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NONCODING-RNAs IN MACROPHAGE POLARIZATION:
THE RELEVANCE OF THE "JUNK" RNA

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Lead me where my trust is without borders

Let me walk upon the waters

Wherever You would call me

Take me deeper than my feet could ever wander

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Overview

The work presented in this thesis is concerned with the function of the noncoding RNAs in macrophage polarization, a biological process thinly regulated. For this reason noncoding RNAs are becoming more and more relevant. Several studies have explored this issue, identifying some microRNAs (small noncoding RNAs) involved in the inflammatory response and macrophage activation. Macrophages are the key cell in innate immunity system that are essential in on set and resolve inflammation. Moreover macrophages sustain tissue integrity during development such as, bone morphogenesis, and adipose tissue generation after damage. They perform these function due to their responsiveness to the surrounding microenvironment. On the base of the different stimuli macrophage may undergo to different polarity, changing in turn the run of the inflammation. Understanding the mechanism underline macrophage plasticity and macrophage re-programming is the aim of this study.

To better elucidate the aim of this work, the thesis has been dived in three macro chapters. Chapter 1 is focused on the characterization of specific miRNA patens in human macrophages, activated *in vitro* towards of a classic (M1) or alternative (M2) phenotype. From the screening is emerged a de novo expressed microRNA, miR-135b in classic activated macrophage, which is able to damp M2 phenotype in favor of the M1 thru the direct targeting of important M2 transcription factors, c-MYC, STAT6 and KLF4.

Chapter 2 emphasizes the role of miR-135b in macrophage activation, analyzing its chromatin occupancy upon TRL triggered. From this investigation has been highlighted the presence of an lncRNA, BLACAT1, miR-135b's host gene. Surprising the induction of these RNAs is not correlated; on the contrary BLACAT1 is induced by anti-inflammatory stimulation. These results glimpse a further step of regulation promoted by lncRNAs

Chapter 3 highlights the role of miRNAs in the gouty arthritis, a pathology characterized by the accumulation on the joint of MSU crystals. Innate immunity cells and in particular macrophage are mainly involved in its progression. Using this paradigm we typified the miRNome on *in vivo* setting. Out the miRNAs modulated with the progression of gout, we identified miR-135b. We have demonstrated that in BMDMs miR-135b is IL- 1R1-

dependent, capable of inhibits the same pathway by the direct target with MyD88, IL-1RAP, and IL-.1R.

Chapter: MicroRNA-135b: the pivot of the macrophage polarization balance.

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INTRODUCTION

Macrophage activation and polarization process

Macrophage activation and polarization

Macrophages are monocyte-derived myeloid cells able to acquire distinct functional phenotypes depending on the microenvironment. They play an essential role in the immunity system, sustaining tissue integrity during development such as, bone morphogenesis, and adipose tissue generation and after damage. Moreover, they are fundamental players in onset and resolve the inflammation, interacting with lymphocytes T and B. It is also clear that macrophages are involved in many chronic diseases such as atherosclerosis, asthma, rheumatoid arthritis. In these pathologies, they could be seen as an important source of inflammatory cytokines (IL12, IL18, IL23, and TNFα [1]) that driven autoimmune inflammation.

The macrophage polarization is a concept introduced by Stein and colleagues in 1992, after the observation that macrophages exposed to IL-4 promote an alternative activation with a different phenotype from the classic one [2]. Afterwards, Mantovani and colleagues have schematically categorized macrophages in "classical" (M1) activated, which are triggered with microbial stimuli (e.g. LPS) alone or together with the TLR eliciting; and "alternative" (M2) cell types that are activated by IL-4/IL-13 [3] (Figure 1. 1). Thus, the M1-M2 nomenclature is derived from the Th1 and Th2 cytokines associated with these macrophage phenotypes, however the activation state is more broadly. Nowadays has been highlighted that "M2-like" phenotype is endorsed by various stimuli such as, glucocorticoids, IL-10, transforming growth factor-β (TGF-β) and immunoglobulin complex [4].

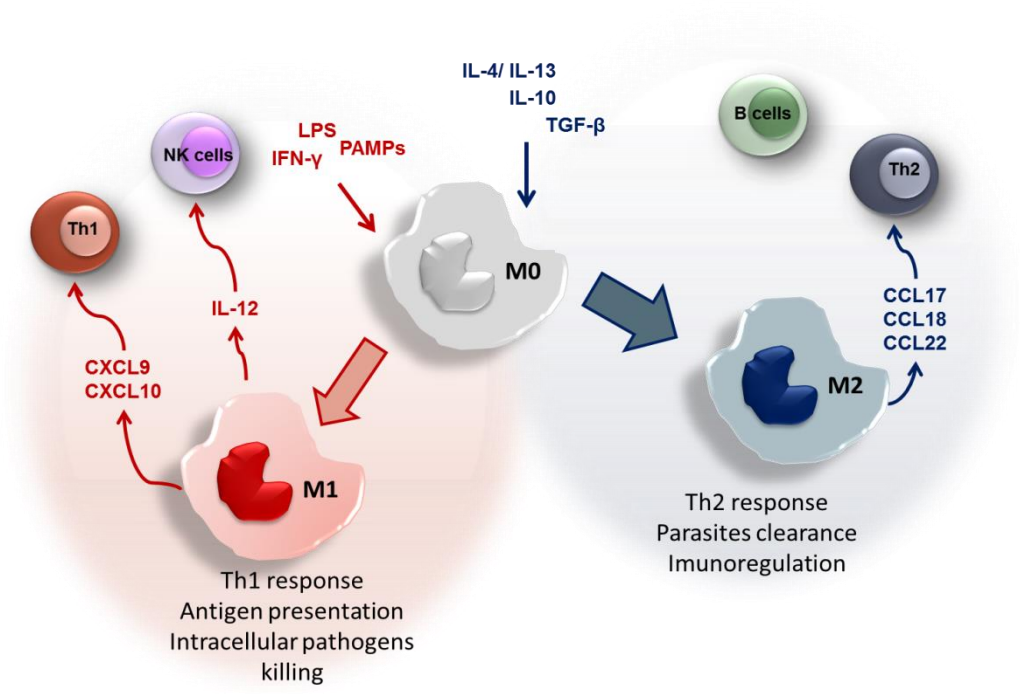

Figure 1. 1 Key properties and functions of polarized macrophages.

Functionally M1 are characterized by the expression of pro-inflammatory cytokines (e.g. IL1β, IL6, IL12, IL23 and TNF) and effector molecules such as reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI) which guide the Th1 responses, and have tumoricidal capacity. Thus, the classical activation could be seen as the first line of defense against intracellular pathogens. While, M2 macrophages are associated to Th2 responses, they produce immune regulatory factors (e.g. IL10 and TGFβ1) that dampen the immune response. In addition, M2 cells ensure pro-tumoural effects, through the production of tumor growth factor, (e.g. EGF, FGF1, TGFβ1), they promote angiogenesis and matrix remodeling (e.g. VEGFA, FGF1 and MMPs) [5].

Regulation of macrophage activation and polarization

Macrophage dichotomy, M1 and M2, is useful representation that denotes the cell plasticity. It is extensively demonstrate that macrophages switch their phenotype and

physiology in response to environmental insults, in a process named re-programming [6]. It was highlighted with i*n vitro* experiments, LPS-activated macrophages become hyporesponsive to a second inflammatory hit [7]. This phenomenon, called endotoxin tolerance, cause the production of anti-inflammatory cytokines such as IL-10 and TGF-β, the gene expression switching from a pro-inflammatory M1 signature to an M2-like anti-inflammatory phenotype [7].

In angiogenesis (a fundamental process that occurs in physiological wound healing and tumor progression), macrophages are recruited to wound or tumor site release angiogenic cytokines and promote the vascular supply. Stress factors produced by hypoxia and altered metabolism stimulate macrophages to switch from an inflammatory to a resolving phenotype. Especially in cancer a high number of macrophages contributes to the ongoing growth, neovascularization, and metastasis of malignant cells [8]. It is clear the cell plasticity is an important phenomenon. The macrophage skewing is a process that ensues *in vivo* under physiological (e.g. ontogenesis and pregnancy) and in pathological conditions (infection, cancer and allergy) [5] and is thinly regulated at transcriptional and post-transcriptional level.

Transcriptional regulation

M1 signaling network

A signaling network underlies the different macrophage phonotypes; however, IRF/STAT signaling is the core pathway in macrophage polarization. Lipopolysaccharide (LPS) and other microbial ligands lead to the activation of TRL4 pathway through two adaptors, MyD88 and TRIF [9]. The signaling through TRIF adaptor activates IFN regulatory factor 1 and 3 (IRF1 and IRF3), promotes expression and emission of type 1 interferon, (IFNα and IFNβ). The released type 1 interferons leads to phosphorylation of STAT1 (signal transducers and activators of transcription 1), supports the expression of inflammatory chemokines such as CCL5, CXCL9 and CXCL10. In addition, STAT1 is activated by IFN-γ (member of the type II interferon) through JAK 1 and 2, and leads to the expression of chemokines, adhesion molecules (ICAM-1) and transcription factors (IRF1 and IRF8) that

mediate the second wave of IFN-γ signaling, leading to CCL5 secretion [10,11]. On the other hand, the signaling pathway downstream TRL4 mediated by MyD88 adaptor guides the activation of NF-κB (nuclear factor kappa B). NF-κB/Rel family is a transcription factor described for the first time Sen and Baltimore in 1986 as a nuclear factor essential for the transcription of immunoglobulin kappa light chain in B cells [12]. The NF-κB family is composed by five members in mammal: p50, p65 (RelA), c-Rel, p52, and RelB. They are present in homo- and hetero-dimeric complexes, which are often maintained in an inactive in cytosol thru I-κB proteins (inhibitor of kappa B kinase) [13]. When I-κBs are degraded, NF-κB is released and translocate to the nucleus, where promotes the synthesis of a large number of inflammatory genes, TNFα, IL1B, cyclooxygenase 2, IL-6, and IL12p40 [14,15]. The NF-κB signaling pathway is also activated by IL-1β.

M2 signaling network

M2 macrophages are activated by IL-4 produced by the Th2 cells, but also by the macrophages themselves [16]. This interleukin is recognized by IL-4Rα1 receptor paired with common gamma chain (γc), leads to STAT6 activation and translocation via JAK 1/3. In addition, this signaling pathway modulates the activation of the transcription factor PPARγ (peroxisome proliferator-activated receptor γ), that cause the inhibition of M1 response by blocking NF-κB and AP-1 [17]. PPARγ regulates the metabolism of fatty acid, promots the aerobic respiration that occurs in the alternative activated macrophages [18]. Moreover, STAT6 works in synergy with Krüppel-like factor 4 (KLF4) and sustains M2 gene expression, inhibiting thus the M1 one. KLF4 is a member of zinc finger DNA-binding transcriptional regulators subfamily (KLFs), which plays important roles in several cell processes, including hematopoiesis. Indeed, KLF4 is a critical regulator of macrophage polarization, mediating the sequestration of NF-κB co-activator such as p300/CBP and associated factors like PCAF. These findings were confirmed by myeloid-specific KLF4-deficient macrophages which shown increased pro-inflammatory gene expression [19]. Another significant transcription factor induced in human alternative activated macrophages is c-MYC, it is a pleiotropic transcription factor with an oncogenic potential involved in frequent processes in non-transformed cells, including lymphocytes, promots cell growth and survival [20]. Oscar M. Pello and colleagues have identified a restrict list of c-MYC-dependent genes in M2 cells. In particular, they assessed a direct c-

MYC interaction with the promoter of ALOX15, MRC1 and SCARB1; and supports also STAT6 and PPARγ [21].

Alternative macrophages are also activated by TGFβ, which is anti-inflammatory cytokines with pleiotropic effects involved in development, carcinogenesis, fibrosis, wound healing and immune responses [22]. TGFβ signaling pathway occurs mainly through phosphorylation of the SMAD proteins. They are composed by 3 functional classes: the receptor-regulated Smad (RSmad); the Co-mediator Smad (Co-Smad); and the inhibitory Smad (I-Smad). The RSmads when phosphorylated form hetero complexes with the Co-Smads. The complex translocates into the nucleus and regulates target genes transcription, such as ARG1 and MGL2 [23]. This complex is antagonized by the inhibitory Smad that competes with R-Smads for receptor or Co-Smad interaction [24]. However, TGFβ reprograms macrophages to M1 phenotype, particularly when the SMAD-dependent pathway is blocked. Indeed TGF-β could activate kinase 1 protein (TAK1), which in turn activates JNK, p38, and NF-κB [25].

Interleukin-10 is anti-inflammatory cytokine that activates M2 macrophages. Its induction is caused by TLRs activation in particular TLR9 and TLR4 in macrophages and dendritic cells [26]. IL-10 is a soluble ligand that binds to a receptor complex composed by IL-10R1 subunit, which is restricted to leukocytes (IL-10Rα in mice) and a signaling subunit ,IL-10R2 (IL-10Rβ in mice), which leads to STAT1 and STAT3 activation *via* Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway [27]. This interleukin inhibits pro-inflammatory responses caused by innate and adaptive immunity, and prevents tissues damaged in case of impaired immune responses . Therefore, IL-10/JAK1/STAT3 axis is an essential homeostatic mechanism that controls the degree and duration of inflammation. Indeed all the major TLR signaling pathways (such as those involving NF-κB, and PI-3K) are targeted by IL-10. For example, TNFα expression is abolish by IL-10 through the up-regulation of Bcl3 (*via* STAT3), which interacts with NF-κB at the TNFα promoter, inhibiting its [28,29]. Macrophages are considered as the most IL-10 responsive cell in the immune system since they express a high level of IL-10R1. In activated macrophages, IL-10 represses the production of pro-inflammatory cytokines (e.g. IL-1, IL-6, IL-12, TNFα) [30], chemokines (i.e. monocyte chemotactic protein 1 (MCP-1) [31], and the production of nitric oxide [32]. Overall, the IL-10

anti-inflammatory effect in macrophages involves ~20% of the LPS-induced genes, including pro-inflammatory mediators such as cell surface receptors, chemokines and cytokines [33]. Due to its relevance in damping the inflammation IL-10 deficiencies are associated with inflammatory diseases. For example, both IL-10 and IL-10R KO mice developed colitis spontaneously and mutations in the IL-10 and IL-10R genes correlate with susceptibility to inflammatory bowel disease (IBD) in human [34-36]. It was also reported that IL-10 inhibits allograft rejection after organ transplantation [37]. These studies highlight the importance of IL-10 in negatively regulating inflammation and autoimmune diseases. On the other hand, increased IL-10 expression has been associated with many chronic bacterial and viral infections. IL-10 represents an important mechanism of immune escape by different pathogens (for instance, the viral IL-10; vIL-10) to suppress the immune responses of the host [38]. Obviously, the IL-10 production control can be used as a therapeutic approach for the treatment of different kinds of infections.

Conclusions on transcriptional regulation

The highlighted transcriptional network stresses that the microenvironment (e.g. growth factors, fatty acids and bacteria) is extremely essential to determine macrophage phenotypes. The activation and macrophage re-polarization are ascribed to 4 phenomena: (1) macrophage are activated and their response is amplified; (2) suppression of the alternate phenotype; (3) the re-polarization process is activated; (4) feedback phenomenon [39]. However, the signaling pathway (schematically represented in Figure 1. 2) works on and in synergy with post-transcriptional regulation.

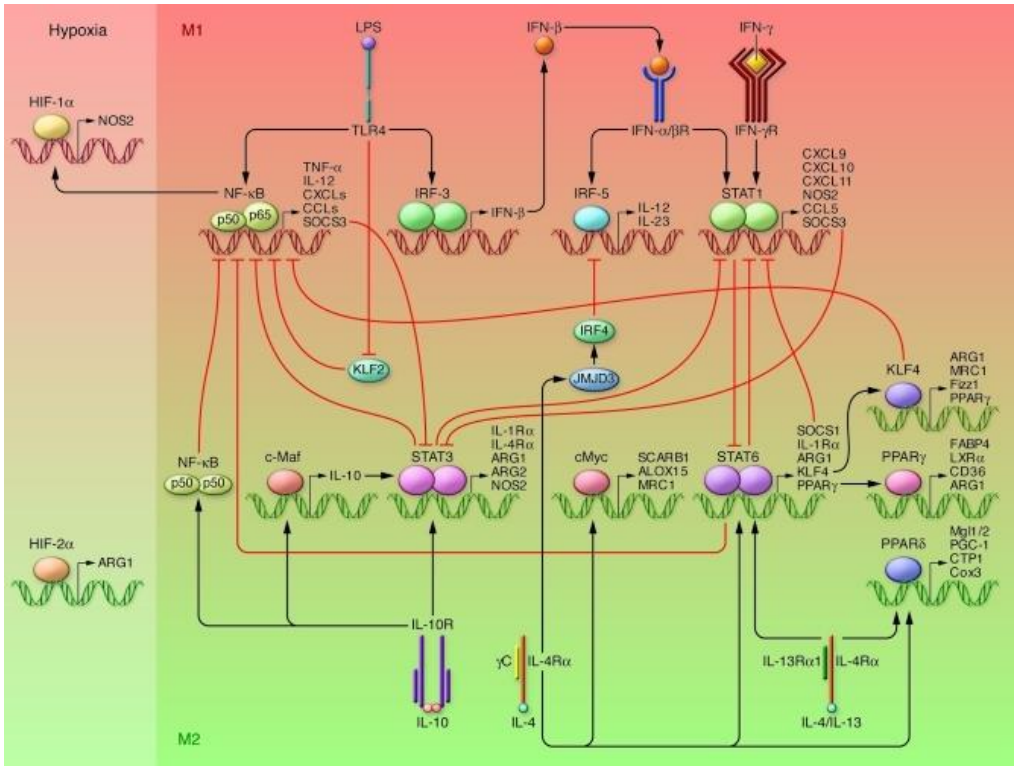

Figure 1. 2 Macrophage transcription network. M1 stimuli trough the TLR4, IFN-α, or IFN-β receptor (IFNAR) and IFN-γ receptor (IFNGR) pathways, promotes NF-κB (p65 and p50), AP-1, IRF3 and STAT1 transcription factors. This leads to the transcription of M1 genes. While M2 stimuli such as IL-4 and IL-13 signal activate STAT6. IL-10R signaling pathway activates STAT3. (Figure adapted from Sica and Mantovani, 2012.

Post-transcriptional regulation: microRNAs

MicroRNAs (miRNAs) are small single-stranded RNAs of 20-24 nucleotides highly conserved among species. They modulate gene expression by the fasten to complementary 3'UTR region of the target mRNA, causing mRNA decay or translation inhibition [40]. Since the first discovered miRNA (lin-4) in Caenorhabditis elegans [41], additional miRNAs have been identified in exponential way across species, more than 700 miRNAs have in mammalian cells (miRNA registry at http://microrna.sanger.ac.uk/sequences). They play critical roles in development such as cell proliferation, differentiation and apoptosis, and their dysregulation causes disease, such as cancer. miRNAs are encoded by genes in primary hairpin RNA transcripts (pri-miRNAs) by RNA polymerase II. This transcript is processed by the protein complex composed by ribonuclease III-type Drosha and its double strand RNA-binding protein

DGCR8 (DiGeorge syndrome critical region gene 8). The obtained pre-miRNAs are actively transported to the cytoplasm by exportin-5, and further processed by Dicer (cytosolic ribonuclease III) in a 22 nt double-stranded miRNA. One strand (the guide strand) is selectively incorporated into the miRNA-induced silencing complex (RISC). The mature miRNA in the RISC interacts with Argonaute (Ago) proteins, and the 3′ UTR (3′ untranslated region) of the target messenger RNAs (mRNAs). The resultant outcome of miRNA- mRNA interaction inside the RISC complex is the translational inhibition, degradation or deadenylation of the target messenger RNAs (Figure 1. 3) [42].

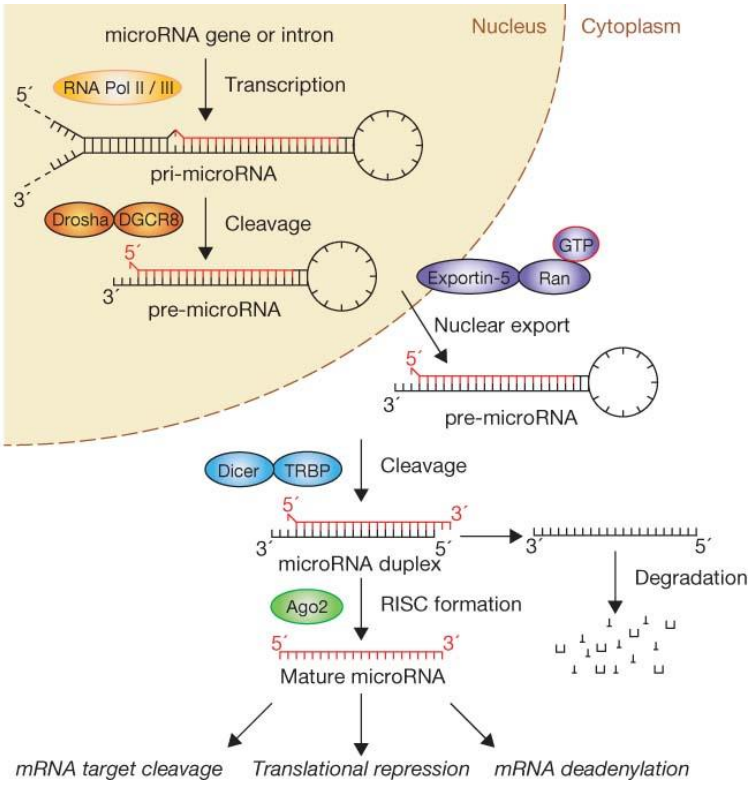

Figure 1. 3 The miRNA processing pathway includes the synthesis of the primary transcript (pri-miRNA) by RNA polymerase and its cleavage by the microprocessor complex Drosha/DGCR8 in the nucleus. The pre-miRNA is exported into the cytosol Exportin-5-Ran-GTP. In the cytoplasm, the Dicer complex cleaves the pre-miRNA into a microRNA duplex. The functional strand of the mature miRNA (red) is loaded 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. Figure from Winter et al., 2009.

microRNAs in the innate immune system

Chen and colleagues pointed out in 2004 the first evidence that miRNAs regulate the immune response with a report which showed selective expression of miR-142a, miR-181a and miR-223 in immune cells [43]. Understanding miRNA profiles is an essential step towards unraveling miRNAs function in macrophage polarization. Different studies tried to evaluate this issue however is emerged an uneven landscape due to species specific and the use of different protocols of macrophage polarization. TLRs signaling and miRNAs regulation in monocytes and macrophages are capable of actuating manifold targeting strategies which modulate expression of key factors in pro- and anti-inflammatory processes [44]. Some miRNAs are associated with M1 cells (miR-155, miR-21, miR-125, miR-9, and miR-146) and are transcribed *via* TLR, whereas some M2 stimuli (glucocorticoids, IL-4, and IL-10) induce the synthesis of miR-511, miR-187 and miR-378 [45].

We discuss more in detail the principal miRNAs involved in this process .

microRNA-155

The first miRNA and most investigated as a pro-inflammatory miRNA is miR-155, which is strongly induced by LPS or Type I interferons in both monocytes and macrophages [46]. miR-155 supports M1 phenotype repressing the negative regulators of pro-inflammatory response, such as SOCS1 establishing an antiviral and antibacterial response [47,48], and BCL6 being involved in atherosclerosis [49-51]. It is well-established that miR-155 sustains TNF-α stability upon LPS stimulation, playing pro-inflammatory function [52]. Moreover, miR-155 is known to suppress M2 phenotype, inhibiting important hallmarks such as C/EBPβ, which controls Arg1, Chi3l3 and the production of IL-10 [53]. The same IL-10 down-regulates miR-155 *via* STAT3 improving the miR-155 target SHIP1 [54,55].miR-155 is self-regulating thru the targeting of AP-1, transcription factor entails in miR-155 transcription. This miRNA shifts the M2 phenotype of TAM (tumor associated macrophages) towards the M1 phenotype and, thereby, enhances the anti-tumoural properties [53]. However, miR-155 is not a mere pro-inflammatory miRNAs, indeed Tang and collogue identified Myd88 as miR-155 target in *H. pylori*-induce inflammation [56].

microRNA-146 family

miR-146 family is composed by miR-146a and miR-146b. miR-146a is an interesting microRNA involved in the innate immunity system, induced by LPS, TNF-α and IL-1β in a NF-κB-dependent manner. Moreover, miR-146a is able to negative regulate TLR signaling by direct targeting of IRAK-1 and TRAF6 [57], being essential in endotoxin tolerance, thus its insufficient induction could lead to a prolonged inflammatory response [58]. miR-146a has been shown to be up-regulated in various inflammatory outcomes such rheumatoid arthritis (RA), and gout [38,59]. Recent evidences have that miR-146a gene single-nucleotide polymorphisms (SNPs) are associated with severe sepsis and other pathologies [60,61]. In addition, our lab identified miR-146b as an IL-10-dependent miRNA with anti-inflammatory activity by direct target of TRL4, Myd88, IRAK-1 and TRAF6 [62].

miR-125 family

miR-125 family includes miR-125a and miR-125b, which share the same "seed" sequence are induced by TLR signaling. Both miRNAs sustain inflammation by targeting the NF-κB negative regulator, TNFα Induced Protein 3 (TNFAIP3, A20), and by IRF4 [63,64]. At the same time miR-125a and miR-125b compromised TNF-α production and transcript stability [65].

miR-511-3p

miR-511-3p is an intronic miRNA of CD206/MRC1(mannose receptor) gene. Highly expressed in M2 and TAM macrophages being co-regulated with the host gene. However it targets Rock2 and IRF4 important transcription factor that activates M2 phenotype [66,67]. miR-511-3p is bona fide M2-associated miRNA capable to modulate different M2 activities, such as cellular proliferation, metabolism and immune responses [68].

Let-7 family

The let-7 is composed by 12 genes, which encode for 9 miRNAs (let-7a to let-7i, miR-98).

Let-7c has been shown to be induced in M-BMM, being critical in the regulation of macrophage polarization thru direct target of C/EBPδ [69]. Another family member, let-7f is up-regulated upon LPS stimulation in murine macrophages. It promotes the production of cytokines such as TNF-α and IL-1β by direct target of A20, a feedback inhibitor of the NF-κB pathway [70].

miR-9, miR-147

Macrophage inflammatory response to infection involves the up-regulation of additional miRNAs, such as, miR-9 and miR-147 [71,72]. These miRNAs can also be induced by TLR signaling, and can negatively regulate activation of inflammatory pathways in myeloid cells. MiR-9 represses NFKB1 [68]. While miR-147 has been shown to attenuate TLR2, TLR3 and TLR4-mediated production of inflammatory proteins such as TNF-α and IL-6 [72].

Conclusions of post-transcriptional regulation

In general, the intracellular network of miRNAs is comparable to the phenomena ascribed to signaling pathway known to be involved in macrophage polarization (Table 1. 1). More in specific way miRNAs can be classified on the base of their activation in M1 or M2 associated, such as miR-155 and miR-146b respectively. However they may provide positive feedback to sustain the macrophage phenotype (e.g. miR-155, miR-187), or negative feedback to limit excessive response (e.g. miR-146a, miR-511), inflammation for M1 or anti-tumoural in case of the M2 phenotype.

