and type IV collagenase secretion in human monocytes. Proc Natl Acad Sci U S A *90*, 4577-4581.

Wahl, S.M., Hunt, D.A., Wakefield, L.M., McCartney-Francis, N., Wahl, L.M., Roberts, A.B., and Sporn, M.B. (1987). Transforming growth factor type beta

induces monocyte chemotaxis and growth factor production. Proc Natl Acad Sci U S A *84*, 5788-5792.

Wang, C., Deng, L., Hong, M., Akkaraju, G.R., Inoue, J., and Chen, Z.J. (2001). TAK1 is a ubiquitin-dependent kinase of MKK and IKK. Nature *412*, 346-351.

Wang, G., Mao, W., Zheng, S., and Ye, J. (2009). Epidermal growth factor receptor-regulated miR-125a-5p--a metastatic inhibitor of lung cancer. FEBS J *276*, 5571-5578.

Weber, A.N., Moncrieffe, M.C., Gangloff, M., Imler, J.L., and Gay, N.J. (2005). Ligand-receptor and receptor-receptor interactions act in concert to activate signaling in the Drosophila toll pathway. J Biol Chem *280*, 22793-22799.

Webster, J.C., Oakley, R.H., Jewell, C.M., and Cidlowski, J.A. (2001). Proinflammatory cytokines regulate human glucocorticoid receptor gene expression and lead to the accumulation of the dominant negative beta isoform: a mechanism for the generation of glucocorticoid resistance. Proc Natl Acad Sci U S A *98*, 6865-6870.

Webster, J.I., Tonelli, L., and Sternberg, E.M. (2002). Neuroendocrine regulation of immunity. Annu Rev Immunol *20*, 125-163.

Williams, L.M., Ricchetti, G., Sarma, U., Smallie, T., and Foxwell, B.M. (2004). Interleukin-10 suppression of myeloid cell activation--a continuing puzzle. Immunology *113*, 281-292.

Williams, M.J. (2007). Drosophila hemopoiesis and cellular immunity. J Immunol *178*, 4711-4716.

Winter, J., Jung, S., Keller, S., Gregory, R.I., and Diederichs, S. (2009). Many roads to maturity: microRNA biogenesis pathways and their regulation. Nat Cell Biol *11*, 228-234.

Witwer, K.W., Sisk, J.M., Gama, L., and Clements, J.E. (2010). MicroRNA regulation of IFN-beta protein expression: rapid and sensitive modulation of the innate immune response. J Immunol *184*, 2369-2376.

Wu, J., and Xie, X. (2006). Comparative sequence analysis reveals an intricate network among REST, CREB and miRNA in mediating neuronal gene expression. Genome Biol *7*, R85.

Xiao, G., Harhaj, E.W., and Sun, S.C. (2001). NF-kappaB-inducing kinase regulates the processing of NF-kappaB2 p100. Mol Cell *7*, 401-409.

Yamada, M., Gomez, J.C., Chugh, P.E., Lowell, C.A., Dinauer, M.C., Dittmer, D.P., and Doerschuk, C.M. (2011). Interferon-gamma production by neutrophils during bacterial pneumonia in mice. Am J Respir Crit Care Med *183*, 1391-1401.

Yamamoto, M., Yamazaki, S., Uematsu, S., Sato, S., Hemmi, H., Hoshino, K., Kaisho, T., Kuwata, H., Takeuchi, O., Takeshige, K.*, et al.* (2004). Regulation of Toll/IL-1-receptor-mediated gene expression by the inducible nuclear protein IkappaBzeta. Nature *430*, 218-222.

Yekta, S., Shih, I.H., and Bartel, D.P. (2004). MicroRNA-directed cleavage of HOXB8 mRNA. Science *304*, 594-596.

Yi, A.K., Yoon, J.G., Yeo, S.J., Hong, S.C., English, B.K., and Krieg, A.M. (2002). Role of mitogen-activated protein kinases in CpG DNA-mediated IL-10 and IL-12 production: central role of extracellular signal-regulated kinase in the negative feedback loop of the CpG DNA-mediated Th1 response. J Immunol *168*, 4711-4720.

Yoneyama, M., Kikuchi, M., Natsukawa, T., Shinobu, N., Imaizumi, T., Miyagishi, M., Taira, K., Akira, S., and Fujita, T. (2004). The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. Nat Immunol *5*, 730-737.

Yu, L., Hebert, M.C., and Zhang, Y.E. (2002). TGF-beta receptor-activated p38 MAP kinase mediates Smad-independent TGF-beta responses. EMBO J *21*, 3749-3759.

Yudt, M.R., and Cidlowski, J.A. (2001). Molecular identification and characterization of a and b forms of the glucocorticoid receptor. Mol Endocrinol *15*, 1093-1103.