Table 1. 1: miRNAs regulate in macrophage polarization

miRNA	Function	Targets
miR-155 [27,47,73]	Promotes M1 phenotype; Inhibits M2 phenotype	SOCS1, SHIP1, BCL6, C/EBPβ, IL-13Rα1, FADD, IKKε, Ripk1, MyD88, AP-1
miR-146a [74, 75]	Promotes endotoxin tolerance; Inhibits M1 phenotype	TRAF6, IRAK1, IL-8, RANTES
miR-146b [74]	Inhibits M1 phenotype	TRAF6, IRAK1
miR-9 [71]	Suppresses M1 activity	NF-κB1
miR-125a [63,64]	Promotes M1 phenotype; Inhibits M2 phenotype	KLF13, A20, IRF4
miR-125b [26]	interfere with M2 activation	IRF4
miR-511-3p [66,67]	negative regulators of the macrophage M2 response.	Rock2, IRF4
let-7c [69,70]	Inhibits M1 phenotype	PAK1, C/EBPδ, A20
miR-187 [76]	Sustains the inhibitory effect of IL-10	TNF-α, NFKBIZ
miR-378	inhibitor of the M2 responses	

AIM OF THE STUDY

Macrophages are critical effectors of onset and resolve the inflammation. Together with dendrite cells macrophages function as antigen-presenting cells (APC), on the other hand they eliminate microbes and tumor cells. They also contribute to tissue repair and remodeling, angiogenesis and so on. They fulfill these functions according to microenvironment stimuli thru the adoption of different spectrum of activation. M1, or classical activated macrophages respond to LPS and IFN-γ, whereas M2, or alternative activation, occurs in response to the cytokines IL-4 and IL-13. This cell plasticity is the key of macrophage efficacy of immune defense. Although, instabilities in macrophage plasticity might compromise immune responses and contribute to different diseases, such as cancer, arthritis and atherosclerosis. Therefore, a profound understanding of regulatory mechanisms undergo macrophage plasticity is critical.

Recently has been demonstrated that post- transcriptional regulation is important in the inflammatory response. The wells know protagonists of this regulation are microRNAs (miRNAs). Some evidences have liked miRNAs to the macrophage activation, sustain one phenotype against the others. The first aim of this work to typify the miRNome of human polarized macrophages to define its contribution on macrophage re-programing process from M1 to M2 phenotype.

Anti-inflammatory cytokine IL-10 that is crucial for damping inflammation induced by Toll-like receptor (TLR) signaling. The second aim of this study was to examine whether IL-10 plays a role in miRNA functions.

RESULTS

miRNome analysis in steady state and activated macrophages.

TLDA TaqMan arrays were used in an attempt to identify miRNA profile on activated macrophages, which might play a role on macrophage polarization. Human monocyte-derived macrophages obtained from three healthy donor buffy coats were cultured for 24h in presence of LPS+IFN-γ (M1) or in IL-4 (M2). The induced expression of M1 and M2 markers was observed to confirm the polarization (data no shown) and 733 miRNAs were profiled. We detected 388 expressed miRNAs in macrophage population. Moreover unsupervised clustering of the miRNA-profiling data revealed distinctive miRNA signatures. The alternative activation (M2) shown an expression pattern extremely similar to the resting macrophages (Figure 1.4A). According with this outcome, 69 miRNAs were differentially expressed among the M1 and M2 phenotypes (Figure 1. 4B). Additionally, 10 miRNAs are highly expressed in classic activated cells, including miR-155 and miR-146b (known as pro-inflammatory miRNAs [46,72]). Furthermore, a limited number of miRNAs were *de novo* expressed in activated macrophages, including miR-135b by LPS+IFN-γ treatment. In agreement with microarray data, in individual miRNA-targeted qPCR assays, miR-135b was induced only by LPS alone or in combination with IFN-γ (Figure 1. 4 C), and displayed delayed kinetics in comparison with has-miR-155 (Figure 1. 4 D), picked at 24h. These data indicated miR-135b as a novel miRNA associated with the classic activated macrophages, which might sustain this phenotype.

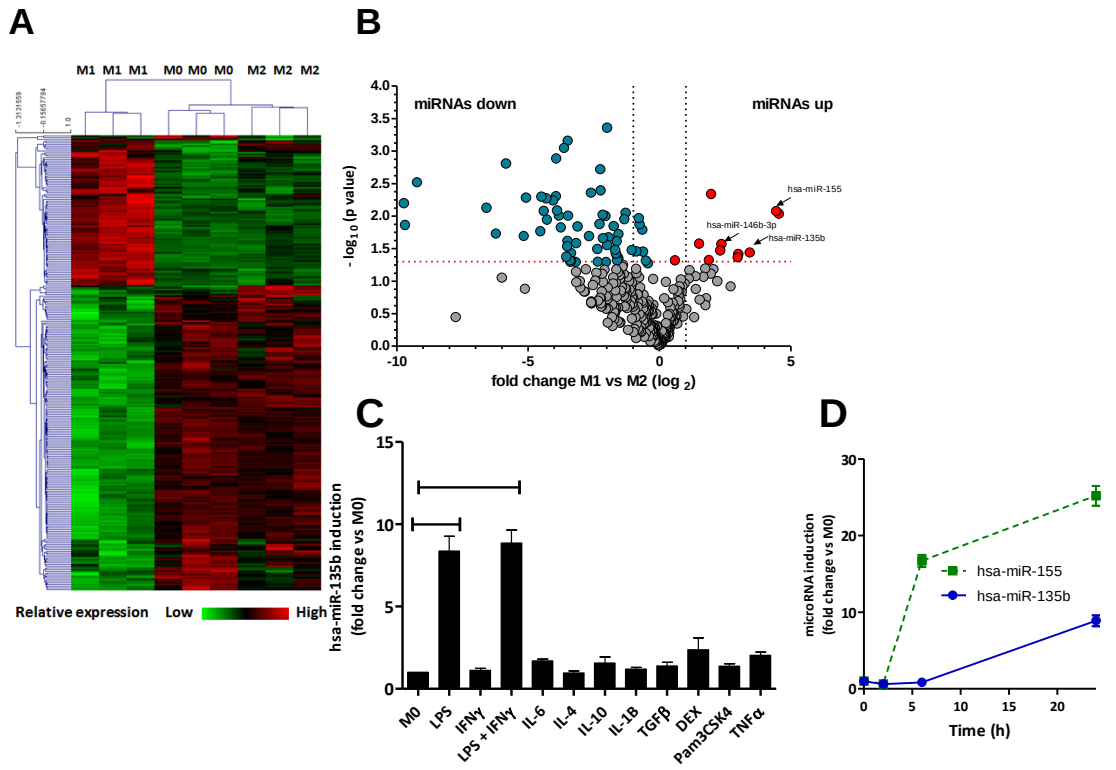

Figure 1. 4 Differentially expressed microRNAs. A, Microarray analysis was conducted on 3 independent preparations of resting macrophages (M0) and macrophages exposed to 100 ng/ml LPS and 20 ng/ml IFN-γ (M1) and to 20 ng/mL of IL-4 (M2) for 24 hours. Cluster analysis of the miRNAs expression (2^{-DCT}) in different macrophage subsets. Red line and green lines shown the expression patterns of up regulated and down regulated miRNAs respectively. **B,** Volcano plot shown a predominant up-regulated miRNAs in M1 macrophages against M2. The x axis shown the difference in relative expression values (log_2 expression values); the y axis represented statistical significance ($-log_{10}$ p-values). Vertical dotted lines represented the threshold for the log_2 fold change (equivalent to a 2 fold change). The red dots corresponded to miRNAs with a significant expression that were up-regulated in M1 macrophages. The blue dots were miRNAs significantly down-regulated in M1 compare with M2 macrophages. **C,** human macrophages were cultured for 24h with 100 ng/ml IL-1β, 20 ng/ml IL-6, 1 μg/ml Pam3CSK4, 20 ng/ml TNFα, 100 ng/ml LPS, 20 ng/ml IFN-γ, 50 ng/ml TGF-β, 50 ng/ml IL-10, 10^{-6} M dexamethasone (DEX) and 20ng/ml IL-4. RNA was extracted and mature miR-135b ware analyzed by qPCR. n =3; mean ± SEM. **D,** kinetic analysis of miR-155, miR-146a and miR-135b in human macrophage stimulated with LPS (100ng/ml) for 2, 6 and 24h**.** Data were expressed as mean ± SEM of expression fold change compared to resting untreated macrophages (M0) of 3 independent experiments. Statistics has been calculated using Bonferroni's multiple test *p<0.05, ****p < 0.0001 compared with resting macrophages.

miR-135b do not activate steady state macrophages

To understand if miR-135b might trigger *per se* macrophages, we transfected resting *in vitro* generated monocyte-derived macrophages (which hypo-expressed miR-135b), with a synthetic microRNA (mimics) for miR-135b and control for 24 h; expression levels of marker genes of macrophage activation were measured by qPCR As shown in **Errore. L'origine riferimento non è stata trovata.**, miR-135b had no modulatory effect, was not able to activate the resting macrophages even though there was a trend of induction forM1 markers (CCL5 and COX2) however results did not reach statistical significance.

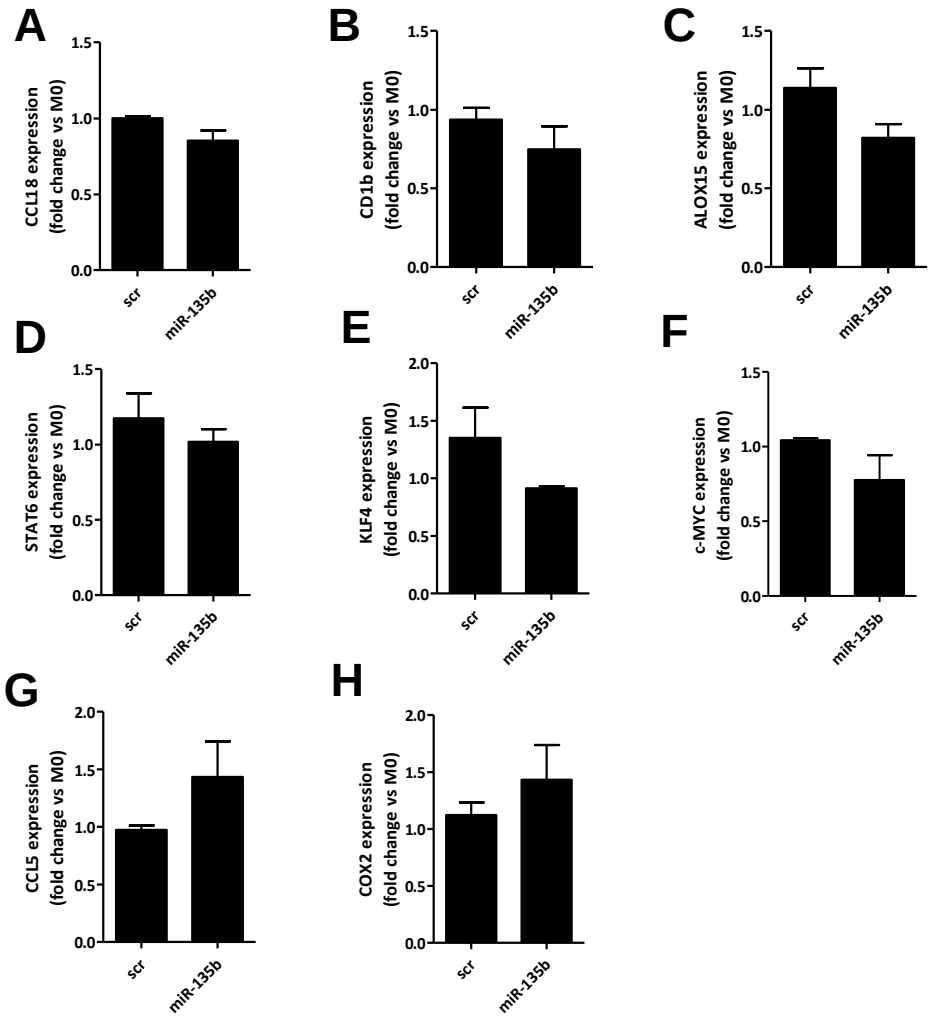

Figure 1. 5 miR-135b is not able to activate steady state macrophages. Resting human macrophages were transfected with 20 nM control mimics or mimics for miR-135b. 24h after transfection, mRNA levels of M2 marker genes (**A-F**) and M1 (**G,H**) were determined by real-time PCR. Data are expressed as fold change n=5; mean ±SEM.

Overexpression of miR-135b enhances the expression of M1 chemokines

To further explore miR-135b influence on activated macrophages we transfected M2 macrophages with control mimics (scr) or miR-135b mimic and after 24h inflammatory chemokines were evaluated by qPCR and ELISA (Figure 1. 6). Enhancer levels of miR-135b increased CCL5, CXCL10 and IL-12p40 secretion levels, compared with control mimics; however they were not comparable to what is produced by M1 macrophages. Thus miR-135b has a moderate effect on the promotion of the M1 phenotypes, but is not able *per se* to divert the M2 phenotype towards M1.

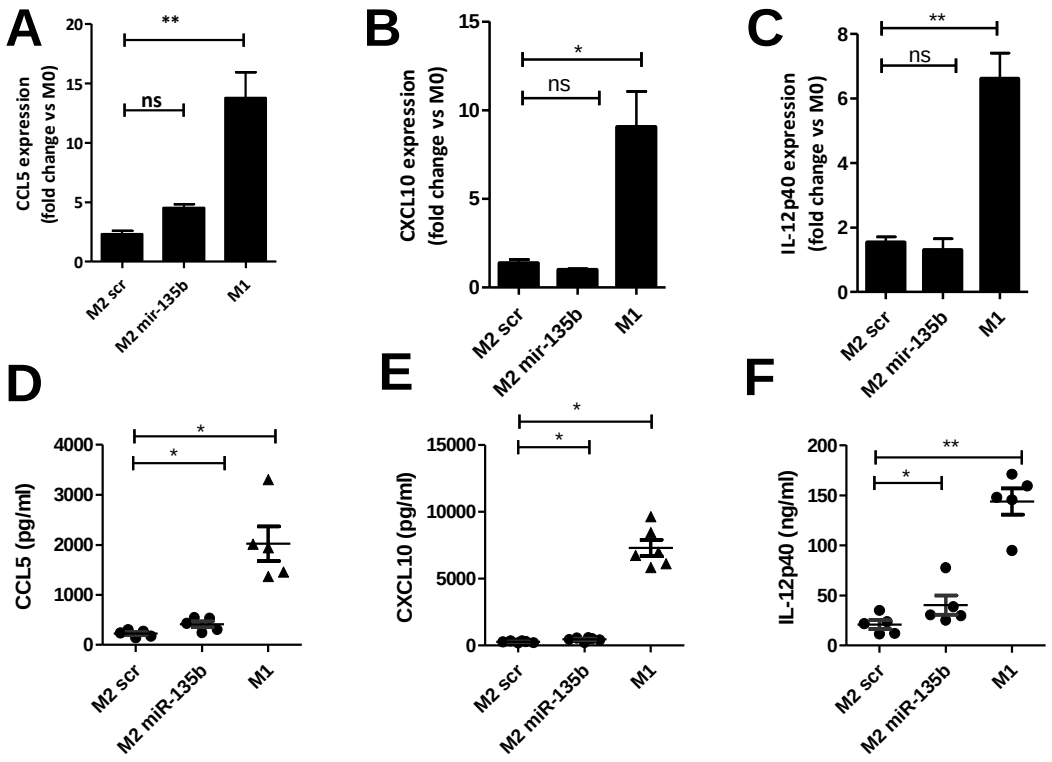

Figure 1. 6 Overexpression of miR-135b enhancer the expression of M1 chemokines. Resting macrophages were transfected with 20nM of control (scr) or miR-135b for 24h, then cells were treated with IL-4 for 24h. Levels of CCL5 (**A**), CXCL10 (**B**) and IL-12p40 (**C**) were determined. n=3; mean ± SEM; ** p<0.01 compare to control (scr). Chemokines secretion were determined in each samples for CCL5 (**D**), CXCL10 (**E**) and IL-12p40 (**F**) by E.L.I.S.A. n=4; mean ± SEM; * p<0.05 compared to control (scr).

On the other hand, M2 marker genes were inhibited by enhancer miR-135b expression (Figure 1. 7 A-F). miR-135b impaired macrophage functionality, thru the inhibition of M2 chemokines CCL17, CCL18 and CCL22, which were reduced as well as in M1 macrophages (Figure 1. 7 H-I). These data confirm that miR-135b damps the alternative phenotype, favoring the pro-inflammatory or classic one.

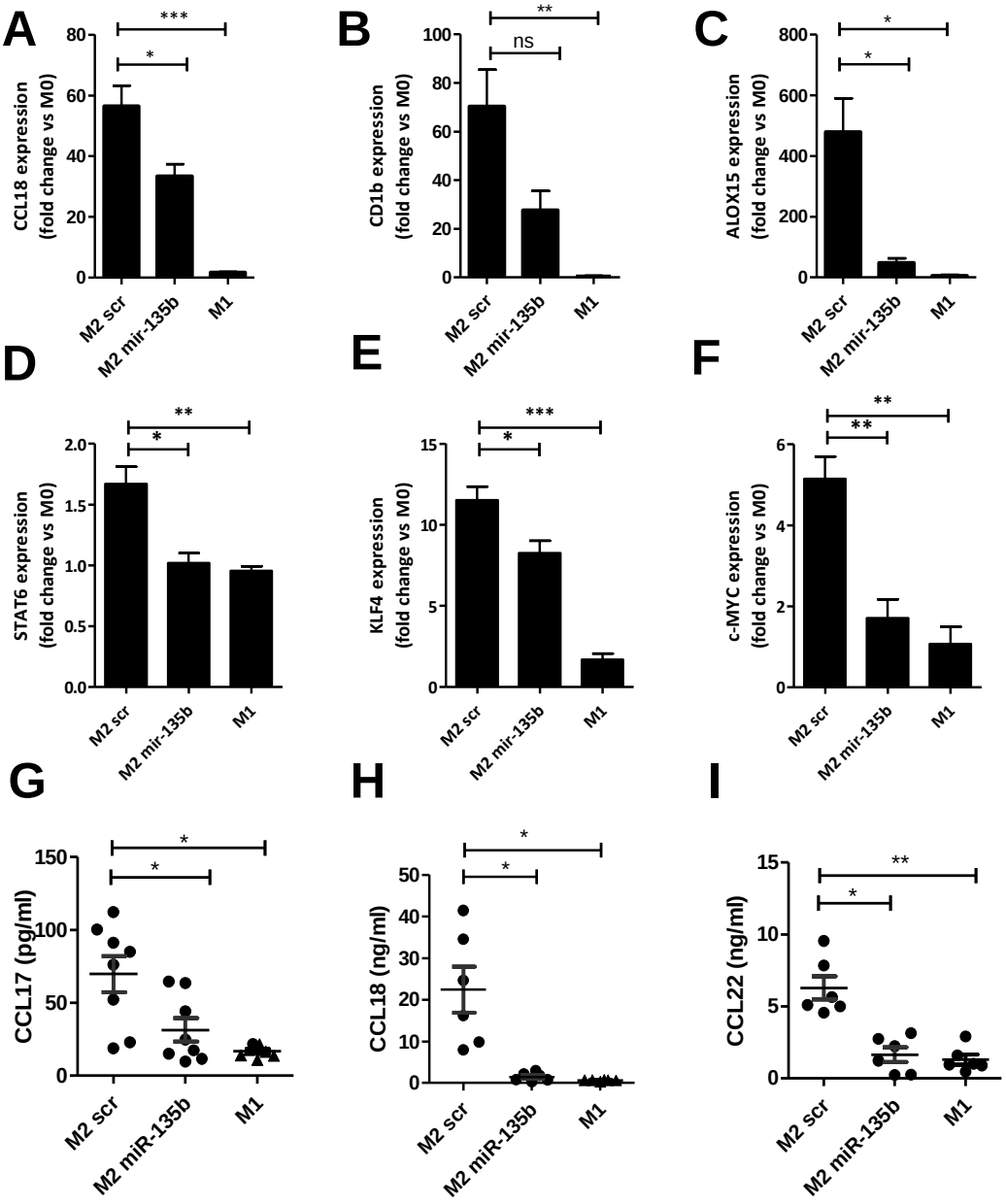

Figure 1. 7 Overexpression of miR-135b inhibits the M2 phenotype Resting macrophages (M0) were transfected with 20 nM control mimics (scr) or miR-135b mimic and after 24h cells were treated with IL-4 for additional 24 hours. Expression levels of M2 markers (**A-F**) were determined by real-time PCR. n=3; mean± SEM; *p<0.05, ** p<0.01, ***p<0.001 compared to M0 transfected with

scramble. M2 chemokines secretion were measured with ELISA (**G-I**). Data are express as n=6; mean± SEM; *p<0.05, ** p<0.01 compared to M0 transfected with scramble.

Gene network of miR-135b

To explore genes and signaling pathways evolved by miR-135b in the macrophage polarization we used an *in silico* approach. We obtained a list of putative target genes with TargetScan (version 4.2, www.targetsacn.org) and miRanda (www.microrna.org) that were integrated with the gene expression datasets produced in our laboratory [77] from M1 and M2 macrophages. The outcome of these analyses was a list of anti-correlated genes (ACT), meaning genes shown up-regulated when targeted miRNAs were down-regulated and conversely. miR-135b ACTs were compared with miR-155 and miR-146a ACT genes to defined restricted gene network ascribable exclusively to miR-135b. The Venn diagram illustrated (Figure 1. 8 A) that the three microRNAs shared 110 ACT genes, the 258 ACT genes specific to miR-135b were further analyzed with Ingenuity Pathway Analysis (IPA). This analysis (Figure 1. 8 B) revealed a significant enrichment for IL-4 pathway signal, which was not shared with miR-155 and miR-146a. These findings support the previous observation that c-MYC, STAT6 and KLF4 are modulated by the overexpression miR-135b as highlighted in Figure 1. 7. The three transcription factors shown a highly targeting score for miR-135b (Figure 1. 8 C-E) identified by Miranda. We validated these interactions performing luciferase reporter assay. HEK293-T cells ware cotransfected with entire 3'UTR vector of c-MYC, STAT6 and KLF4 and with control or miR-135b mimics. As shown in Figure 1. 8 luciferase activity of the reporter 3' UTR of c-MYC, STAT6 and KLF4 were down regulated by miR-135b. This activity was completely restored when the seed sequence was mutated (Figure 1. 8 F-H). We conclude that miR-135b damps M2 phenotype thru the direct target of c-MYC, STAT6 and KLF4 the major transcription factor of IL-4.

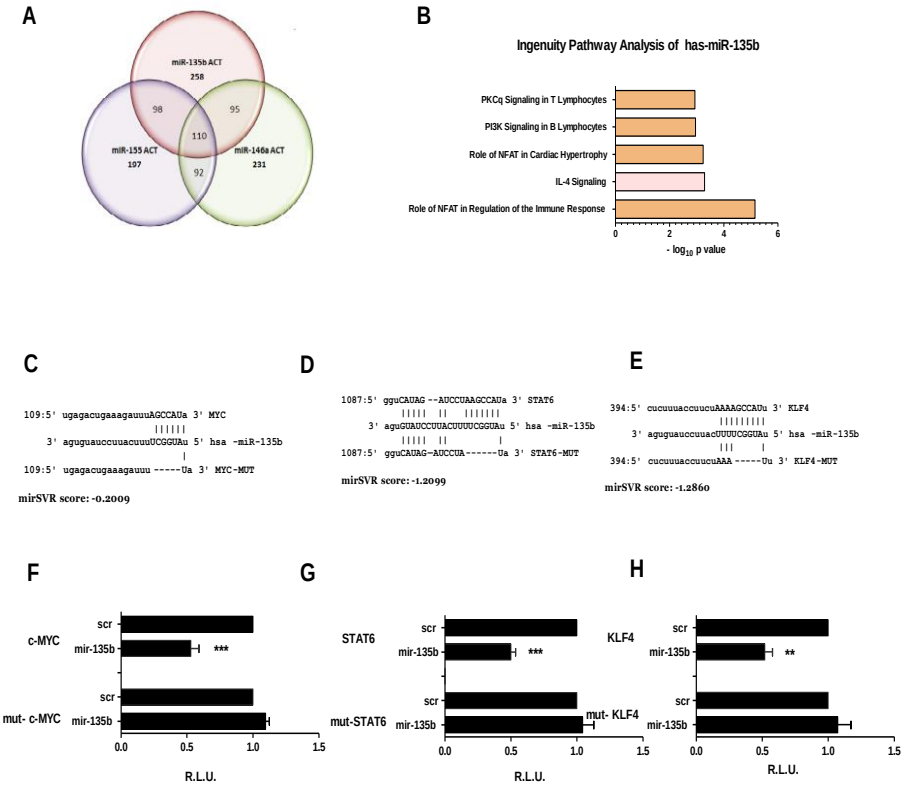

Figure 1. 8 miR-135b targets IL-4 pathway. A, targeting interaction among LPS-induced miRNAs, represented in Venn diagram. miR-135b, miR-155 and miR-146a targets were predicted with TARGETSCAN, Miranda predictions. About 10% of mRNAs are shared by the 3 two miRNAs**. B, C,** IPA analysis of 258 miR-135b anti-correlated target, highlights enrichment in IL-4 signal pathway. This pathway is not shared by the other miRNAs, miR-155 and miR-14a. **D-E**, miR-135b targets IL-4 signaling thru a putative binding with c-MYC, STAT6 and KLF4, with high score of affinity binding identified with Miranda. **G-I,** Human 293T cells were transiently co-transfected with indicated 3'-UTR luciferase construct of c-MYC, STAT6 and KLF4 and with miR-135b mimic or a negative control mimic (scr). Results are expressed as normalized relative luciferase units (RLU). n=4; mean ± SEM. **p < 0.01, ***p < 0.001 compared with control.

MiR-135b inhibits c-MYC, STAT6 and KLF4 protein in human alternative macrophages

To better delineate whether miR-135b/ c-MYC, STAT6 and KLF4 interaction occurs in human macrophages, we transfected resting cells with control or miR-135b mimic and analyzed protein levels after IL-4 treatment. As shown in Figure 1. 9, miR-135b inhibited c-MYC, STAT6 and KLF4 protein, which clearly impacted on the signaling cascade.

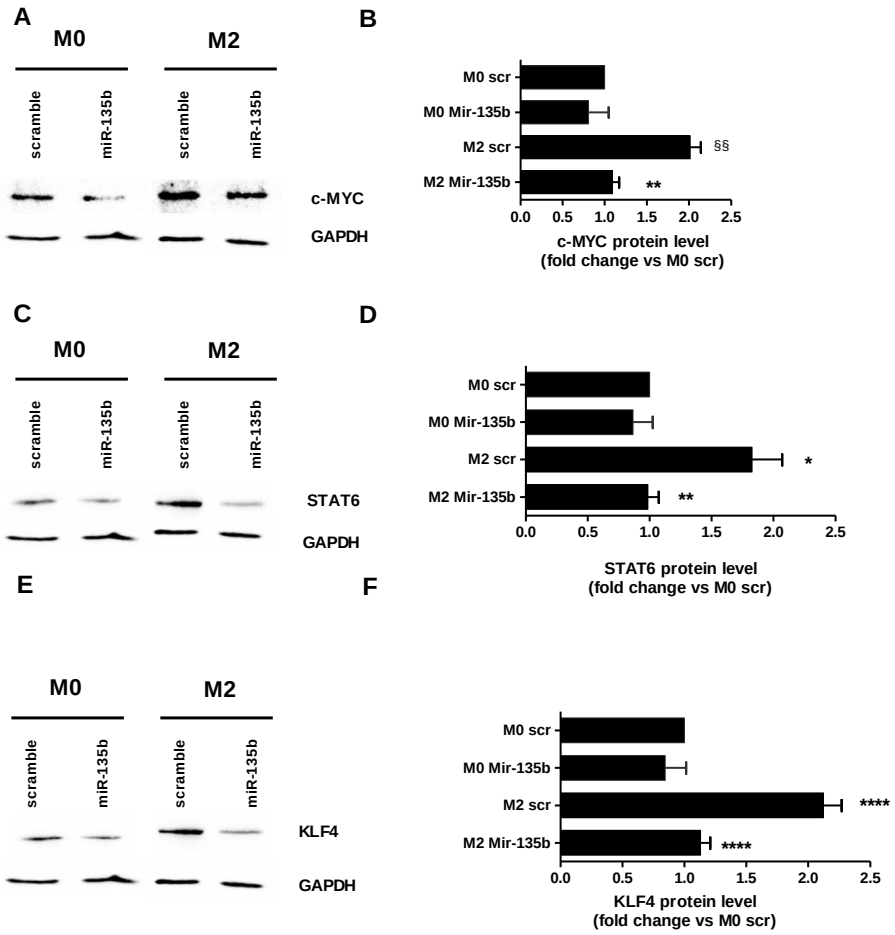

Figure 1. 9 MiR-135b inhibits c-MYC, STAT6 and KLF4 protein in M2 macrophages. Macrophages were transfected with 20 nM control mimics (scr) or mimics miR-135b. After the transfection the cells were treated with 20 ng/ml IL-4, cells were lysed after 24h. Quantification of Western blot data by densitometry. n=3 ± SEM. T-test *p < 0.05, **p < 0.01 compared with the control

The chemokines downstream IL-4 pathway (CCL17, CCL18 and CCL22) diminished when miR-135b is overexpressed, and altered their secretion (Figure 1. 10 A-B); due to the inhibition of STAT6. Furthermore, the effects of miR-135b on c-MYC were notable on the expression of its dependent genes, ALOX15, CD206, CD1B and SCARB1 [21], as revealed in Figure 1. 10C. These outcomes proposed that miR-135b negative regulates the resolve responses induced by interleukin-4 stimulation in human macrophages, recapitulating with the targeting of the transcription factors.

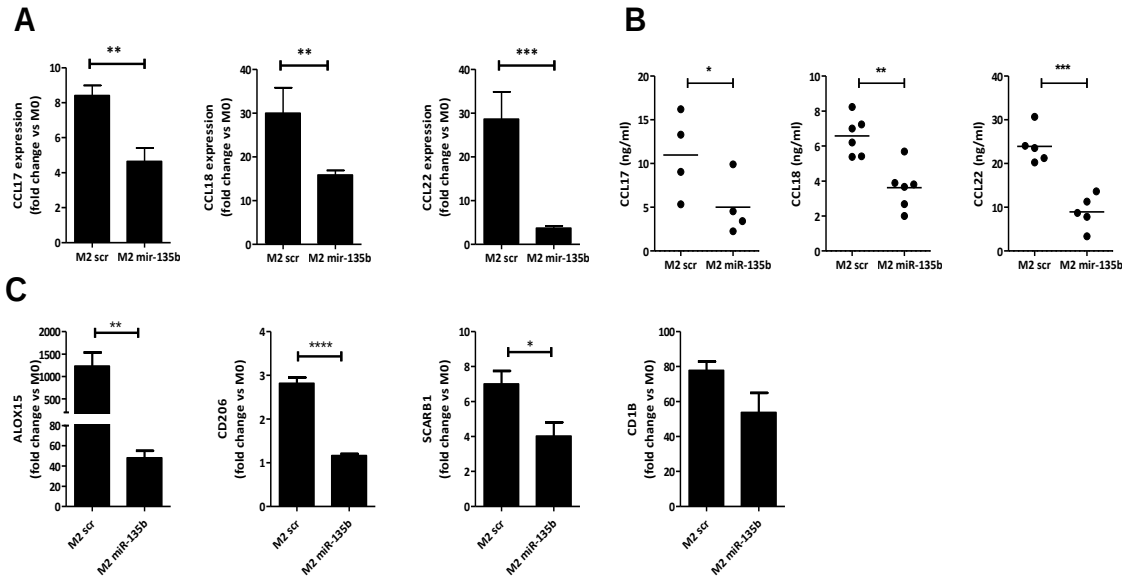

Figure 1. 10 MiR-135b damps M2 polarized phenotype. Macrophages were transfected with 20 nM control mimics (scr) or mimics miR-135b. After the transfection the cells were treated with 20 ng/ml IL-4, cells were harvested, RNA isolated and supernatant collected 24h later. Levels of CCL22, CCL17 and CCL18 were determined compared to M0. Levels of c-MYC dependent genes (SCARB1, MRC1 and ALOX15) were determined compared to M0. n=5; mean ±SEM. *p<0,05 **p < 0.01, ***p < 0.001 n=5; mean ±SEM. The chemokines secretion were determined by E.L.I.S.A. *p<0,05 **p < 0.01, ***p < 0.001.

miR-135b in macrophage plasticity

In order to confirm and extend miR-135b contribution to macrophage polarization, we assessed its expression in the re-polarization process. Human M1 macrophages were cultured with IL-4 (20ng/μl) and M2 macrophages were treated with LPS + IFNγ for 10 hours, we examined the expression of known IL-4- and LPS-induced genes (ALOX15 and IL-12p40 respectively). As shown in Figure 1. 11, M1–to–M2 re-polarization resulted in a sensible decrease of miR-135b expression, while the M2–to–M1 conversion promoted miR-135b induction. These data highlighted that miR-135b expression followed the macrophage plasticity and preserved the inflammatory phenotype.

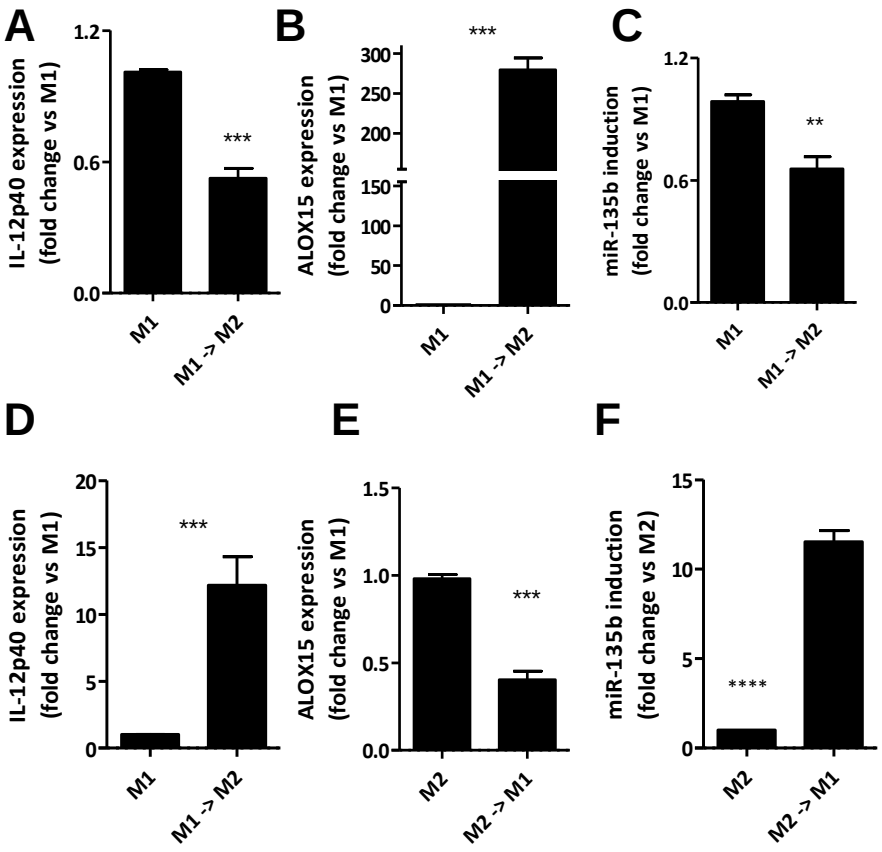

Figure 1. 11 miR-135b in macrophage polarization and re-polarization process. A, expression level of M1 marker IL-2p40 and M2, ALOX15 (**B**) and miR-135b levels in macrophages following M1-to-M2 switch by IL-4 for 10 h and in M2-to-M1 switch by LPS and IFN-γ for 10 h (**D-F**). n = 5; mean ± SEM. **p < 0.01 t-test, compared with resting macrophages.

Inhibition of miR-135b diminishes the expression of M1 markers .

We asked whether the miR-135b inhibition in classical activated macrophages could reverse the phenotype. Therefore, we transfected resting macrophages (M0) with control or inhibitors against miR-135b (α-miR-135b), and after 24 hour we treated the cells with LPS (100ng/ml) plus IFNγ (20ng/ml). As displayed in Figure 1. 12, the miRNA inhibition has a faint impact on M1 markers expression, influencing the levels of CCL5 and CXCL10. On the other hand, the lack of miR-135b promotes the expression of some M2 markers, including c-MYC and STAT6. These observations settle that miR-135b has a repressive role in M2 macrophage polarization.

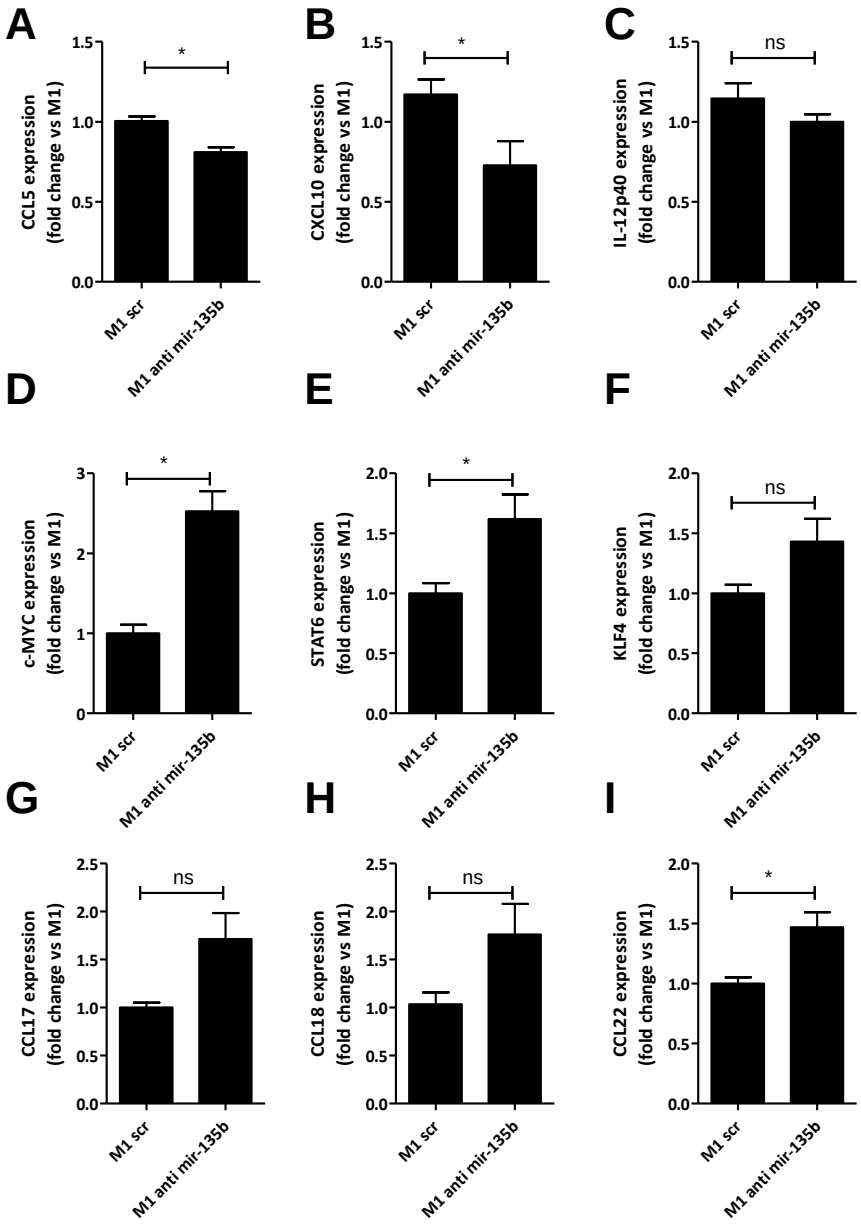

Figure 1. 12 Inhibition of miR-135b diminishes the expression of M1 markers. Resting macrophages were transfected with 20nM of control (scr) or antagomiR for miR-135b for 24h, then cells were treated with LPS for 24h. Cells were harvested and RNA isolated. Levels of CCL5 (**A**), CXCL10 (**B**) and IL-12p40 (**C**) were determined, in addition M2 marker genes were evaluated (**D-I**) . n=3; mean ± SEM; ** p<0.01 compare to control (scr).

IL-10 inhibits miR-135b expression in response to LPS

IL-10 is a pleotropic cytokine crucial in dampening the inflammatory response, preventing an excessive inflammation by down-regulating the pro-inflammatory genes induced by Toll-like receptor activation [78]. McCoy CE and colleagues demonstrated that miR-155 reported to be induced in both mouse and human monocytes/macrophages in response to TLR-stimulation is inhibited by IL-10 stimulation [54]. Therefore, we sought to address whether IL-10 was able to negative modulate miR-135b upon TLR4 activation, with LPS. Thus, we treated resting macrophages with LPS, IL-10 or the combination of both stimuli for 2, 6 and 24h. We measured the levels of miR-135b, miR-146a and miR-155 (Figure 1. 13).

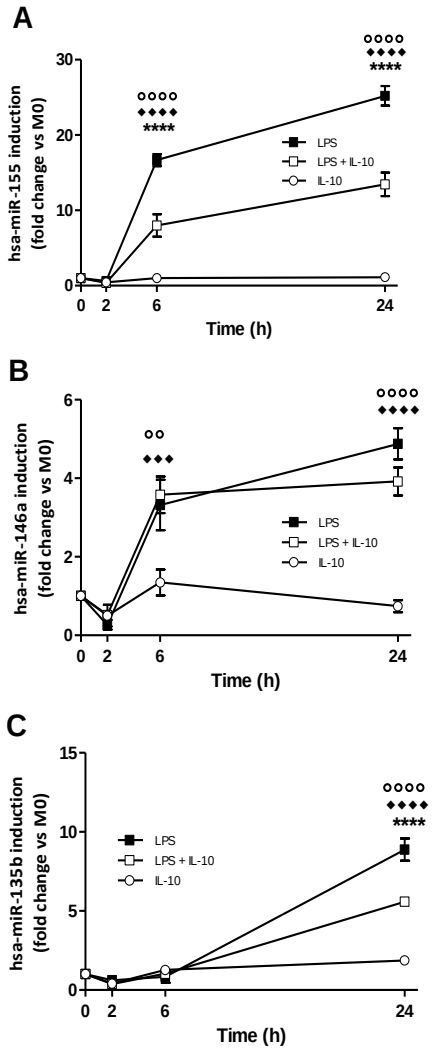

Figure 1. 13 IL-10 inhibits miR-135b expression in response to TLR4 stimulation. Human macrophages were cultured for the indicated times in medium alone or in the presence of 100

ng/ml LPS (black square), 30 ng/ml IL10 (white square) or the combination of both stimuli (white circle). Total RNA was extracted and miR-155, miR-146a and miR-135b expression was assessed by RT-qPCR and normalized to the snRNU6 levels. The results are expressed as fold change respect to resting macrophages (mean ± SEM; *n*= 3). Statistics has been calculated using Bonferroni's multiple test to compare LPS treated to M0 (°°p<0.01; °°°°p< 0.00001); LPS + IL-10 treated sample to M0 (♦♦♦p<0.001, ♦♦♦♦p<0.0001) and IL-10 treated to M0 (***p<0.0001; ****0.00001).

When macrophages were stimulated, the expression of three miRNAs (miR-135b, miR-146a and miR-155) was induced. IL-10 stimulation alone or in concert with LPS did not induce the expression of miR-135b and miR-155, instead, it partially repressed the induction of miRNAs in response to LPS (Figure 1. 13A and C). However miR-146a expression pattern was not influenced by IL-10 as is already reported [54]. These data suggested that IL-10 altered the microRNA expression levels after LPS exposure, and miR-135b might be a novel IL-10 hinder-regulated miRNA gene.

In order to confirm and further investigate the role of IL-10 on miR-135b, we performed a qPCR analysis examined the pri- and the pre-miR-135b at 2, 6 and 24h from the stimulation with IL-10, LPS or in combination. The analysis revealed once again that miR-135b is induced by LPS, shown pri-miR-135b robustly induced at 2h and pre-miR-135b at 6h, in line with the normal processing of the microRNA. Nonetheless, IL-10 strikingly repressed the LPS-induction (Figure 1. 14). Thus, we could speculate that IL-10 counter-regulated miR-135b at transcriptional level.

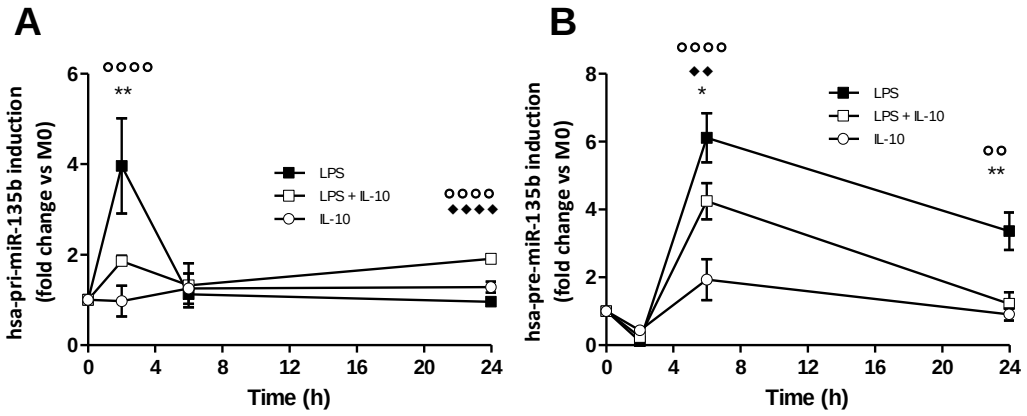

Figure 1. 14 IL-10 inhibits the transcription of miR-135b. Human macrophages were cultured for the indicated times in medium alone or in the presence of 100 ng/ml LPS (black square), 30 ng/ml IL10 (white square) or the combination of both stimuli (white circle). Total RNA was extracted and pre-miR-135b and mature miR-135b expression was assessed by RT-qPCR and normalized to the GAPDH and snRNU6 levels respectively. The results are expressed as fold change against resting macrophages (mean ± SEM; *n*= 3). Statistics has been calculated using Bonferroni's multiple test to compare LPS treated to M0 (°°p<0.01; °°°°p< 0.00001); LPS + IL-10 treated sample to M0 (♦♦p<0.01, ♦♦♦♦p<0.0001) and IL-10 treated to M0 (*p<0.05; **0.01).

Endogenous IL-10 inhibits mature miR-135b in primary BMDM

LPS stimulation beyond induces pro-inflammatory cytokines, prompts IL-10 as negatively feedback modulator. We evaluated that IL-10 outcome on miR-135b in primary BMDM when the cells are treated in combination with LPS, Figure 1. 15A. We also compared the miR-135b expression in primary wild-type and IL-10-deficient BMDM upon LPS treatment. As shown in Figure 1. 15B, LPS normally induced IL-10 secretion; however IL-10-deficient mice were not able to respond. As expected, miR-135b was higher induced (~3-fold) in IL-10 KO mice than wild type upon LPS stimulation. These findings demonstrate that the endogenous IL-10, resulting by LPS stimulation, constrains the expression of miR-135b, checking in this way its expression.

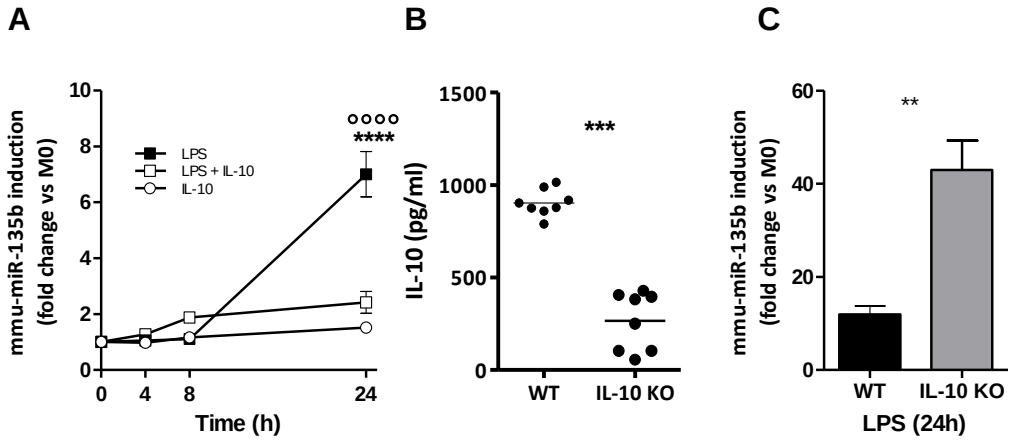

Figure 1. 15 Endogenous IL-10 inhibits mature miR-135b in primary BMDM. A, BMDM were cultured for the indicated times in medium alone or in the presence of 100 ng/ml LPS (black square), 30 ng/ml IL10 (white square) or the combination of both stimuli (white circle). Total RNA

was extracted and miR-135b expression was assessed by RT-qPCR and normalized to the snRNU6 levels. The results are expressed as fold change respect to resting macrophages (mean ± SEM; *n*= 3). Statistics has been calculated using Bonferroni's multiple test to compare LPS treated to M0 (°°°°p< 0.00001); and IL-10 treated to M0 (***p<0.0001). **B**, IL-10 secretion was measured in wild type C57BL/6 and IL-10 knockout mice. Cytokine secretion was determined by E.L.I.S.A. n=7; mean ± SEM; ***p<0.001 compared to control wild type.

miR-135b is highly express in murine classical activated macrophages

We would to address if miR-135b role shown in human macrophages is the same in BMDM. As highlighted in Figure 1. 16 , murine miR-135b (mmu-miR-135b) was highly prompted by TLR4 activation and its expression was dynamic in murine macrophage reprogramming as well as in human macrophages. Indeed, mmu-miR-135b levels were higher when the cells were repolarized from M2 to M1 phenotype (Figure 1. 16 B-C). M1–to–M2 re-polarization decreased miR-135b expression.

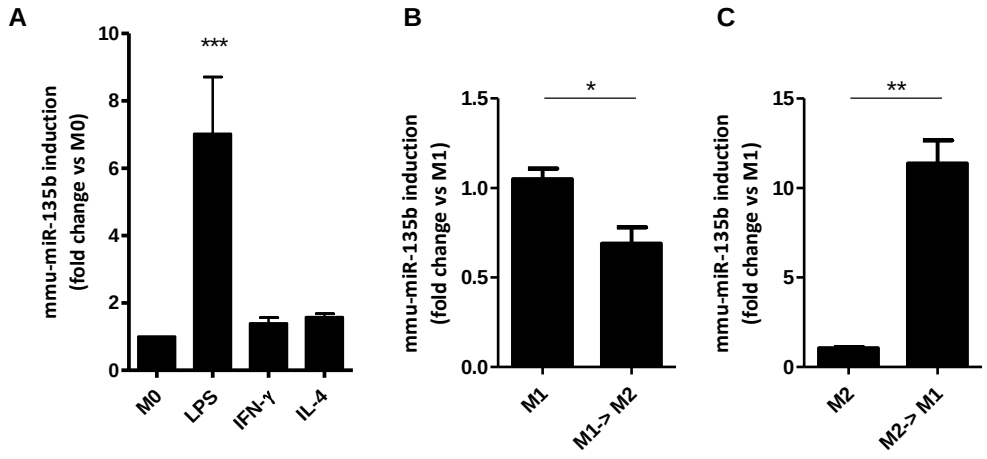

Figure 1. 16 Murine classic activated macrophage highly express mmu-miR-135b. A, bone marrow derived macrophages (BMDM) were cultured for 24h with 100 ng/ml LPS; 20 ng/ml IFN-γ and 20ng/ml IL-4. RNA was extracted and mature miR-135b ware analyzed by qPCR. n =3; mean ± SEM. Statistics has been calculated using t-test ***p < 0.001 compared with resting macrophages. **B,** miR-135b levels in macrophages following M1-to-M2 switch by IL-4 for 10 h. n =3; mean ± SEM. Statistics has been calculated using t-test *p<0.05, compared with M1. **C**, M2-to-M1 switch by LPS and IFN-γ for 10 h. n = 5; mean ± SEM. **p < 0.01 t-test, compared with M2.

miR-135b damps the alternative activated macrophage polarization

We next investigated the role of miR-135b in primary BMDM; thru the enhanced or silenced expression of the miRNA, Figure 1. 17. Boosted mmu-miR-135b expression in M2 macrophages caused the down regulation of c-Myc and Stat6, influencing also Alox15 (Figure 1. 17 A-B); on the other hand promoted the expression of some M1 marker genes (Ccl5 and Nos2). The lack of mmu-miR-135b in M1 macrophages up regulated some M2 marker genes, in particular c-Myc (Figure 1. 17D). These data confirm once again the involvement of miR-135b in macrophage activation and polarization.

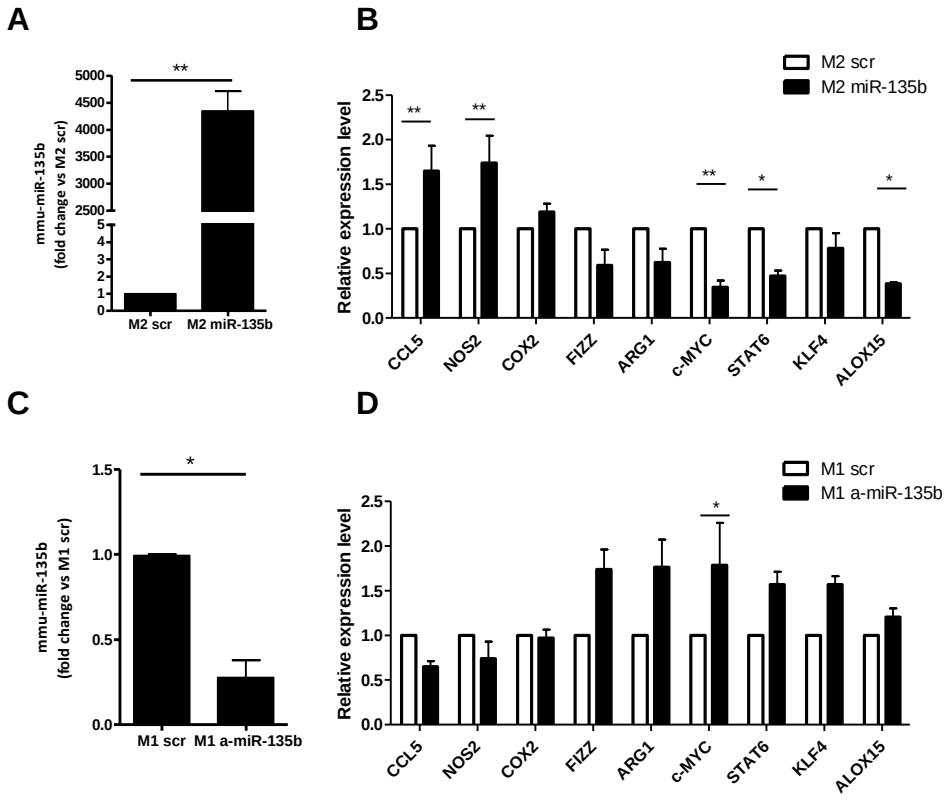

Figure 1. 17 mmu-miR-135b damps the alternative activated macrophage polarization. A,B, BMDM **,** were transfected with 20nM of control (scr) or miR-135b for 24h, then cells were treated with IL-4 for 24h. Levels of M1 markers (Ccl5, Nos2, Cox2) and M2 markers (Fizz, Arg1, C-Myc, Stat6, Klf4 And Alox15) were measured with qPCR. (**C-D**) were transfected with 20nM of control (scr) or antagomiR for miR-135b for 24h, then cells were treated with LPS for 24h. Cells were harvested and RNA isolated. Levels of M1 and M2 markers were determined, n=3; mean ± SEM; *p<0.05; ** p<0.01 compare to control (scr).