Yue, J., and Mulder, K.M. (2000). Activation of the mitogen-activated protein kinase pathway by transforming growth factor-beta. Methods Mol Biol *142*, 125-131.

Zhang, M., Liu, F., Jia, H., Zhang, Q., Yin, L., Liu, W., Li, H., Yu, B., and Wu, J. (2011). Inhibition of microRNA let-7i depresses maturation and functional state of dendritic cells in response to lipopolysaccharide stimulation via targeting suppressor of cytokine signaling 1. J Immunol *187*, 1674-1683.

Zhang, Y., and Dong, C. (2007). Regulatory mechanisms of mitogen-activated kinase signaling. Cell Mol Life Sci *64*, 2771-2789.

Zhao, X., Tang, Y., Qu, B., Cui, H., Wang, S., Wang, L., Luo, X., Huang, X., Li, J., Chen, S.*, et al.* (2010). MicroRNA-125a contributes to elevated inflammatory chemokine RANTES levels via targeting KLF13 in systemic lupus erythematosus. Arthritis Rheum *62*, 3425-3435.

Acknowledgements

Ci sono innumerevoli persone che vorrei ringraziare, a cominciare dalle persone senza le quali non avrei mai neanche potuto cominciare questo percorso.

Ringrazio il prof. Alberto Mantovani, coordinatore della nostra scuola di Dottorato nonchè direttore scientifico dell'Istituto Clinico Humanitas, in cui è stata svolta interamente questa tesi.

Un ringraziamento sentito va al prof. Massimo Locati. Al di là della possibilità di svolgere questa tesi presso il suo laboratorio, Massimo mi ha insegnato cosa vuol dire pensare in maniera scientifica. Molto di più: col tempo mi sono resa conto che se mi ponevo certe domande era solo perchè lui se le poneva prima di me. Di questo, non posso che ringraziarlo. Per quello che sono riuscita a scrivere qui e di quello che potrò fare con questo insegnamento.

Un gigantesco grazie insieme ad un forte abbraccio va a Daniele Gaudiosi. Con lui ho avuto la fortuna di lavorare per qualche mese, all'inizio, ma posso dire di aver imparato da lui quasi tutto quello che so fare ora. Oltre ad avere guadagnato un'amico, che ancora adesso è pronto ad ascoltarmi all'occorrenza.

Sulla stessa linea, ringrazio tanto anche Emanuela Galliera, che è stata per la biologia cellulare quello che Daniele è stato per la biologia molecolare. Anche lei, ancora oggi, un'amica.

Fra i colleghi, tanti sono i nomi che mi vengono in mente. Luca, bioinformatico ma soprattutto grande attore e poeta, oltre che fonte di alcune delle più belle risate nate in Humanitas. Nina, ora lontana in termini di spazio ma sempre stata vicina, al bancone così come durante alcuni pomeriggi dedicati allo shopping. Bedshi, my indian friend: with her I learnt english, I learnt what the real faith means, I saw in her a simple, kind and quiet person and a good friend in the same time; she is really special. Samy, con le sue ansie ma soprattutto con le sue risate. Ivan, con la sua amicizia spontanea e le sue opere d'arte in cucina. Ed Elisa: insieme abbiamo disegnato un progetto, l'abbiamo visto nascere e piano piano realizzarsi, abbiamo cantato e ballato (!) in laboratorio per festeggiare i risultati o per allontanare le ansie; è stata e mi auguro sempre sarà una delle mie più grandi amiche.

Fra i collaboratori, non posso che ringraziare tutto il gruppo dei veronesi, Flavia e Marzia in particolare, nonostante e soprattutto per le ultime "pressioni"; il

gruppo dei romani, Claudia e Valerio soprattutto, che mia hanno insegnato a maneggiare virus; Graziella, Mariacristina e Max.

Tante, tantissime le persone a cui va il mio pensiero al di fuori di Humanitas. Prima di tutto alla mia famiglia: le risate e le distrazioni con Luca, il sostegno della mamma e l'appoggio di papà. Senza di voi non avrei mai potuto essere qui.

A Ubaldo. Non ho parole, e lo sa bene, per dire quello che è stato e che sempre sarà, per me, la sua presenza e il suo appoggio.

A tutti gli amici del gruppo fotografico ma soprattutto a Mario, con la sua forza; ai futuri Dott. Andrea e Dott. chirurgo Matteo, di una dolcezza sconcertante, pronti a chiedere e a curiosare tra le pagine di questa tesi; ai tecnici Marco, Aurelio, Roberto e Maurizio, ognuno di loro ha contribuito alla mia formazione photoshoppista e quindi alla realizzazione delle immagini di questa tesi; e a tutti gli altri, che formano davvero un gruppo inimitabile.

A tutti voi, il mio ringraziamento perchè senza di voi sarebbe stato molto più difficile, e temo impossibile, raggiungere questo incredibile traguardo.