DISCUSSION

In this present study we have demonstrated once again that miRNAs can be considered as master regulators of innate immunity. In particular we have highlighted a restricted miRNAs signature in human *in vitro*-polarized macrophages, 69 miRNAs were differentially expressed among macrophage subsets (M1; LPS + INF-γ, M2; IL-4). From the screening was emerged that resting macrophages and M2 shown a quite similar miRNome. In addition, we have identified a novel microRNA induced by LPS stimulation, miR-135b, both in human and murine macrophages. miR-135b is an oncomiR involved in cancer progression and tissue differentiation, useful also as bio-marker [79,80]. Recently, miR-135b has been reported to be up-regulated in lung tissues upon exposure to cigarette smoke and nanoparticles, through activation of the IL-1α/IL-1R pathway [81]. Interestingly, it suppressed Th2 and Th17 differentiation *via* STAT6 and GATA3 targeting leading to ALCL (anaplastic large cell lymphoma) [57]. Nevertheless, our study identified for the first time miR-135b to be linked to the innate immune response. This microRNA was not able to activate steady state macrophage toward M1 phenotype, however its enforced expression interfered with IL-4 signaling pathway. In addition, miR-135b damped the alternative phenotype favoring the classic one. Consist with these findings, *in silico* analysis highlighted a target enrichment in IL-4 signaling pathway, in particular through interaction with the major transcription factors, c-MYC, STAT6, and KLF4. We have abundantly confirmed these observations in our *in vitro* study thru the enhanced expression of the miR-135b down-regulated in primary macrophages c-MYC, STAT6 and KLF4 at mRNA and protein levels. Interleukin-4 leads to STAT6 activation which controls expression and production of chemokines such CCL17 and CCL22 [82] that we found repressed upon miR-135b overexpression in M2 macrophages. STAT6 works, moreover, in synergy whit KLF4 sustaining M2 gene expression. The inhibition of KLF4 is translated with the increase of pro-inflammatory genes [19], which was confirmed in our study, miR-135b over-expression leads to enhancer M1 chemokines production. c-MYC is a pleiotropic transcription factor, which controls significantly the alternative activation phenotype[20]. It bids directly with the promoter of ALOX15, MRC1, SCARB1 and STAT6 [21]. miR-135b has emerged from our findings as a miRNA that enhanced the classic

polarization and blocked the M2 re-programming thru the targeting of the major transcription factors at the crossroad of macrophage polarization. miR-135b relevance in macrophage re-programming was enforced also by the negative role promoted by IL-10. Interleukin-10 is anti-inflammatory cytokine also induced by TLRs acts as autocrine feedback regulator to resolve acute inflammation. We sought to determine IL-10 role on miR-135b expression. In this study we have confirmed IL-10 role on TRL4-response miRNAs, as found from McCoy and colleagues [54]. IL-10 stimulation in combination with LPS inhibits the expression of miR-135b both in human and murine, with a specific effect. In addition, we demonstrated that miR-135b expression was extremely increased in response to LPS in IL-10 KO macrophages. Overall these data stressing that endogenous IL-10 produced downstream TLR signaling, is a negative feedback system to damp also miR-135b levels.

In summary, we have shown that macrophage phenotypes are characterized by distinct miRNome. Moreover our results highlight a novel miRNA induced downstream TLR4 signaling pathway, miR-135b which enforce the classic activation phenotype and is inhibited by IL-10 to extinguish inflammation. Although miR-135b acts like a pivot in macrophage polarization.

MATERIALS AND METHODS

Reagents.

LPS from *Escherichia coli* serotype 055:B5 was purchased from Sigma, Pam3CsK4, was purchased from Enzo Life Sciences. Human IL-10, IL-1β, IFNγ and IL-4 were purchased from R&D Systems. Dexamethasone was from Sigma-Aldrich. Rabbit anti-STAT6 (#9362) and rabbit anti-KLF4 (#12173) were antibodies from Cell Signaling Technology. Mouse anti-c-MYC (9E10) was purchased from Santa Cruz Biotechnology. Rabbit anti-GAPDH (#5174) was purchased by Cell Signaling Technology.

Macrophage generation and polarization.

Human monocytes were isolated from healthy donor buffy coats by two step gradient centrifugation using Ficoll-Paque (GE Healthcare) and Percoll (Amersham). No adherent cells were discarded, and purified monocytes were cultured in T25 flasks at 10^6 cells/ml in RPMI 1640 (Lonza) supplemented with 10% fetal bovine serum (FBS; Lonza), 1% (v/v) penicillin–streptomycin (Lonza), and 1% glutamine (Lonza). Monocytes were differentiated in macrophages by exposure to 50 ng/ml M-CSF for 6 days. Resting macrophages were then polarized to M1 macrophages using 100 ng/ml LPS plus 20 ng/ml IFNγ or M2 macrophages using 20 ng/ml IL-4 for 24 h before being evaluated. Resting macrophages (M0) were left untreated in culture medium.

Bone Marrow Derived Macrophages (BMDM) preparation

BMDB were obtained from 7-9 weeks old male C57BL/6 wild type and IL-10 KO. Femurs were obtained, muscles connected to the bone were removed and washed in sterile RPMI 1640 and then both epiphyses were removed. The bones were flushed with a syringe filled with RPMI 1640 to extrude bone marrow into a 15 mL sterile polypropylene tube. The obtained BMDMs were cultured in complete IMDM medium supplemented with 10% fetal bovine serum (FBS; Lonza), 1% (v/v) penicillin–streptomycin (Lonza), and 1% glutamine (Lonza). After 7 days of culture, adherent macrophages (BMDM) were recovered and directly used for experiments.

Human macrophage miRNome profiling

TaqMan low density array (TLDA) human miRNA assays (version 2.0; Applied Biosystems) were used to profile the macrophage miRNome in different activate phenotypes. Briefly, cDNA was obtained using the TaqMan reverse transcription kit and Megaplex primer pools A and B (version 2.0). PCR amplification was first performed using TaqMan PreAmp Master Mix (version 2.0). Lastly, TLDA cards A and B were loaded, and PCR was performed on a 7900HT fast real time PCR system (Applied Biosystems). Expressions fold changes each miRNA were evaluated with ΔΔCt method using SDS (version 2.3) and SDS RQ Manager software (version 1.2). U6 was used as an endogenous control.

miRNA isolation and Quantitative RT-PCR Analyses.

Total RNA enriched with small RNAs faction was isolated by TRIzol (Ambion) according to the manufacturer's instructions. For each sample, two independent reverse transcription reactions were performed as followed, using TaqMan miRNA assay (Applied Biosystems). Briefly 10 ng of total RNAs were reverse transcribed with individual stem-loop RT-primers for has-miR-135b (Cat: 002261), has-miR-155 (Cat: 002623), has-miR-146a (Cat: 000468), mmu-miR-155 (Cat: 002571), U6 snRNA (Cat: 001973) and snoR-302 (Cat: 001232). Following, 1 ng of each individually synthesized cDNA in 10μL of final reaction, was used in the qPCR assay with specific TaqMan probes. Real-time PCR was conducted in CFX Connect™ Real-Time PCR Detection System (BioRad). Relative miRNAs expressions were determined by using the ΔΔCt method [83], normalizing the levels with U6 snRNA or snRNA-302.

RNA isolation and gene expression analysis.

Total RNA was purified using TRIzol (Ambion) according to the manufacturer's instructions, and was quantified by its absorption at 260 nm. First-strand cDNA was synthesized from 1 μg of total RNA in 50 μL of reaction mixture using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to manufacturer's guidelines. Quantitative RT-PCR was performed using specific oligonucleotides which are

reported in Table 1. 2 Sybr Green PCR Master Mix (Applied Biosystems) was used in a CFX Connect™ Real-Time PCR Detection System (BioRad). The thermal cycling conditions were composed of 50ºC for 2 min followed by an initial denaturation step at 95ºC for 10 min, 45 cycles at 95ºC for 30s, 60ºC for 30s and 72ºC for 30s. Results were analyzed with CFX software, and relative expression values were calculated according to ΔΔ cycle threshold method normalized using GAPDH as housekeeping.

Table 1. 2 Primers sequence

Human Genes	Forward primer (5'-3')	Reverse primer (5'-3')
ALOX15	CTTGCTCTGACCACACCAGA	GCTGGGGCCAAACTATATGA
CCL17	TGTATATGTCATCTCAGTGC	TAGGGACATCATATCTTAA
CCL18	GAGGCCAGGAGTTGTGAGTT	CATGGTGCAGACGAGGACAA
CCL5	TATTCCTCGGACACCACACC	ACACACTTGGCGGTTCTTTC
CD1b	CAGCAGTTTAGAGGGCCAGG	CAAAAGGAGCAAGGAAGGCAC
CD206	GGGCAGTGAAAGCTTATGGA	CCTGTCAGGTATGTTTGCTCA
CD80	GGGAAAGTGTACGCCCTGTA	GCTACTTCTGTGCCCACCAT
c-MYC	CGTCCTCGGATTCTCTGCTC	GCTGGTGCATTTTCGGTTGT
COX2	CCCTTGGGTGTCAAAGGTAA	GCCCTCGCTTATGATCTGTC
CXCL10	AGCCAATTTTGTCCACGTGT	TGATGGCCTTCGATTCTGGA
CXCL9	TGAGAAAGGGTCGCTGTTCC	TCAAACTGCTTGGCTCACCA
GAPDH	GATCATCAGCAATGCCTCCT	TGTGGTCATGAGTCCTTCCA
IL-10	AGAACCTGAAGACCCTCAGGC	CCACGGCCTTGCTCTTGTT
IL-12p40	CCAAGAACTTGCAGCTGAAG	TGGGTCTATTCCGTTGTGTC
KLF4	ATGCTCACCCCACCTTCTTC	GGTGGTCCGACCTGGAAAAT
STAT6	AACCTTTCTCCTCCGCTTCA	TGAGCGAATGGACAGGTCTT
Murine Genes	**Forward primer (5'-3')**	**Reverse primer (5'-3')**
ALOX15	GAATACCTTGGGCCACTGCT	GTCAGAGATACTGGTCGCCG
ARG1	CAGAAGAATGGAAGAGTCAG	CAGATATGCAGGGAGTCACC
CCL5	TGCTCCAATCTTGCAGTCGT	GCAAGCAATGACAGGGAAGC
c-MYC	TAACTCGAGGAGGAGCTGGA	GCCAAGGTTGTGAGGTTAGG
COX2	AATGAGTACCGCAAACGCTTC	CAGCCATTTCCTTCTCTCCTGTA
FIZZ	GGTCCCAGTGCATATGGATGAG	CACCTCTTCACTCGAGGGACAGT
KLF4	AACATGCCCGGACTTACAAA	TTCAAGGGAATCCTGGTCTTC
NOS2	GCCACCAACAATGGCAACA	CGTACCGGATGAGCTGTGAATT
STAT6	TCTCCACGAGCTTCACATTG	GACCACCAAGGGCAGAGAC

Luciferase Reporter Assay.

HEK-293T cells were plated in 24-well plates in 500 µL of D-MEM medium, supplemented with 10% FBS and 1% of L-glutamine at 16 × 10^4 per well. After 24 h, cells were transfected with 100 ng psiCHECK-2–3′UTR reporter construct and 10pmole of mimic-miR-135b or scramble control (mirVana™, Ambion Applied Biosystems) by using Lipofectamine 2000 (Invitrogen). After 24h from the transfection, cells were lysed, and

luciferase activity of both firefly and renilla, were determined by using the DualGlo Luciferase Assay System (Promega). The enzymatic activities were measured by using a MultiDetection Microplate Reader Synergy 2 luminometer (BioTek). The values of renilla luciferase activity were normalized and were expressed as fold changes relative to the value of the negative control.

Macrophage Transfection.

Purified macrophages were cultured in medium supplemented with 2 mM glutamine and 10% FCS. Were transfected with 10pmole of miRNA mimic (Ambion, Applied Biosystems), or with 10pmole miRCURY LNA microRNA Inhibitor™ (Exiqon), using TransIT-TKO Transfection Reagent (Mirus Bio), according to the manufacturer's protocol. In brief, for transfection in 6-well plates, for each well the following solutions were combined miRNA mimic/ microRNA Inhibitor with 10µl TransIT-TKO mixed 250 µl Opti-MemI. This complex was incubated for 30 min at RT, and dripped into $1.2x10^6$ cells/well seeded. After 24 h, transient transfected cell were stimulated as indicated.

Immunoblots.

Macrophages (5×10^6) were lysed in 40 mM Tris, 1% SDS, 7.5% glycerol and boiled for 10 min at 95 °C. Then 100 µg of total cell lysates were resolved on 10% SDS/PAGE and transferred to nitrocellulose (Hybond; GE Healthcare). Blotted membranes were blocked either with 5% milk in PBST (for c-Myc, STAT6 and GAPDH staining) or in TBST +5% BSA (for KLF4 staining) for 1 hour at room temperature then washed. The blots were incubated with specific antibodies overnight at 4°C or at RT for 1h with the primary antibody of GAPDH. After washing 3x 10 min with the appropriate washing buffer, the membrane was incubated for 1h at RT with a horseradish-peroxidase (HRP)-coupled secondary antibody. The working concentrations and conditions of each antibody are listed in the following Table 1. 3. This was followed by five 3-min washes in TBST at room temperature and incubation in Clarity western ECL substrate chemiluminescent detection reagent (Bio-Rad) for 5 min prior to image acquisition. The chemiluminescent blots were imaged first with the ChemiDoc MP imager (Bio-Rad) and analysed with ImageLab software version 4.1 (Bio-Rad).

Table 1. 3 Antibody dilution for Western blot assay

Primary antibody	Dilution factor	Secondary antibody	Dilution factor
Mouse anti-c-MYC	1:200 in 5% milk PBST	Goat anti-mouse HRP	1:10000 in 5% milk PBST
Rabbit anti-STAT6	1:1000 in 5% milk PBST		
Rabbit anti-KLF4	1:1000 in 5% BSA TBST		
Rabbit anti-GAPDH	1:1000 in 5% milk PBST		

ELISA assay.

Antibodies and detection reagents for ELISA assays were purchased from R&D Systems and used according to the manufacturer's instructions. Samples were diluted so that the optical density fell within the optimal portion of a log standard curve.

Statistical analysis.

Results are expressed as mean ± SEM and statistical significance was based on non-parametric t-test or non-parametric ANOVA followed by post hoc Bonferroni's test performed using the GraphPad Prism 5 software (GraphPad Software).

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Chapter: The “junk” RNA controlled by glucocorticoids: miR-135b and its host gene BLACAT1

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INTRODUCTION

Epigenetics

Epigenetic changes regulate diverse cellular functions including cellular differentiation, cell activation and transformation. Epigenetic mechanisms are classically mediated by post-translational modifications (such as methylation, acetylation, and phosphorylation) of histones and other DNA binding proteins, by methylation and hydroxymethylation of CpG DNA motifs, and by noncoding RNA [1]. These epigenetic variation are dynamically influenced by environmental insults [2], more over the cell specific epigenetic "landscape"' determines accessibility of to the transcription factors [2], such as STATs.

In macrophage polarization histone modifications presents distinct behavior, transcriptional promote or suppress [3]. The balance of positive and negative histone marks at gene promoters and enhancer defines the transcriptional state [3].

Adaptations in gene expression that rule macrophage polarization is caused by the collaboration between gene promoters and regulatory enhancer elements. Promoter regions, located proximal to gene transcription start sites (TSS), while enhancers are TSS-distal cis-elements, contain specific DNA motifs recognized by specific transcription factors. The lineage specific-transcription factors are mostly bound to enhancer regions. The formation of enhancers is therefore cell type-specific than the presence of promoter elements [4]. In the following paragraphs will be highlighted the chromatin organization on enhancer important to define a macrophage phenotype.

Chromatin conformation in M1 macrophage activation

The initial epigenetic landscape at the TRL-leading gene loci is established during macrophage differentiation whereby the myeloid transcription factor PU.1 and C/EBPα bind to regulatory regions (promoters and enhancers) of these genes [5]. PU.1 is already active at the early stage of myeloid differentiation, antagonizing transcription factor GATA-1. It leads towards the monocyte lineage supported by C/EBPα, C/EBPβ and IRF8[6]. From a mechanistic point of view in macrophage activation we can identify three classes

of TFs. The first class (such as Pu.1 and C/EBPβ) open the chromatin to facilitate access of a second group of TFs called 'primers', such as IRF4, that are able to prime for activation regions that are associated with stimulus-dependent gene induction. The third class binds specific set of genes in a stimulus-dependent manner and control gene expression induction (Figure 2. 1). Therefore, the transcriptional potential f is established before stimulation by binding of TF subset to their genomic cis-regulatory regions.

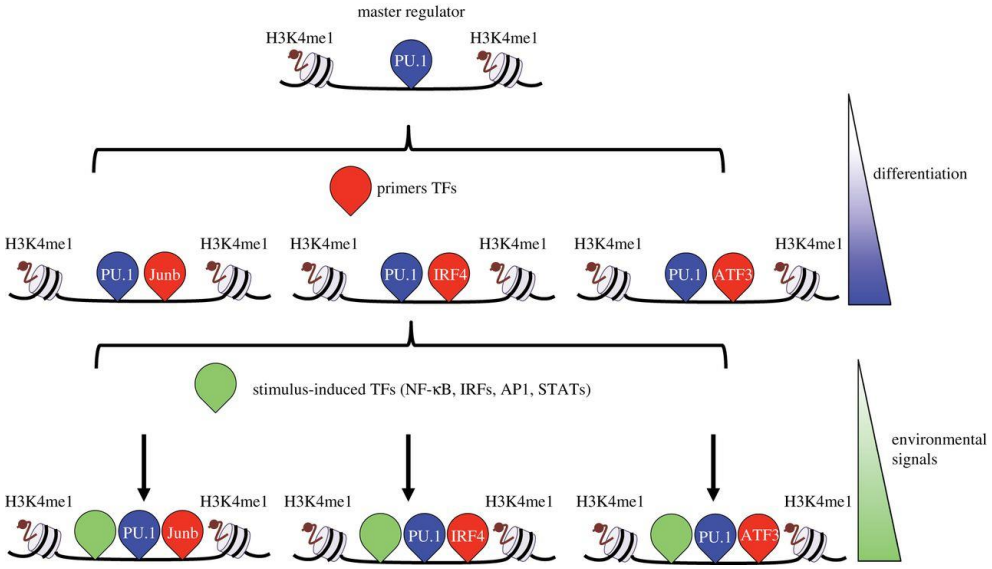

Figure 2. 1 chromatin landscape influenced by Pu.1 The lineage-determining TF Pu.1 binds to regulatory elements and promotes deposition of H3K4me1. This binding is followed by the arrived of a second group of TFs called "primers" that in turn leads to gene transcription of triggered-specific genes, such as NF-κB, AP-1, and STATs that promote a specific set of pro-inflammatory genes. Figure from Ghisletti and Natoli, 2013.

It is broadly recognized that histone modifications constitute a "histone code", characterized by histone acetylation marks and H3K4 enriched in active chromatin regions, while, trimethylation of H3K9, H3K27 and H3K79 are linked to silencing of gene expression [7,8]. A subset of LPS-inducible genes experiences signal-dependent demethylation of trymethylated H3K27 to allow their expression. This activity is promoted by (KDM6B; also known as JMJD3), a specific demethylase induced in itself upon LPS treatment. Removal of the trimethylation H3K27 occurs during cellular

differentiation [9], demonstrating that macrophage activation has some landscapes similar to differentiation. The dynamics that allow the transcription of significant in macrophage polarized genes recognized that their loci phenotypes exist in three broad states, characterized by the variation of the "histone code". Firstly, the chromatin is inaccessible, characterized by the presence of negative marks, such as H3K9me3 and H3K27me3. Then, the chromatin is partial open ("poised" state) characterized by the presence of activating histone marks, occupancy of gene repressors such as BCL6 (B cell leukemia 6), some cases there is a prebound RNA polymerase II (pol II) close to TSS. However the transcription is controlled by concurrent presence of repressive histone marks such as H3K9me3 and H3K27me3, co-repressor complexes. Finally, an open chromatin configuration characterized by active histone marks, and ongoing transcription [10]. In addition, on other important histone modifications, is the nucleosome remodeling by the ATP-dependent complex BAF (also termed SWI/SNF), which facilitates recruitment of transcription factors such as NF-κB, and release of paused pol II [11]

Chromatin conformation in M2 macrophage activation

Macrophages are activated by TLR ligands or TNF in transient manner followed by a state of tolerance [12]. Epigenetic mechanisms that regulate activation toward tolerance need to be further clarified, however it is known that tolerized genes display reduced chromatin accessibility, followed by and diminished recruitment of transcription factors such as p65. On the contrary, non-tolerized genes maintain an open chromatin state.

Alternative activated macrophage responses are mediated by histone demethylase JMJD3, which promotes expression of the key M2 transcription factor, IRF4 [13]. Different studies have highlighted the important role of JMJD3, which is induced IL-4 in a STAT6-dependent manner [14], in alternative activation with ChIP and ChIP-seq analysis. JMJD3 is also express by TLR-inducible in macrophages *via* an NF-κB-dependent pathway. Since H3K27 trimethylation is implicated in the silencing of gene expression, by regulating a set of genes such as Bmp2 and Hox [15]. Has been revealed JMJD3 is dispensable for M1, in

contrast is essential for M2 macrophage. Indeed the expression of the M2 marker genes encoding Arg1, Ym1, Fizz1, MR and IL-13 was impaired in JMJD3 deficient mice [13].

The Junk RNA and the immunity system

The central dogma of the biology formulated by Crick in 1958 [16], which says that DNA is transcribed into messenger RNAs (mRNAs), followed by protein translation, is multifaceted. Only 2% of the human genome encodes for proteins, while a broadly 90% of the genome is being actively transcribed, producing the previously known "junk RNA" The discovery of this "junk RNA", nowadays named non-coding RNA (ncRNA) transcripts, has opened a novel perspective on the RNA centrality in gene regulation; it is evident that mRNA transcription does not inevitably lead to protein translation. Some of the ncRNAs are associated with mRNA splicing (small nuclear RNAs; snRNAs) and translational machinery, such as ribosomal RNA, transfer RNA, RNase P, SRP-7S. One of the most abundant class of ncRNAs are microRNA that regulate gene expression in a post-transcriptional way [17]. Nowadays a particular class of ncRNA are established, long non-coding RNAs, lncRNAs which are greater than 200bp.

MicroRNAs

MicroRNAs (miRNAs) which are small ncRNAs capable of silencing target mRNA expression through the RNA-induced silencing complex (RISC), causing mRNA decay or translation inhibition [17]. Evidence of their function are arising, even though less is known about their own regulation and expression activity. Recently, evidence for post-transcriptional control of miRNA activity has been accumulating [18,19]. In contrast to the initially thought of a linear and universal miRNA processing pathway, multiple discoveries led to the recognition of miRNA-specific differences that open a plethora of regulatory process.

Transcriptional regulation

Generally, miRNAs are transcribed in primary transcript (pri-miRNA, containing a cap as well as a poly-A tail) is generated by RNA polymerase II or III. Both RNA polymerases are differently regulated and recognize specific promoter and terminator elements. For

instance selected miRNAs are controlled by transcription factors, such as c-Myc or p53 [20,21] or depends on the methylation of their promoter [22]; clearly the miRNA transcription control is not an universal step .

Pri-miRNA editing

Primary transcript editing is as a post-transcriptional change of RNA sequences by deamination of adenosine (A) to inosine (I), altering the base pairing and structural properties of the transcript, influencing the further processing as well as the target recognition abilities. A-I editing is mediated by ADAR (adenosine deaminases acting on RNA) 1 and 2 and was described for miR-22, miR-223 and miR-99a [23,24].

Pri-miRNA processed by DroshaDGCR8

The obtained primary transcript is processed in the nucleus by the Microprocessor, a complex composed by Drosha (a RNase III endonuclease) and the cofactor, DiGeorge syndrome critical region gene 8 (DGCR8/Pasha), into a precursor-miRNA (pre-miRNA) of ~70-100 nucleotides. Pasha contains two double-stranded RNA-binding domains and is essential for miRNA processing. Indeed a single nucleotide polymorphism in a miRNA precursor stem can block Drosha processing (e.g. miR-125a) [25]. In addition, primary transcripts are processed co-transcriptionally by Drosha and this process precedes splicing of the protein-encoding or non-coding host RNA that contains the miRNAs [26].

In mammalian there are accessory proteins associated with Drosha, which includes the DEAD-box helicases p68 (DDX5) and p72 (DDX17) DEAD box helicases P68 and P72 and the heterogeneous nuclear RNA complex (hnRNP) proteins [27]. These RNA helicases (p72 and p68) facilitate Drosha processing of a subset of pri-miRNAs [28]. Another way to influence the pri-miRNA processing is through the interaction of p68/p72 with other proteins. For instance p68 with SMAD protein, which facilitates the processing of pri-miR-21, or p53 associated with p68 enhancing the process of miR-16-1, miR-143, and miR-145, in response to DNA damage [29,30]. The p68/p72 complex is also used to decrease the processing a restrict number of miRNA (miR-16, miR-125a, miR-143, miR-145, and miR-195) upon stimulation of estrogen receptor alpha (ERα) [31]. (Figure 2. 2)

Intronic microRNA precursors bypass Drosha

An alternative pathway for miRNA biogenesis without Drosha-mediated cleavage is used by "mirtrons" that are intron-derived microRNAs. miRNAs are released from their host transcripts by the action of the splicing machinery and lariat-debranching enzyme, which yield pre-miRNA-like hairpins [32].

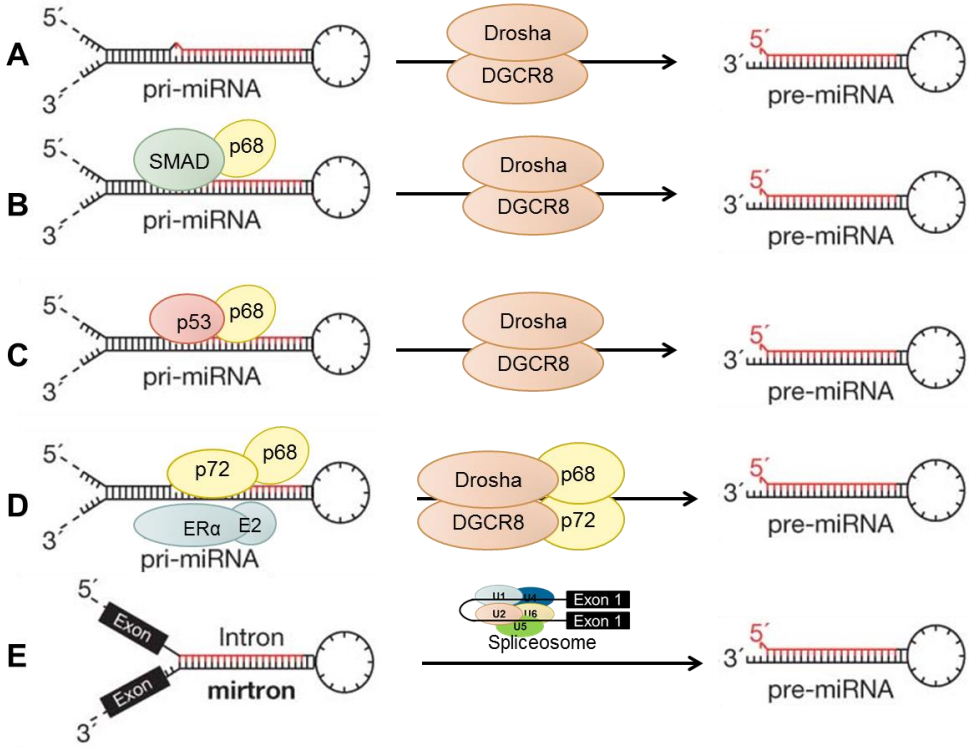

Figure 2. 2 Regulation of pri-miRNA processing The microprocessor complex Drosha–DGCR8 cleaves the pri-miRNA, releasing the pre-miRNA. (b) Some miRNAs require additional specificity factors (for example p68 and p72) for efficient cleavage. (c) Interaction of pri-miR-18a with hnRNP A1 facilitates cleavage of this specific miRNA by Drosha. (d) TGF-beta signaling induces SMAD binding to the miR-21 precursor and enhances its efficient processing by Drosha. (e) Splicing can replace Drosha processing if the released and debranched intron (mirtron) has the length and hairpin structure of a pre-miRNA.

Exportin-5–Ran-GTP mediates pre-miRNA export

After the nuclear processing the obtained pre-miRNA is recognized by the Exportin-5 (XPO5) and exported with Ran-GTP in the cytosol [33]. Exportin-5 protects also pre-miRNAs

from digestion recognizes them independently of the sequence or the loop structure, however the length of the double-stranded stem and presence of 3′ overhangs are important for proper recognition of pre-miRNAs [33-35].

microRNA processing in the cytosol

In the cytosol, the pre-miRNA is cleaved in a double-stranded RNA duplex (approximately 22 nt) by RNase III endonuclease, DICER, by the binding with PAZ domain to the 3′ overhang of the precursor-miRNA [36]. This interaction allows the pre-miRNA recognition and correct processing. Deletion of Dicer decreases or abrogates

the production of mature miRNAs [37]. The resulted duplex RNA is incorporated into the miRNA-induced silencing complex (miRISC), which includes the Argonoute proteins (Ago 1-4). Ago proteins regulate miRNA abundance post-transcriptionally, and loss of endogenous Ago2 diminishes the expression and activity of mature miRNAs [38]. In the miRISC one strand of the duplex is selected (the guide strand), while the opposite strand (miRNA*) is eliminated. The guide strand, bound to the GW182 family members in miRISC, regulates target miRNAs, in processing bodies (P-bodies) [27,39]. Recently has been shown that Ago2 is phosphorylated by p38 MAP kinase under cellular stress conditions, abetting its localization to P-bodies [40]. Ago2 pool has been identified at the nucleus by the RAN-GTP shuttle protein Importin-8 (Imp8), which is also required for miRNA-guided cytoplasmic regulation of a subset of mRNAs[41]. The re-import of miRNAs into the nucleus is particularly relevant because growing evidences that miRNAs regulate gene expression in the nucleus at the transcriptional level (e.g. miR-373 targets the promoter of E-cadherin) [42,43].

LncRNAs

DNA microarray technology revealed that the genome encodes at least as many lncRNAs as the known protein coding genes [44]. Currently, lncRNAs are defined as RNA larger than 200 bp devoid of coding potential.

XIST and H19, were discovered in the 1980s and 1990s accidentally by searching cDNA libraries for clones of interest (24, 25). However, the 21st century, with the advent of the human genome project and the technology progression (e.g. RNA sequencing; RNA-Seq), allows the identification of novel ncRNA species. LncRNAs were considered as "junk" RNA and now their functional roles remained mostly elusive and debated[45] [46]. It is observed to be key regulators at transcriptional and translational levels, effecting cell identify and function [47]. In addition many lncRNAs are restrained in sub-nuclear compartments [48,49], suggesting that such lncRNAs may be involved in a potential function in the located compartment.

lncRNA in gene regulation

In general, lncRNAs exert their function by regulating gene expression driving the formation of ribonucleic protein complexes. This connection was elucidated in 1975 by Paul & Duerksen, how found that biochemically purified chromatin contained twice as much RNA as DNA, raising the idea that RNA may influence chromatin structure and gene regulation [50]. LncRNAs orchestrate genetic regulatory outcomes, participate in chromosome dosage compensation, imprinting and other process (XIST, H19, and AIR) . (2; 6).

Xist is associated with chromatin regulation occurs during X chromosome dosage compensation in mammals (Figure 2. 3B). Importantly, this lncRNAs physically interacts with the Polycomb repressive complex 2 (PRC2), causing the PRC2 and H3K27me3 localization to the inactive X chromosome [51]. This type of regulation is indicated as *cis*-, meaning that lncRNAs contribute to local control of gene expression by recruiting histone

modification complexes to specific areas of the genome. Another silencing lncRNA is the metastasis-associated in lung adenocarcinoma transcript, MALAT1. Studies have shown that MALAT-1 regulates alternative splicing by interacting with the SR (serine/arginine) rich family that regulates alternative splicing [52]. Contrariwise, lncRNAs can recruit PRC2 and regulate distantly located genes throughout the genome; *trans*-regulation. One of these RNA is HOTAIR was the first of such RNAs recognized. HOTAIR physically associates with the PRC2 and modulates of hundreds of sites throughout the genome [53]. (Figure 2. 3C).

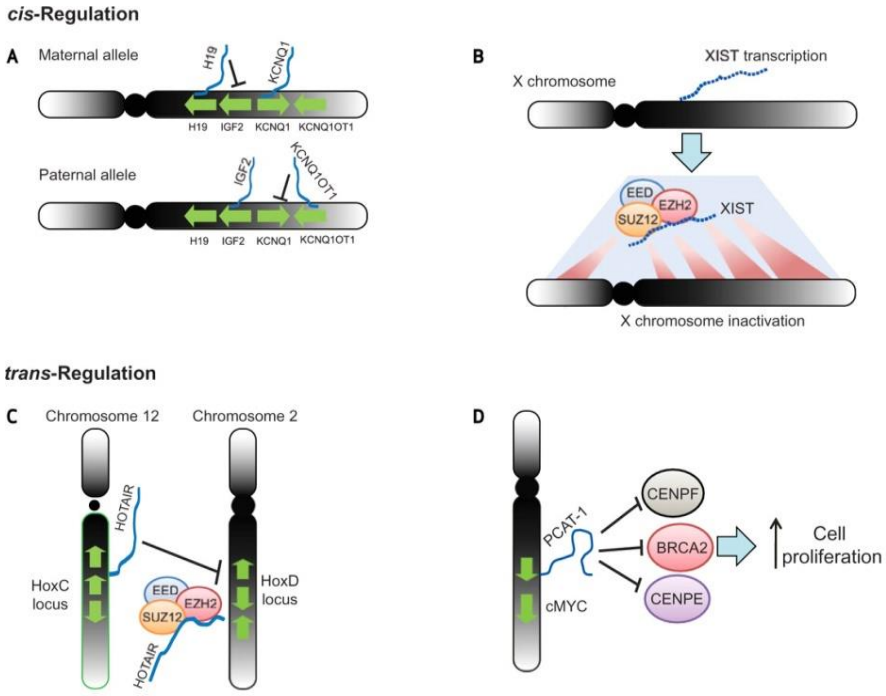

Figure 2. 3 LncRNAs in gene regulation. **A, B** cis-regulation of gene expression results in local control of genes neighboring, or on the same chromosome. For instance XIST transcription facilitates inactivation of an individual X chromosome in female by recruiting the Polycomb Repressive Complex 2 (PRC2). **C, D** lncRNA trans regulation that results in control of genomically-distant genes. For instance HOTAIR is transcribed from the HoxC cluster on chromosome 12 but represses the HoxD locus via PRC2-mediated epigenetic modifications .

Enhancer- associated lncRNA

Even though several lncRNA mediate gene silencing, some lncRNA function as enhancers of transcription. Genome-scale mapping of histone modifications and enhancer binding proteins have identified associated lncRNAs. This class of lncRNA called enhancer RNAs (eRNAs) are bidirectional, lacking polyA tail, and having very low copy number [54]. Moreover are marked by a H3K4me1 histone signature denoting enhancer regions (21), rather than the H3K4me3/H3K36me3 signature classically associated with lncRNAs. They have important functional roles [55] beyond to be simple byproduct of transcription in enhancer regions.

Recently a novel class of lncRNA has been identified, the translational regulatory lncRNA (trlncRNA). They are up-regulated in paired clinical breast cancer primary and lymph-node metastasis samples [56].

Long non-coding RNAs in immunity

Recent evidences have revealed functional roles for long non-coding RNAs (lncRNAs) in the regulation of inflammatory mediator or cytokine expression. Many lncRNAs were found to be dynamically regulated in macrophages under TLR ligand *via* the Myd88-NF-κB pathway. One of these is the long intergenic non-coding RNA (lincRNA)-Cox2, was found to act as a master regulator of gene expression. In Pam3CSK4 treatment of BMDMs, silencing of lincRNA-Cox2 induced up-regulation of Ccl5, but down-regulation of Il-6. These results suggest that lincRNA-Cox2 mediates both activation and repression of immune responses [57]. Moreover, the TLR4 pathway up-regulates lnc-IL7R, a lncRNA that overlaps the 3′-UTR of the human interleukin-7 receptor α-subunit (IL7R). This lncRNA negatively regulates E-selectin, VCAM-1, IL-8, and IL-6 expression following LPS stimulation [58].

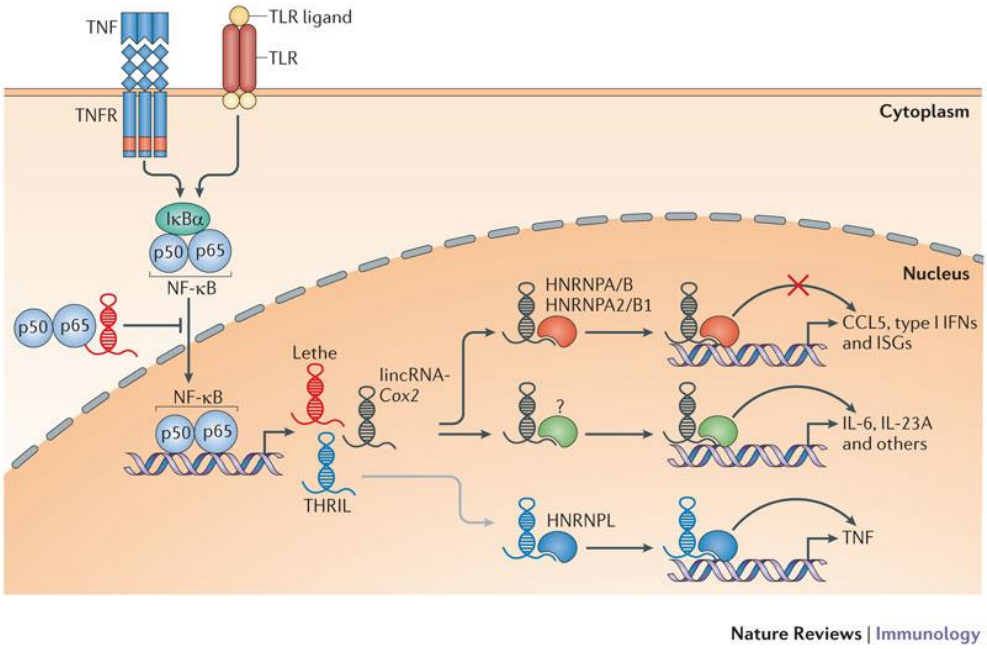

Figure 2. 4 lncRNAs in innate immunity. Figure from Carpenter et al., 2014

Lethe, a pseudo gene lncRNA of Rps15a, is selectively induced *via* NF-κB or glucocorticoid receptor agonist, and interacts with NF-κB subunit RelA to inhibit RelA DNA binding and target gene activation [59]. Finally, a lincRNA THRIL is induced by TNF in human monocytes through its interactions with HNRNPL130. Overalls, these studies emphasize the importance of lncRNAs in regulating gene expression in macrophages and highlight yet another layer of complexity in gene regulation (Figure 2. 4). Further analysis of their molecular functions could provide important insights into gene regulation, inflammation and human diseases.

AIM OF THE STUDY

It has become clear that chromatin landscape plays an important role in macrophage polarization and function, and determines the magnitude and specificity of the macrophage transcriptome. Exploration of epigenetic regulation of macrophage polarization and function is outstanding to explore with the additional epigenetic mechanisms, such as the role of noncoding RNA.

We have shown that macrophage are characterized by distinct miRNome in steady state and in activated phenotype. We have identified a novel miRNA induced downstream TLR4 signaling pathway, miR-135b which enforces the classic activation phenotype and damps the alternative one. Although miR-135b results as a hinge microRNA in macrophage polarization. Understanding whether miR-135b locus is accessible and activated in macrophage upon stimulation could open on other way to stabilize a macrophage phenotype in itself. Although, the aim of this study was to gain insight the epigenetic landscape that rules miR-135b expression in classic activated macrophages.

RESULTS

hsa-miR-135b is transcribed from within the intronic sequence of a long non-coding RNA, BLACAT1

Human miR-135b mapped on the negative strand of human chromosome 1 (chr1q32.1) in the intron of BLACAT1 (bladder cancer associated transcript 1; Figure 2. 5A) which was near LEMD1 (the murine host gene of miR-135b). Thus BLACAT1 was not interspecies conserved, and it was identified by Wang He and colleagues as a novel lncRNA associated with polycomb repressive complex 2 (PRC2) with a pro-oncogenic activity in bladder cancer and in gastric cancer [60,61]. We sought to define whether both RNAs are co-transcribed in classic activated macrophages by qPCR.

We evaluated the kinetic of BLACAT1 and miR-135b in M1-activated macrophages compared with the expression of miR-155 and its host gene and lncRNA, MIR155HG. As presented in Figure 2. 5 BLACT-1, is expressed in early hours while is down-regulated at 24h, while LPS induced miR-135b at 24h; the same kinetics occurs for miR-155 and its host gene, which is known to be co-expressed. Based on these results we supposed that miR-135b and its host gene are co-transcribed and they could share the same promoter.

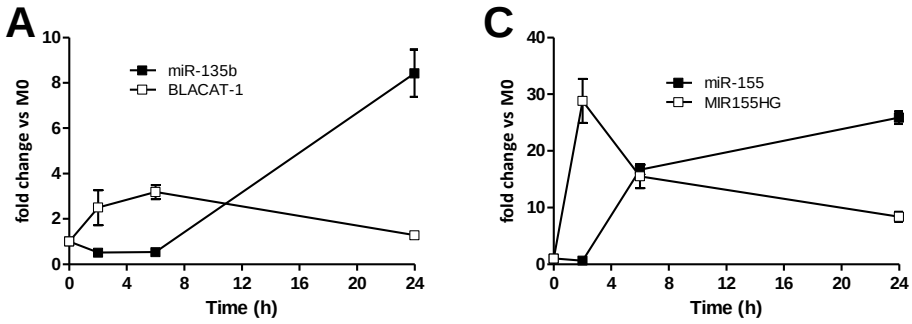

Figure 2. 5 hsa-miR-135b is transcribed from within the intronic sequence of BLACAT1. A, human macrophages were triggered with LPS (100ng/ml) at 2, 6 and 24h and BLACT1 and mature miR-135b were analyzed by qPCR. **B**, human macrophages were triggered with LPS (100ng/ml) at 2, 6 and 24h and MIR155HG and mature miR-155 were analyzed by qPCR. n =3; mean ± SEM. *p<0.05, **p<0.01; ****p < 0.0001 compared with resting macrophages.

miR-135b TSS identification

To clarify the relation between BLACAT1 and miR-135b we evaluated the locus chromatin landscape. Firstly, with an *in silico* approach using Human Epigenome Browser (http://epigenomegateway.wustl.edu/; Figure 2. 6). We found that miR-135b/BLACAT1 locus was extremely variable in all the selected cell lines (lung fibroblast, breast epithelia cells, colon smooth muscle cells, pancreatic cells, K562 and monocytes) in spite of miR-155 locus, which had a conserved active promoter shared between miR-155 and its host gene MIR155HG (Figure 2.6A). The enhancer/promote of miR-135b was not defined an active enhancer was designated proximal to BLACAT1 exon 2. To address the miR-135b transcriptional star site (TSS) we performed an *in silico* analysis of miR-135b genome sequence upstream 10 kb, using ProScan v.1.7. This analysis revealed two TSS located respectively at ~1kb and ~8kb from pre-miR-135b. Both putative regions conserved binding sites for TATA boxes and some transcription factors, such as NF-κB. We performed 5'-rapid amplification of cDNA ends (RACE) to address the miR-135b TSS. We obtained a PCR product of about 1350 bp, overlapping the putative region proximal to miR-135b.

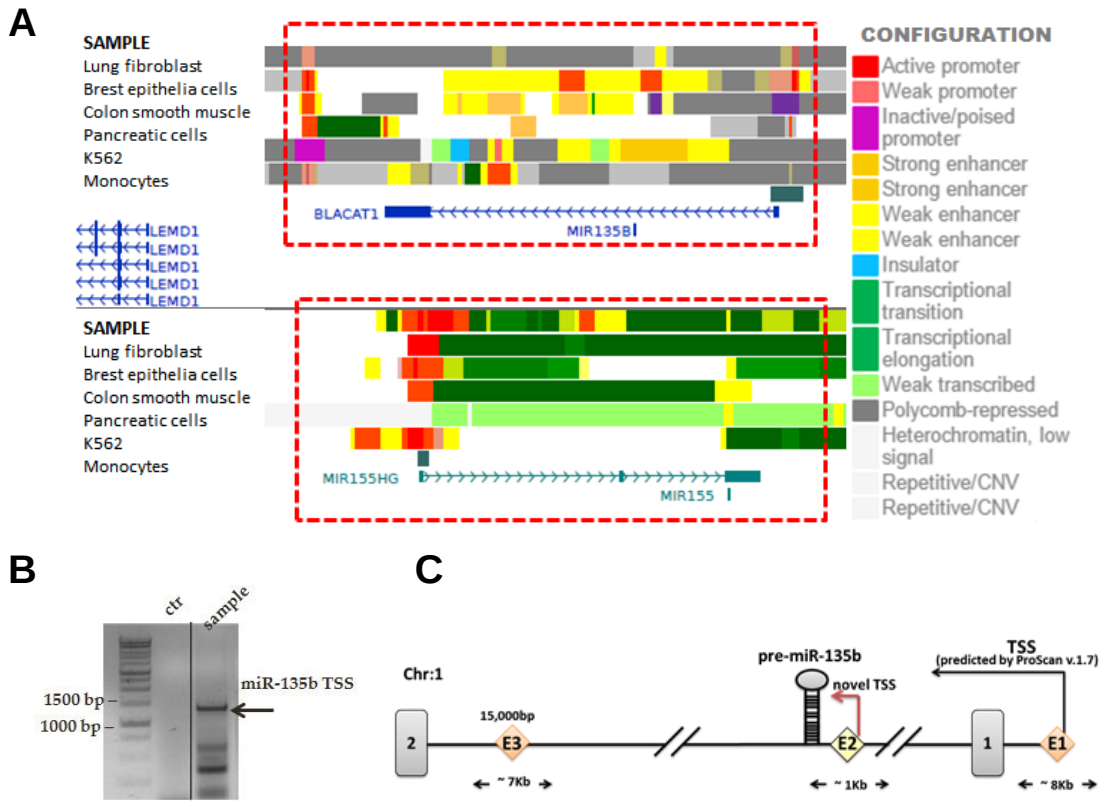

Figure 2. 6 Epigenetic modifications of BLACAT1/miR-135b locus. A, Genomic heatmap of cell line datasets covering BLACAT1 genomic region (upper panel) and MIR155HG (has-miR-155 host gene) displaying the chromatin state (e.g. Active promoter; red, enhancer; yellow and polycomb repression; grey), using Human Epigenome Browser and ProScan v.1.7. **B**, 5′RACE mapping of intronic miR-135b novel TSS.**C**, genomic organization of the miR-135b/BLACAT-1 locus with the putative TSS.

Epigenetic modifications of BLACAT1/miR-135b locus upon LPS treatment

We sought to address the miR-135b chromatin status in macrophages resting and activated. We performed ChIP analysis in resting and activated macrophages triggered with LPS for 2h. Therefore, we analyzed the "histone code" on the putative enhancer region proximal to the exon1 of BLACAT1 (E1), the putative enhancer region close to miR-135b TSS (E2), and the regulatory element active in monocyte (E3). We evaluated H3K4me3 (green), H3K27ac (blue); H3K27me3 (red) and for Polycomb 2 (SUZ12; cream, and EZH2; pink; Figure 2. 7). The ChIP analysis shown that pre-miR-135b region (E2) was the most active among the others three in both resting (M0) and LPS-induced macrophages (M1). There was an enrichment of PolyComb repressive complex 2 in this region in steady state macrophage, identified by increased occupancy of SUZ12, EZH2 and trymethylated histone H3 in K27, H3K27me3 , which was removed by the inflammatory treatment (Figure 2. 7A). In agreement with these data, there was a striking increase of acetylated histone H3 in K27 (H3K27ac) in M1 compared to M0 with trymethylated H3 lys4 (H3K4me3), confirming an open chromatin configuration (Figure 2. 7). The putative enhancer, E3, endured an active state under LPS stimulation; while the putative enhancer region (E1) flanking TSS was not engaged by any of the antibodies exanimated. Overalls, these data show that the proximal pre-miR-135b enhancer is activated by LPS triggered and also the second region nearby the BLACAT1 exon 2 is influenced. On the contrary, MIR155 is characterized by a single promoter region that is engaged by polycomb in steady state macrophage, repression that is completely removed upon LPS triggering (Figure 2. 5).

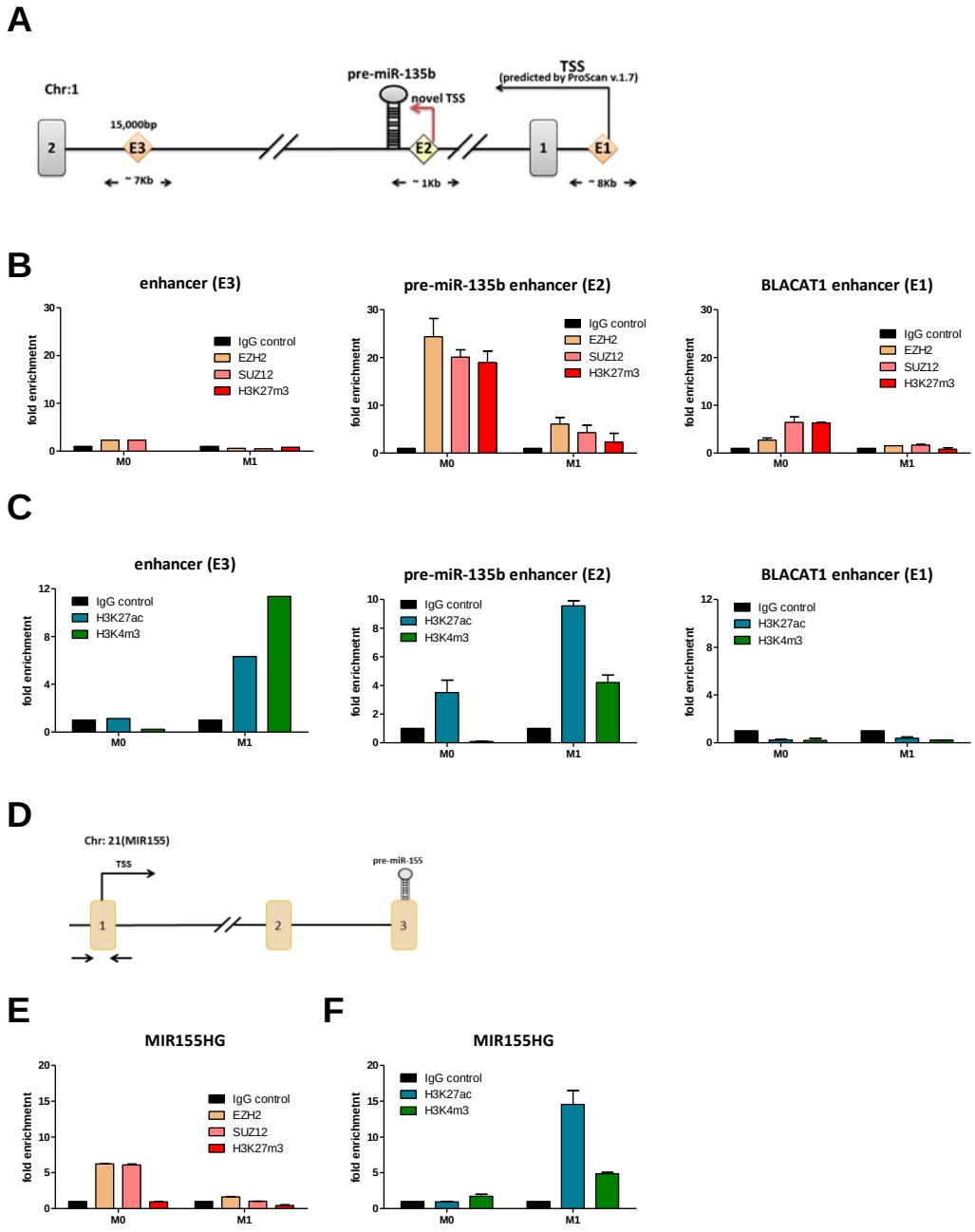

Figure 2. 7 Epigenetic modifications of BLACAT1/miR-135b enhancer region upon LPS treatment. A, schematic representation of BLACAT1/miR-135b locus, putative enhancer element (E1/2/3) are marked. Arrows shape show the enhancer region amplified by specific primers in qPCR. amplification of immunoprecipitated chromatin. **B**, CHIP analysis for PolyComb and repressive histone modification, H3K27me3, on the putative enhancer regions in resting and M1 macrophages. **C**, CHIP analysis of the putative enhancer regions in resting and M1 macrophages, using antibodies for active histone modification H3K4me3 and H3K27ac. **D**, schematic representation of MIR155HG/miR-155 locus. Arrows shape show the promoter region amplified by specific primers in qPCR amplification of immunoprecipitated chromatin. **E**, CHIP analysis for PolyComb and H3K27me3, on the promoter regions in resting and M1 macrophages. **F**, CHIP analysis of the promoter regions in resting and M1 macrophages, using antibodies for active

histone modification H3K4me3 and H3K27ac. Results of qPCR are analyzed with the ΔΔCt method. Values of each immunoprecipitated sample fold enrichment to their respective IgG control. Mean ± SEM (n = 3).

Correlation between host gene BLACAT1 and miR-135b in activated macrophages

The correlation between miR-135b and BLACAT1 is still an open question, to further address this issue we measured BLACAT1 and miR-135b levels in classical and alternative activated macrophages. As shown in Figure 2. 8A, there was no association with miR-135b and BLACAT-1 expression in macrophages stimulated with pro-inflammatory stimuli for 24h (LPS, IL-1β, IL-6, Pam3CSK4 and TNFα) with a Pearson correlation index of R =0,38. Again, the Figure 2.8 B shows no correlation between the lncRNA and miR-135b in alternative activated macrophages (R= 0,43) caused by the IL-10 triggered, which down-regulates miR-135b (as we have identified in Figure 1. 13), while BLACAT1 was induced. In addition, stimulation with glucocorticoids (dexamethasone; DEX) promoted the lncRNA expression and down regulated miR-135b (Figure 2. 8B), suggesting a novel inhibitory effect promoted by dexamethasone on the "inflammatory" miR-135b.

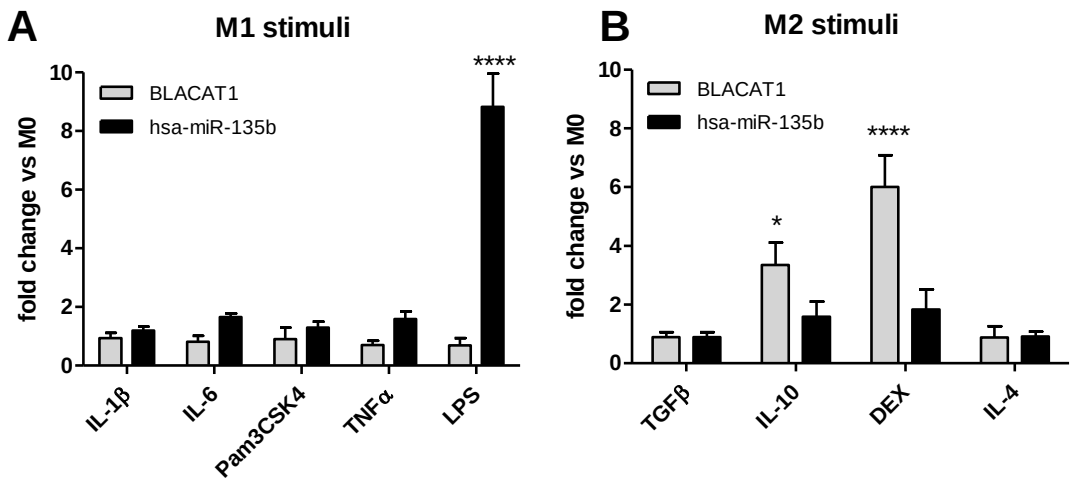

Figure 2. 8 Expression of host gene BLACAT1 and mature hsa-miR-135b. A, human macrophages were cultured for 24h with 100 ng/ml IL-1β, 20 ng/ml IL-6, 1 μg/ml Pam3CSK4, 20 ng/ml TNFα,100 ng/ml LPS **B,** macrophages were treated for 24h with 50 ng/ml TGF-β, 50 ng/ml IL-10, 10^{-6} M dexamethasone (DEX) and 20ng/ml IL-4. RNA was extracted and BLACAT (grey bar) and mature miR-135b (black bar) ware analyzed by qPCR. n =3; mean ± SEM. *p<0.05, ****p < 0.0001 compared with resting macrophages.

miR-135b is inhibited by glucocorticoids

In order to determine whether glucocorticoids have negative effect on miR-135 as well as on miR-155 during the TLR4-mediated macrophage inflammatory response [62], we analyzed miR-135b levels in human macrophages; we pretreated the cells with a synthetic glucocorticoid, dexamethasone (Dex; 10^{-6} M) for 1 h and then we stimulated them with LPS (100ng/ml) for 24 h. As expected, Dex blocks the NF-κB pathway, reduced the expression and the secretion of TNF-α, IL-6 and IL-12p40 (Figure 2. 9A-B). We further investigate with the same sample miR-155, miR-146b, and miR-135b expression levels. The three miRNAs were induced downstream of TLR4 pathway, and were significantly down-regulated by the combined action of dexamethasone and LPS. This profound miRNAs inhibition was well highlighted in time dependent manner (Figure 2. 9 D), while BLACAT1 is up-regulated by dexamethasone treatment, being unaffected by LPS (Figure 2. 9E). Overalls these data display that glucocorticoids inhibit miR-135b expression, revealing an imbalance expression between the microRNA and its host gene, BLACAT-1 that is significantly induced by glucocorticoids .

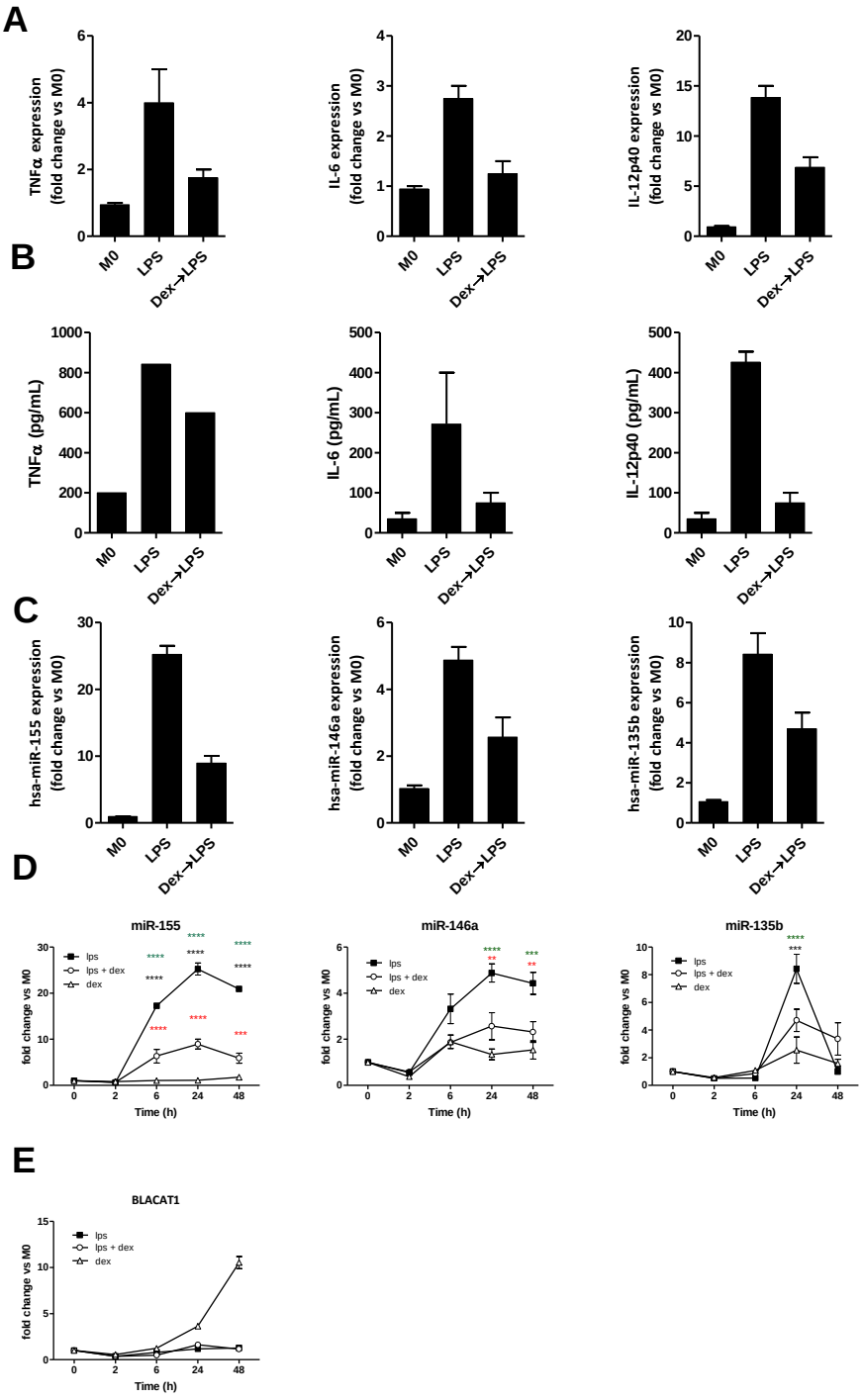

Figure 2. 9 Dexamethasone regulates miR-135b. Human macrophages were primed with Dex (10^{-6} M) and treated with LPS (100 ng/ml) for 24h **A-B**, TNF-α, IL-6, and IL-12p40 expression and protein levels were measured with qRT-PCR and ELISA respectively. Mean ± SEM (n=3). **C**, miR-155, miR-146a and miR-135b expression levels in qRT-PCR, data were normalized to the endogenous control U6. Mean ± SEM, n=3. **D**, miR-155, miR-146a and miR-135b expression levels in qRT-PCR in time course. **E**, BLACAT1 expression level, Mean ± SEM, n=3 *$p < 0.05$, **$p < 0.01$, ***$p<0.001$.

lncRNA BLACAT1 blocks miR-135b in DEX-activated macrophages

BLACAT1/linc-UBC1 is a nuclear LncRNA, which physically interacts with PolyComb repressive complex 2 (PRC2) mediating a transcription regulation [61]. Thus, we sought to determine whether BLACT1 was able to regulate the expression of miR-135b upon dexamethasone treatment. We measured the expression level of miR-135b in macrophages upon dexamethasone (24h) after silenced BLACAT1 with LNA™ longRNA GapmeR (lna-BLACAT1). As illustrated in Figure 2. 10, the expression of miR-135b is completely restored after the inhibition of the LncRNA in dose response manner. These data suggest that BLACT1 has a regulatory role on miR-135b, with a epigenetic control.

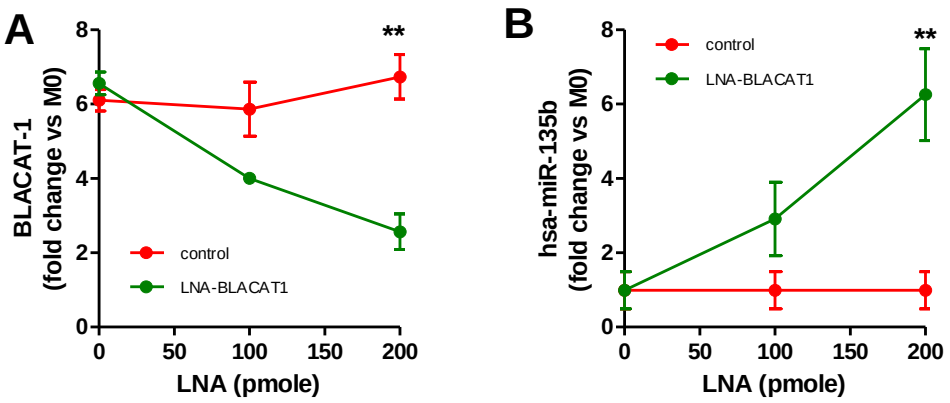

Figure 2. 10 lncRNA BLACAT1 blocks miR-135b in DEX-activated macrophages. Human macrophages were transient transfected with LNA™ longRNA GapmeR of BLACAT1 at 100 and 200 pmole. After 24h the cells were treated with Dex (10^{-6} M) and BLACAT1 (**A**) and miR-135b (**B**) levels were evaluated qRT-PCR. Mean ± SEM (n=3). **p < 0.01.

Epigenetic modifications of BLACAT1/miR-135b enhancer region upon DEX treatment.

To further investigate the miR-135b inhibition promoted by BLACAT1 upon glucocorticoids. We evaluated in ChIP assay the chromatin occupancy in human macrophages stimulated with glucocorticoids after 2h, supposing a repressed chromatin at the active enhancer E2.

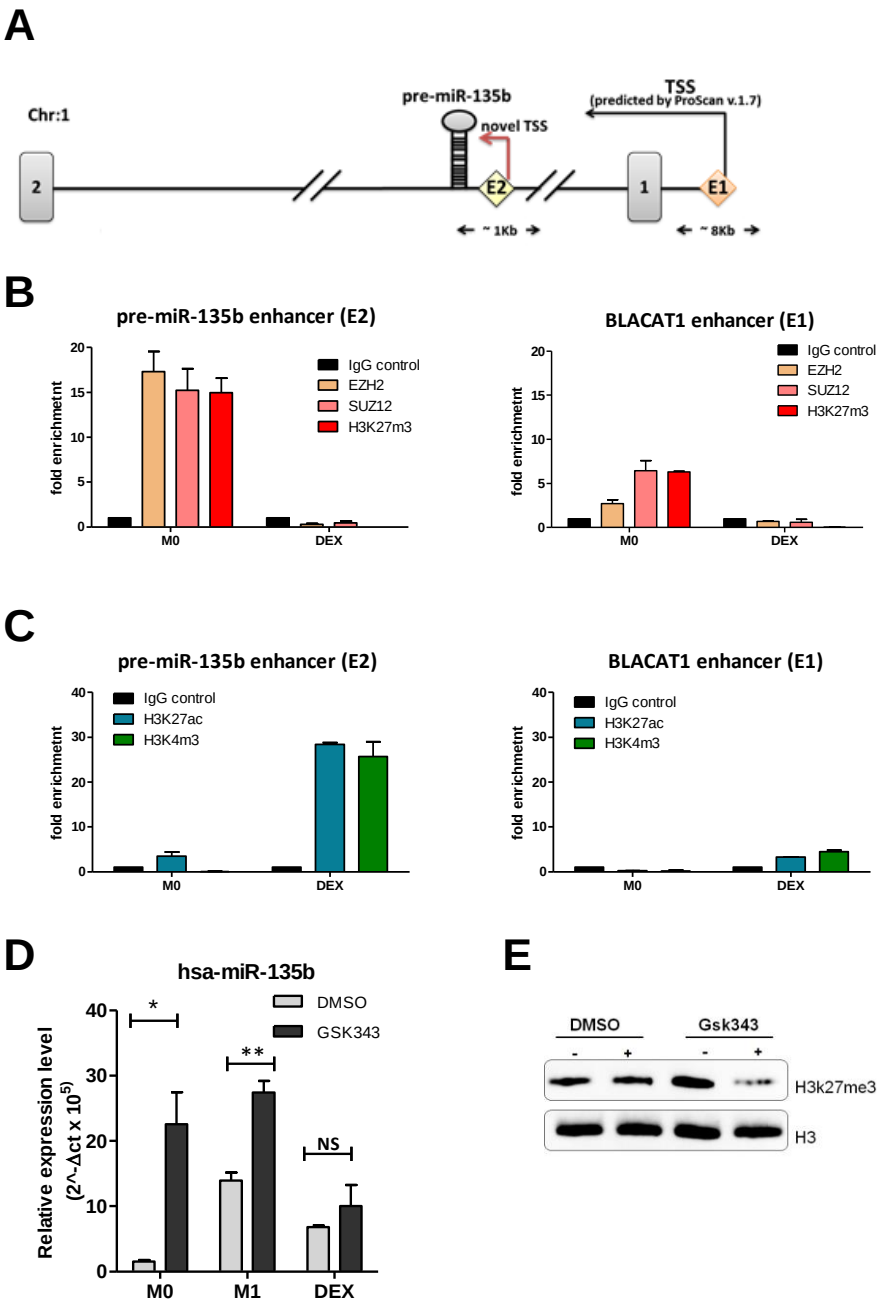

Figure 2. 11 Epigenetic modifications of BLACAT1/miR-135b enhancer region upon DEX treatment. A, schematic representation of BLACAT1/miR-135b locus, putative enhancer element (E1/2/3) are marked. Arrows shape show the enhancer region amplified by specific primers in qPCR. amplification of immunoprecipitated chromatin. **B**, CHIP analysis for PolyComb and repressive histone modification, H3K27me3, on the putative enhancer regions in DEX-induced macrophages. **C**, CHIP analysis of the putative enhancer regions in DEX-induced macrophages, using antibodies for active histone modification H3K4me3 and H3K27ac. Results of qPCR are analyzed with the ΔΔCt method. Values of each immunoprecipitated sample fold enrichment to their respective IgG control. Mean ± SEM (n = 3) **D,** miR-135b relative expression levels in resting and activated macrophages treated 24h with 200 nM GSK 343 (EZH2 synthetic inhibitor) or control

(DMSO). Mean ± SEM (n = 3), * p<0.05 ** p<0.01. **E,** representative immunoblotting of nuclear extracts from resting macrophages treated 24h with 200 nM GSK343 or DMSO.

Surprisingly, dexamethasone did not entail the recruitment of the PolyComb repressive complex 2 (SUZ12 and EZH2) on the pre-miR-135b enhancer (E2) as illustrated in Figure 2. 11. On the contrary, upon dexamethasone stimulation the main enhancer was highly engaged by the active histone modification H3K4me3 and H3K27ac (Figure 2. 11C). As shown in Figure 2. 11D, resting and classical activated macrophages treated with GSK 343, an EZH2 inhibitor, expressed high levels of miR-135b, while Dex-induced macrophages were not influenced by GSK 343, stressing that the locus was not repressed by PRC 2.

Glucocorticoids inhibit miR-135b processing through MCPIP1

To address the glucocorticoids regulation on miR-135b, we speculate a post-transcriptional regulatory event. We measured the levels of pri-miRNA, pre-miRNA and mature miR-135b upon dexamethasone and LPS stimulation, as control. The Figure 2. 12 displayed how dexamethasone up-regulated pri-miR135b with a remarkable accumulation of the transcript at the late hours (24 and 48h) in spite of LPS stimulation whereby pri-miRNA was induced at the early hours and following repressed. Dexamethasone induces likewise BLACAT1, however, the pri-miR-135b augmentation was not translated in mature miRNA (Figure 2. 12C); on the contrary, glucocorticoids repressed pre-miR-135b expression (Figure 2. 12**Errore. L'origine riferimento non è stata trovata.**B). Overall, dexamethasone blocks the miR-135b processing, probably in concert with lncRNA BLACAT1 accumulation.

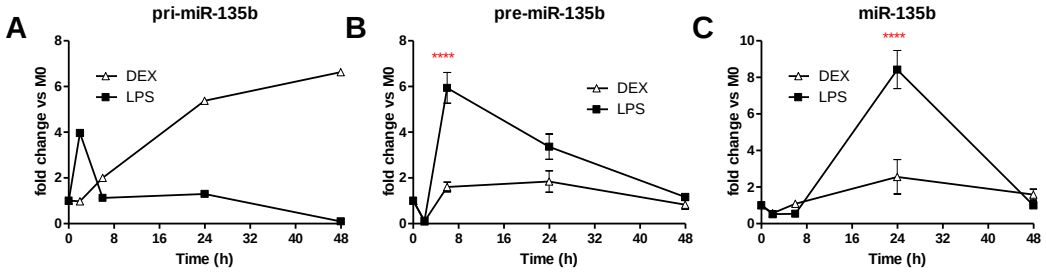

Figure 2. 12 Dex inhibits miR-135b processing. Human macrophages were cultured for the indicated times in medium alone or in the presence of 100 ng/ml LPS or dexamethasone (DEX 10^{-6} M) Total RNA was extracted and pri-miR-135b (**A**), pre- (**B**) and mature miR-135b (**C**) expression was assessed by RT-qPCR and normalized to the snRNU6 levels. The results are expressed as fold change respect to resting macrophages (mean ± SEM; *n*= 3). * p<0.05 ** p<0.01, **** p<0.0001

Hiroshi I Suzuki and colleagues identified MCP-induced protein 1 (MCPIP1) as an immune regulator ribonuclease which suppresses miR-135b biosynthesis in cancer [63]. Furthermore, MCPIP1 expression dynamically changes during inflammation and has been shown to promote the mRNA decay of several inflammatory genes such as IL-6 [64]. We examined the potential involvement of MCPIP1 in the regulation of miR-135b in Dex-

induced macrophages, lacking the LncRNA. We silenced, i*n vitro*, BLACAT1 in human macrophages using LNA™ longRNA GapmeR and subsequently treated the cells with dexamethasone or LPS measuring MCP1PI expression.

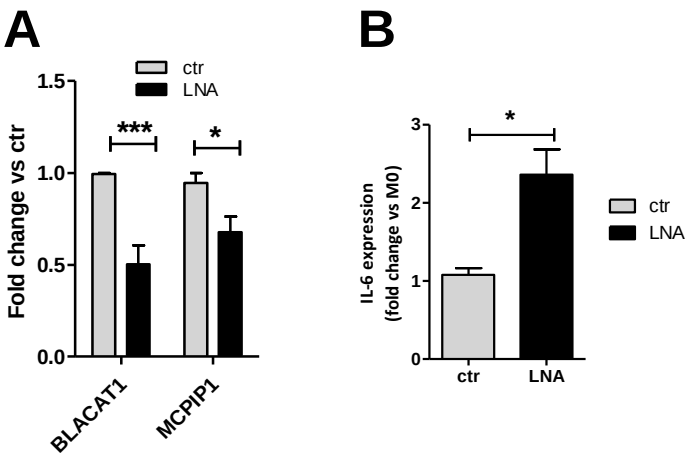

Figure 2. 13 MCPIP1 expression is impaired by BLACAT1 inhibition. Human macrophages were transient transfected with LNA™ longRNA GapmeR of BLACAT1 at 200 pmole. After 24h the cells were treated with Dex (10^{-6} M) and BLACAT1 and MCPIP1 mRNA (**A**) and IL-6 (**B**) levels were evaluated qRT-PCR. Mean ± SEM (n=3). *p<0.05; ***p < 0.001.

In accordance with our previous results the lack of BLACAT1 further suppressed MCPIP1 expression (Figure 2. 13A), with a followed IL-6 mRNA induction (Figure 2. 13 B). These data emphasize that miR-135b expression is compromised by the presence of MCPIP1 stabilized by BLACAT1; upon dexamethasone treatment. An open question that remains is how BLACAT1 impacts on MCPIP1 expression.

BLACAT1 role in resolving macrophages

Confirmed the negative interplay between miR-135b and BLACAT1, we sought to investigate the impact of BLACAT1 on pro-resolving macrophages. We silenced the lncRNA with LNA™ longRNA GapmeR upon dexamethasone stimulation and we

analyzed some pro- and anti-inflammatory markers. BLACAT1 reduction in DEX-treated macrophages impacts on the expression of CCL5 (Figure 2. 14 E) and again IL-6 (Figure 2. 14 H), but the M2 marker genes TGF-β (Figure 2. 14 I), IL-10 (Figure 2. 14 L) were induced by the lack of BLACT1 at 2h.While the other analyzed genes were not influenced by the lncRNA silencing. The interesting subject emerged by these data is that BLACAT1 regulates M2 genes mapped on the same chromosome, chr1. Further investigation are needed to clarify the activity of this lncRNA.

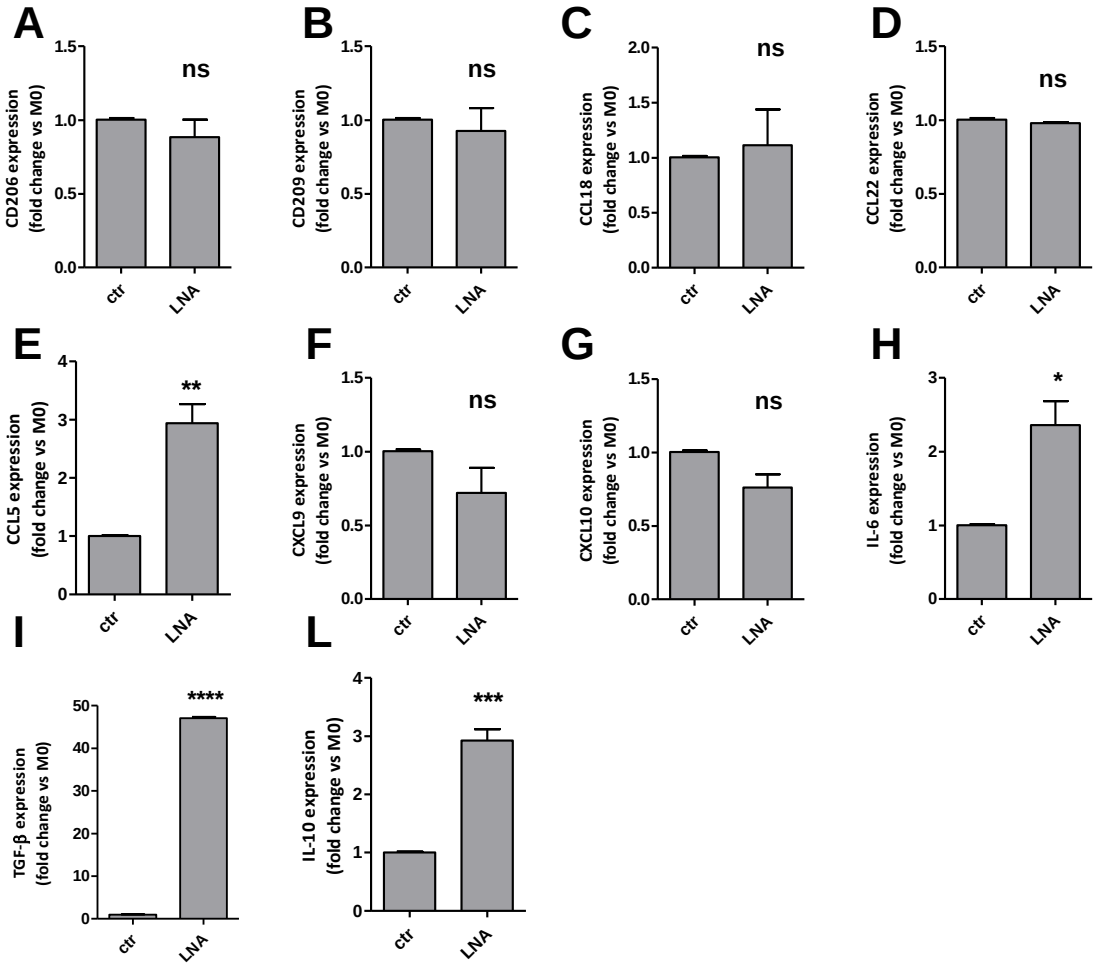

Figure 2. 14 BLACAT1 dependent genes. Human macrophages were transient transfected with LNA™ longRNA GapmeR of BLACAT1 at 200 pmole. After 24h the cells were treated with Dex (10^{-6} M) and indicated genes levels were evaluated qRT-PCR. Mean ± SEM (n=3). *p<0.05; **p<0.01; ***p < 0.001 ****p<0.0001 t-test.

DISCUSSION

It is becoming largely accepted that the "junk" RNAs are relevant and attract interest. We have point out the activated macrophage miRNome, in particular the role of miR-135b in damping the alternative phenotype. For its implication and verified role we have analyzed the chromatin occupancy on miR-135b. The *in silico* analyses shown that the miRNA reside in the intron region of BLACAT1. The majority of intronic microRNAs reported to play an antagonistic or synergetic role as an enemy or a partner of their host genes [65]. So we sought to define the relation between BLACAT1 and miR-135b. Firstly, we revealed that BLACAT1/miR-135b locus is extremely complex in terms of transcriptional control and cell specificity. As highlighted in public CHIP-seq data merged with ProScan v.1.7 output, there is an evidence of three putative enhancer regions on the locus. We identified the miR-135b TSS with 5'RACE at about 1kb distance, located on the enhancer shared by both miR-135b and BLACAT1. Indeed, ChIP experiments, for different markers of chromatin activity, indicate a well-defined landscape in this specific region. We revealed that LPS treatment leads to miR-135b up-regulation causing a chromatin opening conformation on the enhancer region, with an enrichment of H3K4me3 and H3K27ac; discharging PolyComb 2. These results are in agreement with our previous findings, indicating miR-135b as a novel M1 associated microRNAs.

Furthermore, exploring the BLACAT1 and miR-135b relation, we remarkably identified a different types of responses for both RNAs upon LPS or anti-inflammatory stimuli such as glucocorticoids and IL-10. In particular, the lncRNA, BLACAT1 is highly induced in macrophages treated with dexamethasone (synthetic GCs) and IL-10, while mature miR-135b was found to be down-regulated. Supposing a chromatin repression on the miR-135b enhancer upon glucocorticoids, we performed a Chip analysis to evaluate the EZH2 and SUZ12 occupancy. In support to this speculation, BLACAT1 is associated with repressor complex polycomb 2 acting as molecular scaffold [61].

Surprisingly, we found that GCs treatment induced an open chromatin, which is not translate with mature miR-135b. Therefore PRC2 is not engaged in this locus with the help of BLACAT1, this aspect in agreement with the *trans*-acting of BLACAT1. Overalls miR-

135b/BLACAT1 transcriptional landscape highlights an antagonistic role with the RNAs. Furthermore, we highlighted that glucocorticoids inhibited the biogenesis of miR-135b, finely though the accumulation of the pri-miRNA. We speculate that BLACAT1 promotes this upon dexamethasone treatment. We found that inhibition of BLACAT1 leads to the over-expression of miR-135b, in concert with the inhibition of MCPIP1. MCPIP1 (MCP-induced protein 1) is an immune regulator ribonuclease which suppresses miR-135b biosynthesis antagonizing DICER[63] in cancer. We confirmed MCPIP1 and miR-135b anti-correlation also in pro-resolving macrophages.

In summary, in the present study, we discovered that miR-135b and its host gene, BLACAT1 are under control of the same enhancer proximal to pre-miR-135b. However there is a stormy relation between the two noncoding RNAs, in particular upon anti-inflammatory stimuli, such as glucocorticoids. BLACAT1 is extremely up-regulated in this condition, whereas miR-135b processing is block whit a following lack of the mature form. This is ascribable to MCPIP1 expression that is inhibited by the absence of the lncRNA. These data strongly suggest that the expression of miR-135b is closely associated with its lncRNA, BLACAT1. Open questions remain regarding how MCPIP1 and BLACAT1 interact to influence the miR-135b pathway. Moreover BLACAT1 inhibition cause up-regulation of CCL5, IL-6, TGF-β and, IL-10 upon GC treatment, with a possible chromatin *trans*-regulation. For these propose further analysis are required.

MATERIALS AND METHODS

Reagents.

E. coli lipopolysaccharide (LPS, 055:B5 strain) was purchased from Sigma, IL-10 from R&D System and Dexamethasone was acquired from Sigma-Aldrich. GSK 343, a EZH2 inhibitor was purchased from Sigma.

Macrophage generation and polarization.

Human monocytes were isolated from healthy donor buffy coats by two step gradient centrifugation using Ficoll-Paque (GE Healthcare) and Percoll (Amersham), as described in the precedent chapter. Resting macrophages were then polarized to M1 macrophages using 100 ng/ml LPS or M2-like macrophages using 10-6 M dexamethasone for indicated time . Resting macrophages (M0) were left untreated in culture medium.

miRNA isolation and Quantitative RT-PCR Analyses.

Total RNA and small RNAs faction were isolated by TRIzol (Ambion) and reverse transcribed. qPCR for has-miR-135b (Cat: 002261), has-miR-155 (Cat: 002623), has-miR-146a (Cat: 000468), U6 snRNA (Cat: 001973) were performed in CFX Connect™ Real-Time PCR Detection System (BioRad). Relative miRNAs expressions were determined by using the ΔΔCt method [66], normalizing the levels with U6 snRNA.

RNA isolation and gene expression analysis.

Total RNA was purified and reverse transcribed to manufacturer's guidelines. Quantitative RT-PCR was performed using specific oligonucleotides which are reported in Table 2. 1. in a CFX Connect™ Real-Time PCR Detection System (BioRad). Results were analyzed with CFX software, and relative expression values were calculated according to ΔΔ cycle threshold method normalized using GAPDH as housekeeping.

Table 2. 1 Real time PCR primers

Genes	Forward primer (5'-3')	Reverse primer (5'-3')
ALOX15	CTTGCTCTGACCACACCAGA	GCTGGGGCCAAACTATATGA
CCL18	GAGGCCAGGAGTTGTGAGTT	CATGGTGCAGACGAGGACAA
CCL5	TATTCCTCGGACACCACACC	ACACACTTGGCGGTTCTTTC
CD206	GGGCAGTGAAAGCTTATGGA	CCTGTCAGGTATGTTTGCTCA
COX2	CCCTTGGGTGTCAAAGGTAA	GCCCTCGCTTATGATCTGTC
CXCL10	AGCCAATTTTGTCCACGTGT	TGATGGCCTTCGATTCTGGA
CXCL9	TGAGAAAGGGTCGCTGTTCC	TCAAACTGCTTGGCTCACCA
GAPDH	GATCATCAGCAATGCCTCCT	TGTGGTCATGAGTCCTTCCA
IL-10	AGAACCTGAAGACCCTCAGGC	CCACGGCCTTGCTCTTGTT
CD209	GGCAGAAGTGTGGGTAGACAG	AGGGCTAGGTCCCAAAAGAC
CD226	GCCCGAAAATAAGCCGACTG	TGCTCCTTTATGAGGATGGGC
TGF-β	GGGCTACCATGCCAACTTCT	GACACAGAGATCCGCAGTCC
IL-6	TGCGATGGAGTCAGAGGAAA	ACTAGGGGGAAAAGTGCAGC
MCPIP1	GTCTGACGGGATCGTGGTTT	TCAGGGGGCATAAACTTGTCA

Macrophage Transfection.

Purified macrophages were cultured in medium supplemented with 2 mM glutamine and 10% FCS. Were transfected with indicated concentration of BLACAT1 LNA™ longRNA GapmeR (Exiqon), using TransIT-TKO Transfection Reagent (Mirus Bio), according to the manufacturer's protocol.

ChIP analysis

Sheared chromatin from 5 × 10^6 human macrophages was immunoprecipitated overnight (ON) at 4 °C with 10 μg of the following antibodies: αH3K27me3 (Merck-Millipore, Billerica, MA, USA; cat: 07-449); αH3K4me3 (Merck-Millipore; cat: 07-473); non-specific rabbit IgG (Santa Cruz Biotechnology Inc, Dallas, TX, USA; cat: sc-2027); αEzh2 (Merck-Millipore; cat: 17-662); αSUZ12 (Merck-Millipore; cat: 07-472); αH3K27ac (Merck-Millipore; cat: 07-473);non-specific mouse IgG (Santa Cruz Biotechnology Inc; cat: sc-2025). Quantitative real-time PCR (qPCR) was performed in triplicate by using -specific primers (TableS1). Signals obtained from the ChIP samples were normalized on signals obtained from input sample . Results were expressed as fold enrichment relative to untreated cells.

Immunoblots.

Protein were extracted from macrophages as previously described, by SDS-PAGE electrophoresis. Then were transferred to nitrocellulose (Hybond; GE Healthcare). Blotted membranes were stained for MCPIP1 and β-ACTIN using a concentration of 1:200 overnight. The labeled primary Abs using a secondary Abs conjugated to HRP, which was detected using ECL reagent.

In silico analysis

Evolutionary sequence conservation was examined using the UCSC Genome Browser track (http://genome.ucsc.edu/) and ECR browser (http://ecrbrowser.dcode.org). Identification of transcription factor–binding sites was performed using Genomatix suite programs Mat Inspector (http://www.genomatix.de), and Transfac matrix Database (v.7.0) track on UCSC Genome Browser.

Rapid Amplification of cDNA Ends (5′-Race)

5′-RACE PCR was performed according to the manufacturer's protocol (Invitrogen).
RNA was prepared from a pool of human macrophages by ligating the RNA with a 5′ RACE adapter and a single-stranded cDNA was generated. For nested PCR specific primers were chosen to obtain a sufficient amplification product. Primers were designed using Primer3 software, and BLAST (Table 2). Amplified products were separated on an agarose gel and visualized by ethidium bromide staining.

ChIP Assay.

Sheared chromatin from 5 × 10^6 macrophages was immunoprecipitated overnight (ON) at 4 °C by using polyclonal antibodies against polymerase II (N-20; sc-899; Santa Cruz Biotechnology), H3 (ab1791; Abcam), H3K27ac (ab4729; Abcam), H3K4me1 (39297; Active Motif), H3K27me3 (07-449; Millipore-Upstate). Signals obtained from the ChIP samples were normalized on control IgG (12-370; Millipore-Upstate) in quantitative real-time PCR (qRT-PCR), performed in triplicate by using specific primers (Table).

Statistical analysis.

Results are expressed as mean ± SEM and statistical significance was based on non-parametric t-test or non-parametric ANOVA followed by post hoc Bonferroni's test performed using the GraphPad Prism 5 software (GraphPad Software).

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Chapter: MicroRNA-135b in gouty arthritis

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INTRODUCTION

Gout pathogenesis

Gout is an arthritis caused by deposition of monosodium urate (MSU) crystals within joints and periarticular tissues after chronic hyperuricaemia. This pathology affects 1-2% of adult population being the first cause of arthritis in western countries [1]. In acute gouty arthritis, macrophages phagocyte MSU activating NLRP3 inflammasome thru the priming with TLR2/4. The activated NLRP3 leads to caspase-1, which in turn cleaves pro-IL-1β to produce active IL-1β. IL-1β is the master cytokine produced by MSU stimulation. Its secretion induce the production of other pro-inflammatory mediators, including, tumor necrosis factor-α (TNF-α), interleukin-8 (IL-8) and interleukin-6 (IL-6).

Inflammasome

The inflammasome is a cytosolic protein complex, which includes an intracellular sensor (typically a Nod-like receptor, NLR), the procaspase-1 and the adaptor molecule ASC. Inflammasome activation mediates the production and secretion of interleukin 1β (IL-1β) and IL-18 via caspase-1. The inflammasome belonging to the NLR family include NLRP1, NLRC4 and NLRP3. The NLRP3 (NLR family, pyrin domain containing 3) inflammasome is the most well characterized of the family ones. Its activation required two steps, firstly the "priming" stimulus, provided through activation of a TLRs (e.g. with LPS via TLR4, triggering the NFκB) [2]. This priming step induces the up-regulation of NLRP3, pro-IL-1β and pro-IL-18. After, NLRP3 is activated and caused the maturation of caspase-1, which cleaves pro-IL-1β and pro-IL-18 to mature and releasable IL-1β and IL-18 [3]. Gain-of-function mutations in NLRP3 cause a higher production of IL-1β even in non-stimulated cells (Figure 3. 1).

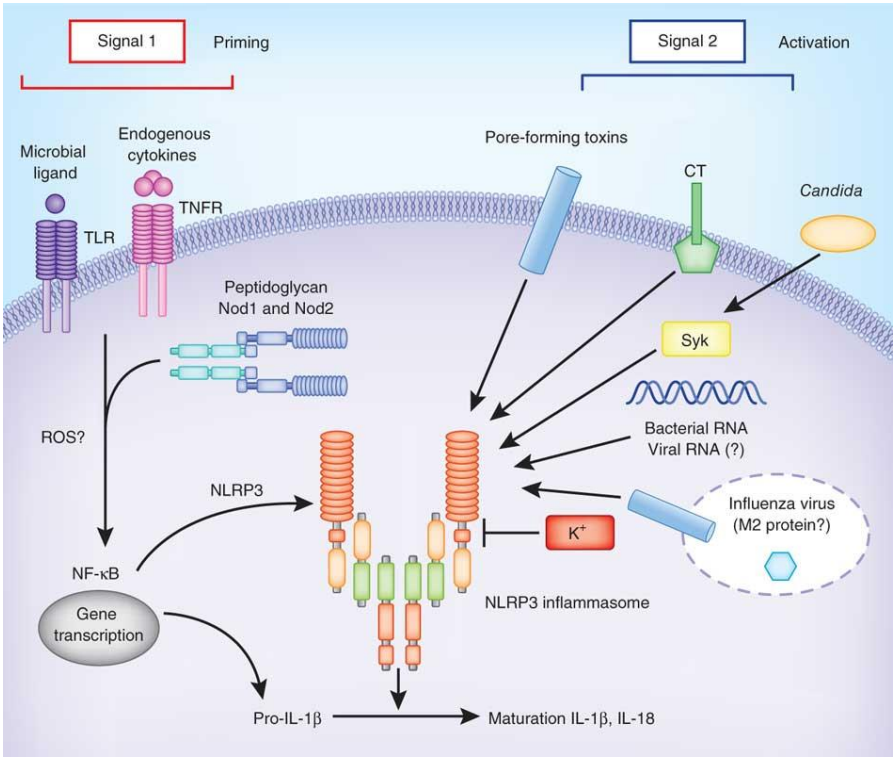

Figure 3. 1 NLRP3 inflammasome activation. Activation of caspase-1 via NLRP3 requires two signals. Signal 1 is represented by microbial molecules or endogenous cytokines and is required for the upregulation of NLRP3 and pro-IL-1β. Signal 2 activates the NLRP3 inflammasome. Activation by S. aureus, S. pyogenes, and S. pneumoniae and V. cholerae is mediated by pore-forming toxins. Other bacterial toxins can also induce the activation of the NLRP3 inflammasome, such as cholera toxin (CT) or C. difficile toxins TcdA and TcdB (not shown here); C. albicans induces activation of the NLRP3 inflammasome through the kinase Syk, although the mechanism involved is unclear. Influenza virus can induce the activation of the NLRP3 inflammasome, but it is controversial whether this is due to a pore-forming activity mediated by M protein or to sensing of viral RNA species in the cytosol. Cytosolic bacterial RNA induces activation of the NLRP3 inflammasome. TNFR, receptor for tumor-necrosis factor. (From: Franchi, Muñoz-Planillo & Núñez, 2012)

Interleukine-1β pathway

Il-1β is a pivotal inflammatory cytokine involved in cell proliferation, differentiation and apoptosis [4] It is a member of the interleukin-1 family: IL-1α, IL-1β, IL-1 receptor antagonist, IL-18, IL-33 and IL-F.

IL-1β binds to IL-1 receptor type 1 expressed by a wide variety of cells is shared with IL-1α. IL-1R1 signals through the binding with IL-1R accessory protein (IL-1RAP) that recruits the cytosolic MyD88 all the way to Nf-κB activation. Pro-IL-1β is cleaved by the caspase-1, but can also be cleaved in caspase-1 independent process [5]. However its

production and release depends on the cell types, monocytes in vitro present constitutively activated caspase-1, therefore they do not need a second hit to release IL-1β. Macrophages instead need the two signals [6]. IL-1β mediates pro-inflammatory effects by promote the expression of IL-6 and CSF, also induces increased expression of adhesion molecules such as ICAM-1 and VCAM-1 [7] (Figure 3. 2).

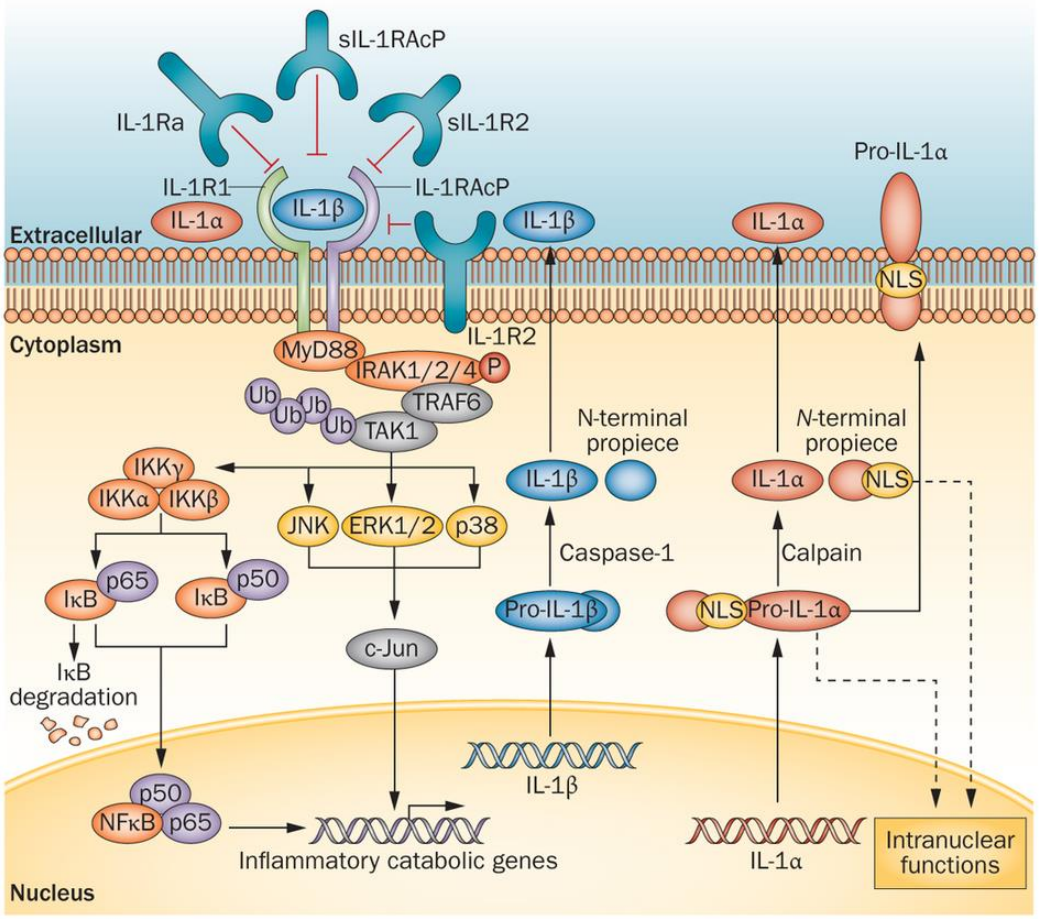

Figure 3. 2 Interleukine-1β pathway

miRNAs and gout

miRNAs are evolutionarily-conserved endogenous non-coding RNAs in involved in many diseases, including gouty arthritis [8]. Hyperuricaemia is a major risk factor of gout, currently has been shown that miR-34a and miR- 448 are involved in the production of uric acid [9,10]. Moreover, miRNAs could influenced the gout arthritis though the regulation of TLR2 and TLR4 on the macrophages (e.g. miR-155, miR-146 and miR-21) [11].

The NLRP3 inflammasome is a central regulator in the inflammatory process, leading the production of IL-1β. Activity that is reduced by the direct miR-223 targeting [12]. IL-1β is also central in the regulation of immune and inflammatory responses. However, there is no miRNA identified to target IL-1β from current literature.

AIM OF THE STUDY

Gout is an autoinflammatory disorder associated with deposition of MSU crystals in joints and periarticular tissues. In acute gouty arthritis, macrophages phagocyte MSU activating NLRP3 inflammasome thru the priming with TLR2/4. The activated NLRP3 leads to caspase-1, which in turn cleaves pro-IL-1β to produce active IL-1β. Although, IL-1β is the master cytokine produced by MSU stimulation. Its secretion induce the production of other pro-inflammatory mediators, including, tumor necrosis factor-α (TNF-α), interleukin-8 (IL-8) and interleukin-6 (IL-6).

In our previously study we have proved the importance of miRNAs in macrophage activation and polarization increasing this landscape. Pointing out miRNA profile is an essential step towards discover novel miRNA functions in this thinly regulates process. Different studies tried to evaluate this issue however is emerged an uneven scenery due to species specific and the use of different protocols of macrophage polarization [13]. To further understand miRNAs implication on this pathology, to settle the base for an effective treatment, we sought to typify the miRNome of murine miRNAs in all the phases of gout arthritis. And to define its relevance in the inflammation progression.

RESULTS

miRNome expression in the in vivo MSU-crystal model

We performed this study to determine whether miRNAs contribute to the progression of particle-induced synovitis, a model broadly use to analyses the implication of the inflammasome and IL-1β signaling pathway. Wild type mice were injected with MSU crystals (100 µg/knee joint), 380 miRNAs were profiled with TaqMan miRNA Arrays on total RNA from periarticular tissue after 3, 6, 15 and 24 hours from MSU injection. A restrict number of miRNAs (~30) was significantly up-regulated in the late time point, 6h as it is shown in Figure 3. 3 as shades of red in the heatmap. While another limited miRNAs group (~19) was induced at 3h from the MSU injection followed by a down-regulation (green shades).

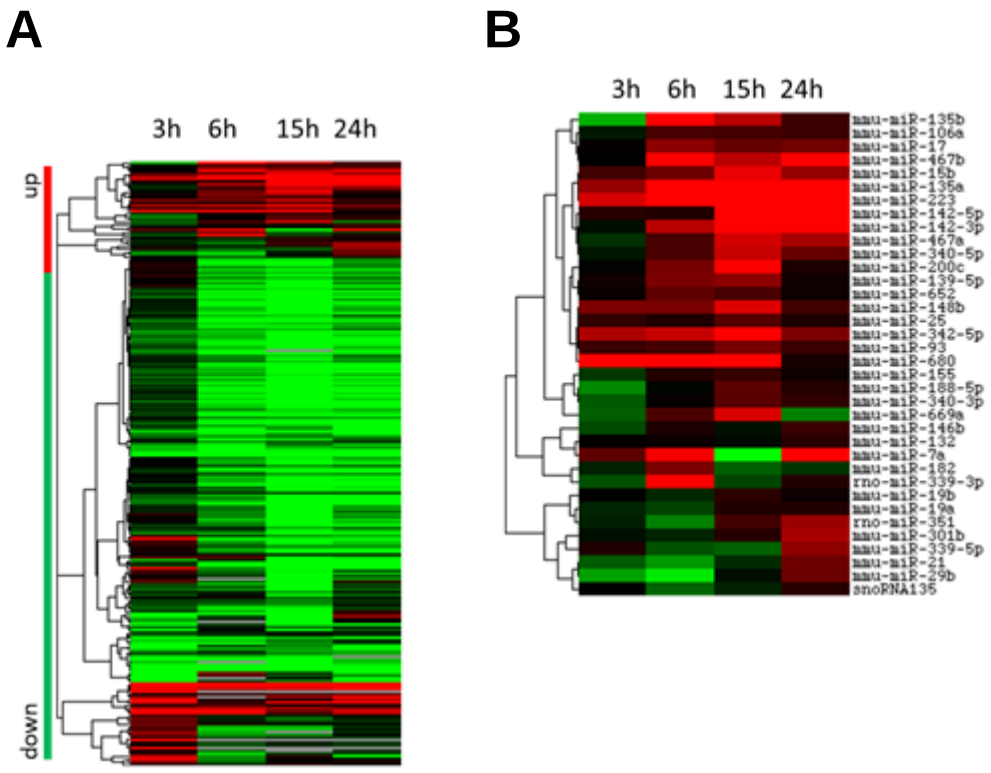

Figure 3. 3 Deregulated miRNome up MSU-gout model. A, Differentially expressed miRNAs ($p < 0.05$) were analyzed by hierarchical clustering of the $\log_2$ value of each miRNA at 3, 6, 15 and 24h after MSU intra-articular injection . Red: up-regulation; green: down-regulation; black: no change.

B, miRNAs that are up-regulated by the MSU treatment The legend on the right displays the microRNA represented in the corresponding row.

Interestingly, miR-135b is among the 30 miRNAs highly expressed, followed by others miRNAs such as miR-223, miR-155 and miR-135a (Figure 3.4). miR-135b was induced at 6 hours and followed repressed. MSU crystals promoted miR-223 and miR-135a expression in this *in vivo* experiment. These modulations were in line with the climax of acute inflammation in this model [14]. However, it was imperative to describe which cells were in charge in this arthritis model. To address this issue we treated mice with MSU crystals (100 μg/knee joint) and after 12 hours neutrophils and macrophages were isolated from synovial fluid, and we measured miR-135b expression (Figure 3. 4B). MSU triggered both cell types caused the induction of miR-135b, which was mainly produced by macrophages. Overalls, we defined a restrict miRNA signature in the model of MSU-induced arthritis which followed the disease progression. We observed miR-135b (M1-associated miRNA which we identified) expressed by periarticular tissue after MSU injection, suggesting a possible role in the gout progression.

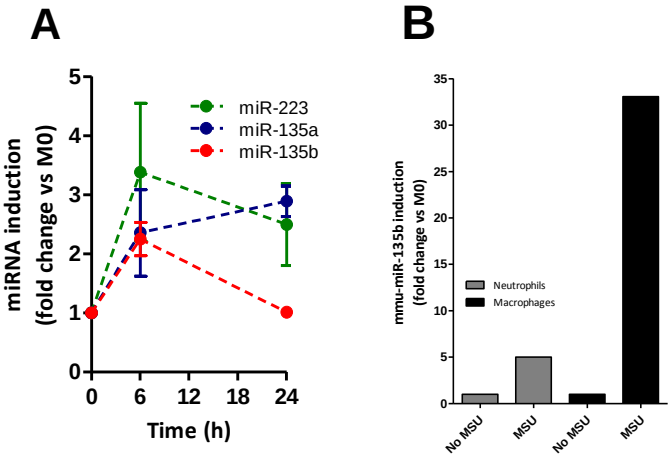

Figure 3. 4 miR-135 is overexpressed in synovial fluid and periritonial macropahges from mice treated with monosodium urate (MSU) crystals. **A**, single taqman assay qPCR was perfermed to define the expression of miR-135b and other inflmmatory microRNAs (miR-135a and miR-223) in vivo experiment. **B**, neutrophils and macrophages where isolated from synovial fluid after MSU

injection, miR-135b expression levels were measuerd. Results are representative of nine independent experiments. Data are shown as the mean ± SEM.

MSU crystals induce miR-135b expression in BMDM

In order to further address miR-135b levels *in vitro* model of MSU-induced gout, we treated the primary BMDM with MSU (150μg/ml) after LPS (10ng/ml) priming for 15 hours and we measured mRNA levels and protein secretion of IL-1β, IL-6 and CXCL1 (the major actors of the MSU-inflammation; Figure 3. 5) at 3, 6 and 15 h. We analyzed mature miR-135b and miR-155 levels (as negative control) in these sample and in BMDM triggered with LPS (100ng/ml) at 6 and 15 hours. As highlighted in Figure 3.6 A, miR-135b was up-regulated by LPS and priming +MSU stimulation, at the contrary, miR-155 induction was caused only by LPS triggering (Figure 3. 6B).

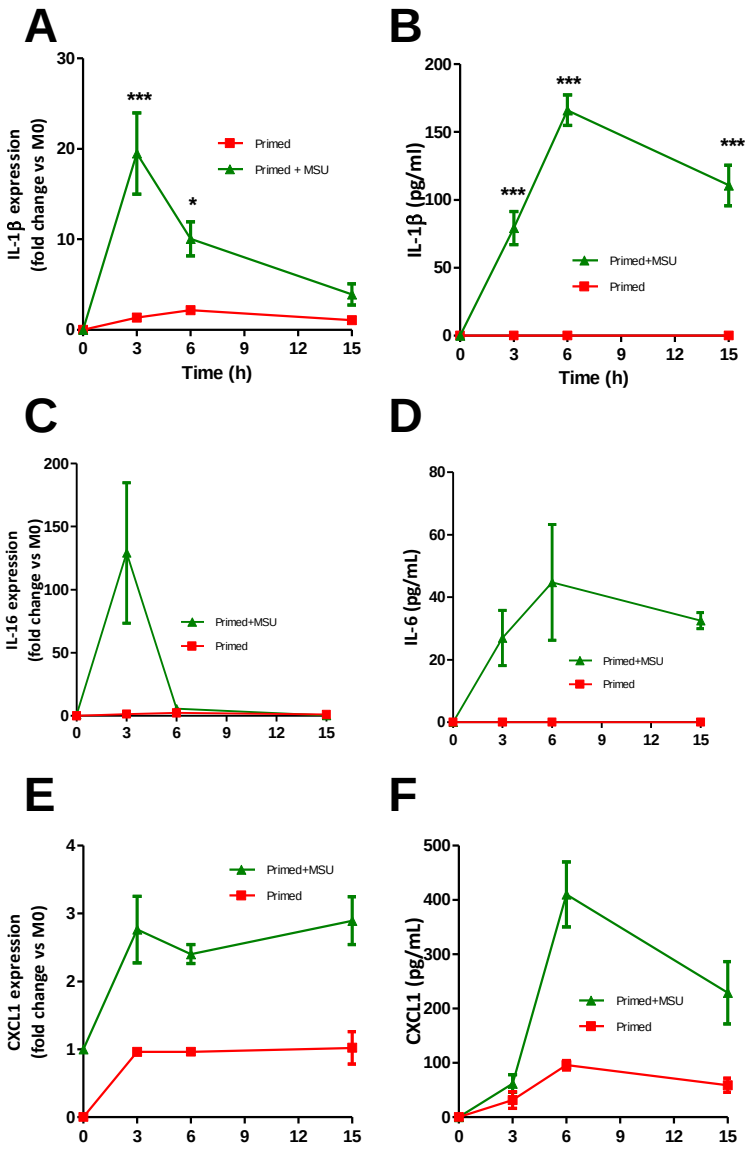

Figure 3. 5 of MSU-induced inflammatory response kinetics (*in vitro*). BMDM were primed with LPS (10ng/ml) for 15h and treated with MSU crystals (150µg/ml), inducing the expression of pro-inflammatory **(A-E)** and the secretion of inflammatory chemokines (**F-I**) at 3, 6, 15, and 24 hours after MSU treatment. Results are express as mean ± SEM n=3. ∗ = P < 0.01 against resting macrophages

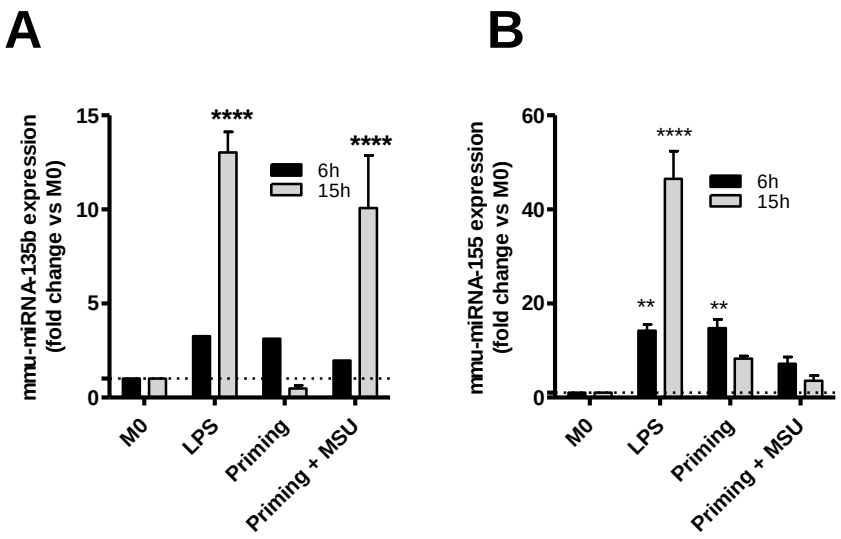

Figure 3. 6 Monosodium urate (MSU) crystals induce miR-135b expression in BMDM. A, mm-miR-135b expression levels in murine macrophages were primed with LPS (10ng/ml) for 15h and treated with MSU crystals (150µg/ml), or treated with LPS (100ng/ml) at 6 and 15h. **B,** expression levels of miR-155. Bar are mean ± SEM. n=4 **p<0.01,****p<0.0001.

miRNA-135b expression is regulated by IL-1R1 during Gout arthritis (*in vitro*)

MSU-induced gout has been shown to be dependent on the activation of the IL-1R1 pathway [15]. We found that MSU effects on miR-135b in BMDM were synergized by IL-1β, and demonstrated that MSU-crystal-induction was directly promoted by IL-1β and its signaling pathway. We treated BMDM isolated from IL-1R1 knockout mice and C57BL/6 wild-type with MSU crystals after 15h of LPS priming, for 6 and 15h. miR-135b in IL-1R1 KO was down-regulated in all the tested conditions were it was shown to be induced. While, MSU alone had no effect both in wild type and in IL-1R1 knockout (Figure 3. 7B). IL-1R1 wild-type mice shown nearby to 4-fold induction, which was lower than the fold induction observed in MSU in vitro model. The absence of IL-1R1 leaded to a significant reduction of miR-135b at 6 and 15 hours (Figure 3. 7). Overall, miR-135b expression in MSU-induced gout is conditioned by IL-1R1 signaling.

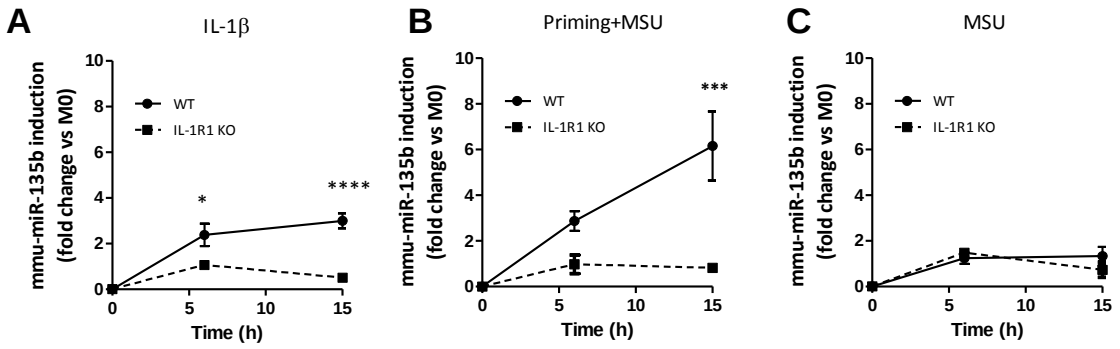

Figure 3. 7 miRNA-135b expression is regulated by IL-1R1 during Gout arthritis. BMDM obtained by C57BL/6 wild-type or IL-1R1 KO were or treated with IL-1β (**A**), or primed with LPS (10ng/ml) for 15h and treated with MSU crystals (150μg/ml) (**B**) or with MSU alone (**C**). Results are expressed as fold change against resting macrophages. mean ± SEM. n=4 *p<0.05, ***p<0.001; ****p<0.0001.

miR-135b putative regulates IL-1R signaling pathway

Although, we shown that IL-1R1 , the mayor signaling pathway involved in gout arthritis, regulated miR-135b expression; however its mode of action and role remained to be elucidated. We used TargetScan (version 4.2, www.targetscan.org) DIANA (http://www.microrna.gr/microT-CDS) and miRanda (http://microrna.org) prediction algorithm, obtaining a list of putative targets, which were analyzed with Ingenuity Pathway Analysis (IPA) and merged with IL-1R pathway signal. Importantly, we identified TOLLP, IL-1R1 and IL-1RAP as putative target in mouse, while MYD88 and IL-1β were found just in human, however miR-135b seed is extremely conserved between murine and human. To define miR-135b binding region on murine MYD88 and IL-1β we used RNA hybrid algorithm with a cut off of 19 kcal/mol.

We tested whether miR-135b could directly repress its putative mRNA targets through 3′ UTR interactions. Thus, the full- or partial-length 3′ UTRs of the murine genes IL-1β (target used as positive control[16]), IL-1R1, IL-RAP and MYD88 were cloned into a reporter vector (psiCHECK2), downstream of firefly luciferase. The plasmid was co-transfected with control and miR-135b mimic in NIH/3T3 cells. IL-RAP 3′UTR showed a reduction to 40 % of total luciferase reporter activity, in presence of miR-135b, which is completely

restored when the seed is mutated. The same result for MYD88 and the control IL-1R1 (Figure 3. 8 A-C). While IL-1β 3′UTR did not display any significant reduction of luciferase levels, if compared with the control (Figure 3. 8 D). These results support the hypothesis that miR-135b exerts a negative feedback control on the IL-1 signaling pathway in the late phase MSU inflammation.

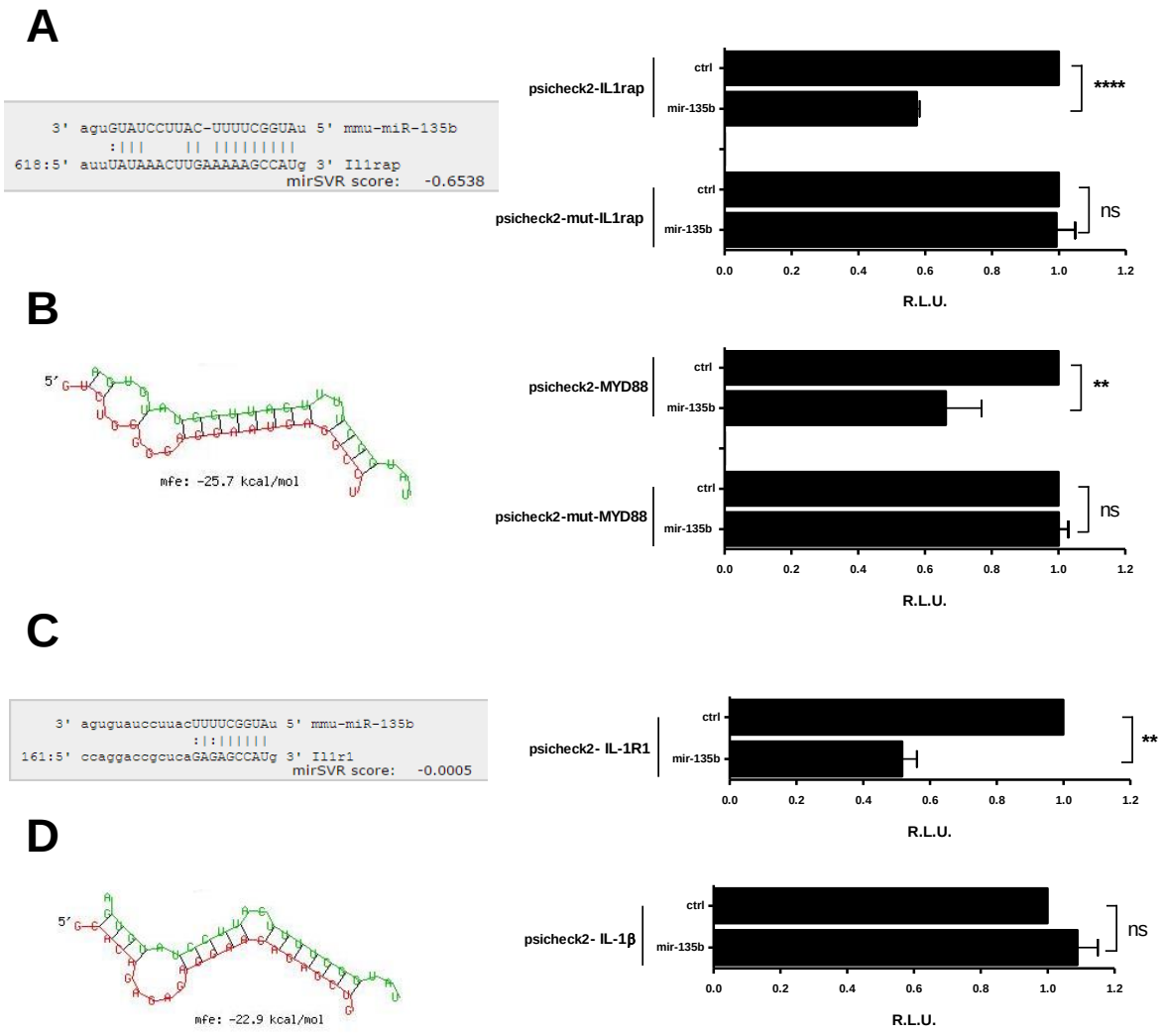

Figure 3. 8 miR-135b direct targets in IL-1β pathway, NIH/3T3 cells were transiently co-transfected with indicated 3′-UTR luciferase construct (the seed sequence or RNA hybrid outcomes are placed nearby) of IL-1RAP (**A**), MyD88 (**B**), IL-1R1 (**C**) and IL-1β (**D**) and with miR-135b mimic or a negative control mimic (scr). Results are expressed as normalized relative luciferase units (RLU). n=4 ; mean ± SEM. **p < 0.01, ***p < 0.001 compared with control

To further investigate if this interaction we transfected resting cells with control mimics or mimics for miR-135b, 15 hours later they were stimulated with IL-1β (20ng/ml). As shown in Figure 3. 9 A-C, enhancer expression of miR-135b inhibited the target mRNAs, MYD88, IL-RAP and IL-6, while the silencing of miR-135b causes a faint induction of those genes. These data highlighted the negative role of miR-135b on the pathway of IL-1β.

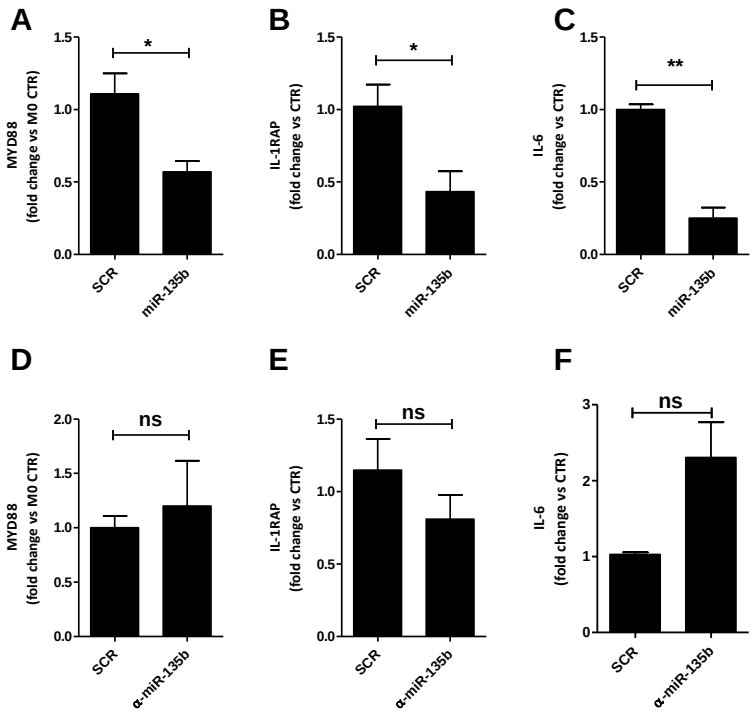

Figure 3. 9 miR-135b inhibits MyD88, IL-1RAP and IL-6 expression. Resting macrophages were transfected with 20nM of control (scr) or miR-135b for 24h, then cells were treated with IL-1β for 24h. Levels of MyD88 (**A**), IL-1RAP (**B**) and IL-6 (**C**) were determined. n=3; mean ± SEM; *p<0.05; ** p<0.01 compare to control (scr)**.** Resting macrophages were transfected with 20nM of control (scr) or antagomiR-135b for 24h, then cells were treated with IL-1β for 24h. Levels of MyD88 (**D**), IL-1RAP (**E**) and IL-6 (**F**) were determined. n=3; mean ± SEM.

Enhancer expression of miR-135b inhibits MSU crystal-induced pro-inflammation

To define miR-135b role on MSU- induced inflammation, we transfected MSU treated macrophages with control or miR-135b mimic for 3, 6 and 15 hours. Figure 3. 10 shown how miR-135b was able to reduce gene expression of MYD88 and IL-1RAP, while IL-1β mRNA expression was unchanged (Figure 3. 10D). MiR-135b influenced drastically the

cytokine IL-6 mRNA expression (Figure 3. 10C) and had a negative impact also on the secretion of the major pro-inflammatory mediators such as IL-1β, TNFα and KC, Figure 3. 11 (A-D). However, the lack of miR-135b had no influence on secretion of the same protein (Figure 3. 10 E-H).

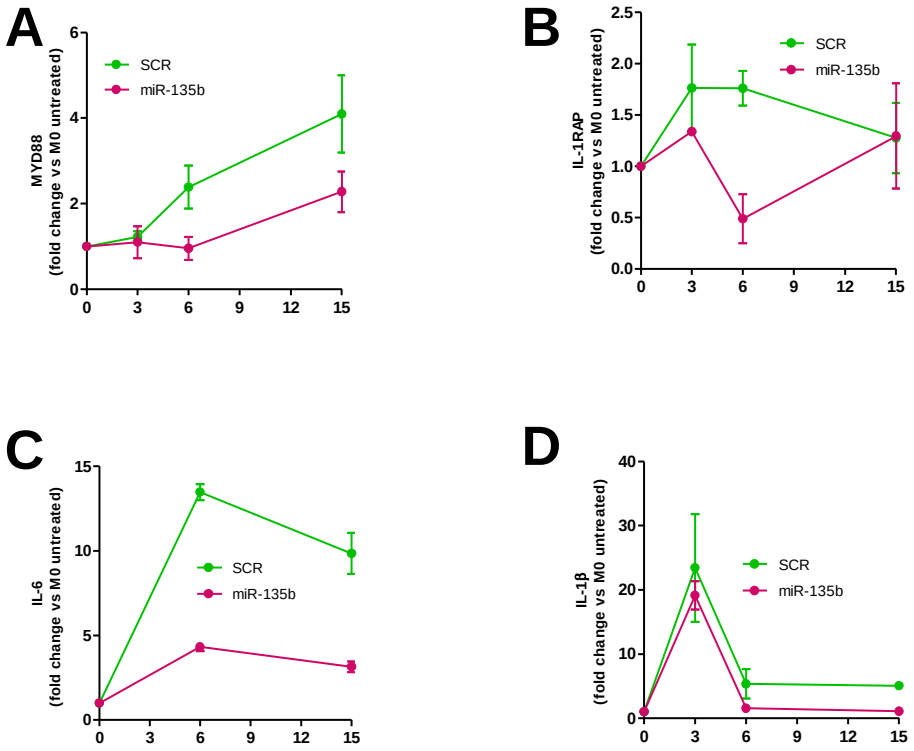

Figure 3. 10 Enhancer expression of miR-135b inhibits MSU crystal-induced pro-inflammation. Resting macrophages were transfected with 20nM of control (scr) or miR-135b for 24h, and then cells were treated with MSU. Total RNA was collected at different time point and MyD88 (**A**), IL-1RAP (**B**), IL-6 (**C**) and IL-1β expression levels were determined. n=3; mean ± SEM;

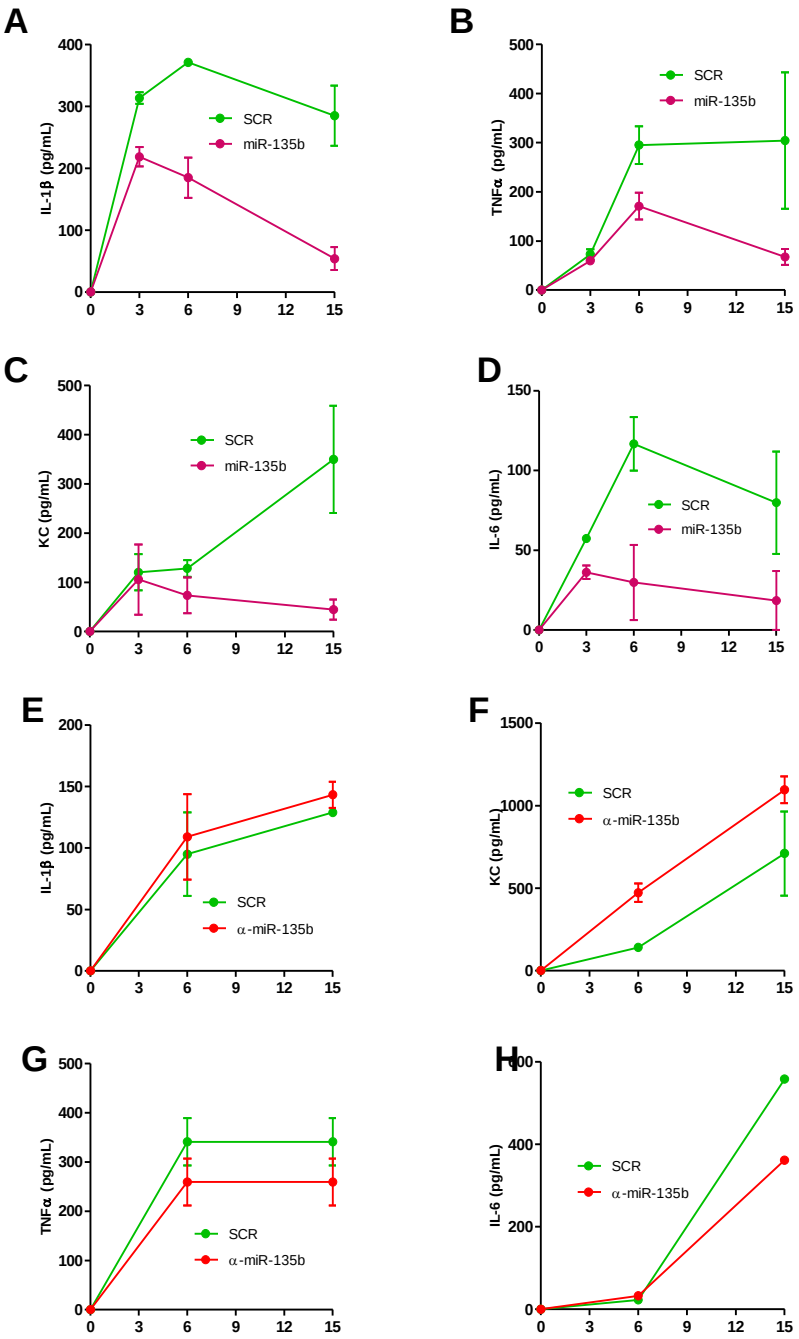

Figure 3. 11 Enforced miR-135b levels inhibits MSU crystal-induced pro-inflammation. A-D, Resting macrophages were transfected with 20nM of control (scr) or miR-135b for 24h, and then cells were treated with MSU. Supernatant of each sample was collected at different time point and IL-1β, TNF-α, KC, IL-6 secretion was evaluated. Macrophages were transfected with scr and antago-miR-135b and IL-1β, TNF-α, KC, IL-6 secretion was evaluated (**E-H**). n=3; mean ± SEM;

DISCUSSION

Gout is an autoinflammatory disorder associated with deposition of MSU crystals in joints and periarticular tissues. Innate immunity cells, in particular macrophage, are mainly involved in its progression. We performed a low-density TaqMan-based array on periarticular tissue from mice injected with MSU crystals for 3,6,15 and 24h. Our results showed that out of MSU crystals induced miRNAs there is increased levels of miR-135b, which we found to be essential in the retention of a pro-inflammatory phenotype. We found that it is induced by inflammatory stimuli, LPS in human macrophages. Its expression is influenced by exposure to nanoparticles cigarette smoke [16-18]. Surprisingly, we found that its expression was closely associated with IL-1R1 pathway. Indeed its impaired expression is due to the lack of IL-1R1.

Due to the expression kinetic that miR-135b has in both MSU-induced model and IL-1β treated macrophages, which is highly expressed in the late hour and immediately inhibited in the followed. We speculate a negative regulatory effect of miR-135b in the progression of gouty arthritis and IL-1β pathway. Although, we explore the putative targeting of miR-135b in the IL-1β pathway, we confirmed a direct interaction between miR-135b and MyD88 and IL-1RAP in the present study. The targets expression was gradually decreased by MSU crystals stimulation in late hours; stressing the anti-correlation with miR-135b. In addition, enhancing miR-135b expression in murine macrophage reduces the target mRNAs and proteins. Moreover, our data highlight that miR-135b has an impact not only on IL-1β pathway, but also contributes with the outcome of the MSU stimulation.

Overalls, in this study we examined the role of miRNAs in MSU gouty arthritis. We have identified miR-135b up-regulated *in vivo* model. The inflammatory phenotype of the acute gouty arthritis *in vitro* shown the induction of miR-135b in macrophages. These data emphasize the role of miR-135b that modulates inflammation in acute gout. Once again,

expression that is in turn negative modulated in function of its kinetics. This landscape could be due to the induction of MICP1 upon IL-1β treatment.

MATERIALS AND METHODS

Reagents.

LPS from *Escherichia coli* serotype 055:B5 and Uric acid were purchased from Sigma. Murine IL-1β was purchased from R&D Systems.

MSU crystal–induced gout

MSU crystals were prepared under pyrogen-free conditions by dissolving 5 mg/ml uric acid (Sigma-Aldrich) in 0.01M NaOH (pH 7.1) solution. The supersaturated uric acid solution was filtered (0.45 μm) and maintained at room temperature for 48 hours. Crystals were washed with 100% ethanol and sonicated to decrease their size.

Mice were given an intra-articular injection of 100 μg of MSU crystals and killed after 0, 6, and 15 hours. The knee cavity was washed with phosphate buffered saline (PBS) and the number of leukocytes was determined. Periarticular tissues were used for evaluation of cytokines and myeloperoxidase (MPO) activity. Total leukocytes were determined in a Neubauer chamber after staining with Turk's solution. Differential cell counts were determined using standard morphologic criteria and were performed on May-Grünwald-Giemsa–stained slides.

Purification of Murine Macrophages.

Mouse macrophages, obtained from 6- to 8-wk C57BL/6 wild type or IL-1R1 KO were plated in six-well plates at a density of 1 to 2 × 105 /cm2 . Femora and tibiae of hind legs were flushed with PBS solution and cells were re-suspended in IMDM medium with L-glutamine, 10% (vol/vol) FCS, 100 U/mL penicillin, and 100 mg/mL of streptomycin and cultured for 7 d with 10 ng/mL macrophage colony-stimulating factor (MCSF). Macrophages were detached using PBS solution containing 10 mM EDTA, washed, and resuspended in IMDM medium supplemented with, 10% heat inactivated FCS, 100 U/mL of penicillin, and 100 mg/mL of streptomycin.

miRNA isolation and Quantitative RT-PCR Analyses.

Total RNA enriched with small RNAs faction was isolated by TRIzol (Ambion) according to the manufacturer's instructions. For each sample, two independent reverse transcription reactions were performed as followed, using TaqMan miRNA assay (Applied Biosystems). Briefly 10 ng of total RNAs were reverse transcribed with individual stem-loop RT-primers for has-miR-135b (Cat: 002222), has-miR-155 (Cat: 000391), has-miR-146a (Cat: 002246), mmu-miR-155 (Cat: 000391), U6 (Cat: 001973) and snoR-302 (Cat:). Following, 1 ng of each individually synthesized cDNA in 10 μL of final reaction, was used in the qPCR assay with specific TaqMan probes. Real-time PCR was conducted in CFX Connect™ Real-Time PCR Detection System (BioRad). Relative miRNAs expressions were determined by using the ΔΔCt method [19], normalizing the levels with U6 or snRNA302.

RNA isolation and gene expression analysis.

Total RNA was purified using TRIzol (Ambion) according to the manufacturer's instructions, and reverse transcribed. Quantitative RT-PCR was performed using specific oligonucleotides which are reported in Table 1. Sybr Green PCR Master Mix (Applied Biosystems) was used in a CFX Connect™ Real-Time PCR Detection System (BioRad).

Table 3. 1 Primers used for qPCR

Genes	Forward primer (5'-3')	Reverse primer (5'-3')
MyD88	GCCCGAAAATAAGCCGACTG	TGCTCCTTTATGAGGATGGGC
IL-1β	TGCCACCTTTTGACAGTGATG	AAGGTCCACGGGAAAGACAC
IL-6	TGCGATGGAGTCAGAGGAAA	ACTAGGGGGAAAAGTGCAGC
TNF-α	GATCATCAGCAATGCCTCCT	TGTGGTCATGAGTCCTTCCA
IL-1RAP	GAGACAAAAGACCTGAAGCA	TGCGGCCGCTAAACTATGGAACT

Luciferase Reporter Assay.

NIH/3T3 cells were plated in 24-well plates in 500 μL of D-MEM medium, supplemented with 10% FBS and 1% of L-glutamine at 16 × 10^4 per well. After 24 h, cells were transfected with 100 ng psiCHECK-2–3′UTR reporter construct and 10pmole of mimic-miR-135b or

scramble control (mirVana™, Ambion Applied Biosystems) by using Lipofectamine 2000 (Invitrogen). After 48 h from the transfection, cells were lysed, and luciferase activity of both firefly and renilla, were determined by using the DualGlo Luciferase Assay System (Promega). The enzymatic activities were measured by using a MultiDetection Microplate Reader Synergy 2 luminometer (BioTek). The values of renilla luciferase activity were normalized and were expressed as fold changes relative to the value of the negative control.

Table 3. 2 primers for luciferese construct

Gene	primer sequence (5'-3')
IL-1B for	CCGCTCGAGCGGCGGAAGAGCAGACAGCCAAC
IL-1B rev	CCGCTCGAGCGGGGAAGAGCAGACAGCCAACC
IL-1R for	ATAAGAATGCGGCCGCTAAACTACTGTGCTGGTGCTTCTTCTG
IL-1R rev	CCGCTCGAGCGGTTTGACAGGAGGAACCCAGT
IL-1RAP for	ATAAGAATGCGGCCGCTAAACTAACCAGGAGAAGTCGCAGGAA
IL-1RAP rev	ATAAGAATGCGGCCGCTAAACTACCTTTGCCTTTGATTGAGAG
MyD88 for	ATAAGAATGCGGCCGCTAAACTAACTACGGTTTTAGATCTGCT
MyD88 rev	CCGCTCGAGCGGTGGACAAAAGTGGGGTGCCT

Macrophage Transfection.

Purified macrophages were cultured in medium supplemented with 2 mM glutamine and 10% FCS. Were transfected with 10pmole of miRNA mimic (Ambion,Applied Biosystems), or with miRNA-inhibitor (Exiqon), using TransIT-TKO Transfection Reagent (Mirus Bio), according to the manufacturer's protocol. After 24 hours, were stimulated as indicated.

ELISA assay.

Antibodies and detection reagents for ELISA assays were purchased from R&D Systems and used according to the manufacturer's instructions. Samples were diluted so that the optical density fell within the optimal portion of a log standard curve.

Statistical analysis.

Results are expressed as mean ± SEM and statistical significance was based on non-parametric t-test or non-parametric ANOVA followed by post hoc Bonferroni's test performed using the GraphPad Prism 5 software (GraphPad Software).

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CONCLUSIONS

Form the central dogma of the biology formulated by Crick in 1958 [1], to the detection of "junk RNA", a new prospective has been open. Macrophages are essential cell of the innate immunity system important in onset and resolve inflammation. Moreover they are important in the cross-talk with adaptive immunology. Macrophages may undergo to different phenotype due to the microenvironment. Especially in the course of a chronic inflammation there is an anomalous prolongation/amplification of the macrophage challenge to lead homeostasis. In this landscape favor macrophage activation and re-programming. The identification of the underlying molecular mechanisms on the base of this process may suggest new approaches to interfere with chronic inflammation and other inflammatory disease, such as cancer. The aim of this presented work was to characterize the epigenetic mechanism involved in macrophage polarization. We have pointed out the impact of miR-135b a *de novo* expressed miRNA in M1 macrophages, and for the first time associated with macrophages in an inflammatory diseases such as gouty arthritis. Indeed we demonstrated that miR-135b locus is activated by the inflammatory stimulus, LPS, which discharge the repressor complex polycomb2. Although we can classified miR-135 as M1-associated or induced, which damps M2 phenotype in favor of the M1 thru the targeting of important the transcription factor, c-MYC, STAT6 and KLF4, sustaining the inflammation. In addition miR-135b expression is inhibited by the anti-inflammatory cytokine IL-10, to highlight its pro-inflammatory role. We have shown in the model of gouty arthritis, miR-135b is induced during the progression of the inflammation by macrophages. However it provides a negative feedback to limit excessive macrophage response to MSU crystal, thru the targeting of the IL-1β pathway. These results confirm the relevance of miR-135b as an important hinge of macrophage polarity, flanked by other miRNAs such as miR-155.

At the light of these observations, the identification of the underlying process that regulates the expression of miR-135b will be essential. miR-135b is located in the intron of the lncRNA, BLACAT1. We have glimpsed that the induction of miR-135b and BLACAT1 is not correlated, on the contrary BLACAT1 is induced by anti-inflammatory stimulation,

influencing miR-135b. Although we can speculate a novel type of regulation beyond IL-10, used by the macrophages to control the expression of this miRNA. Probably with the action of the MCP-induced protein 1 (MCPIP1), which is induced downstream LPS and IL-1β pathway and has been shown to suppress miR-135b biosynthesis in cancer [2].

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