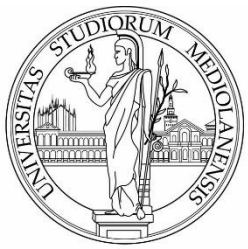

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Ciclo XXX

Role of microRNAs in Macrophage Polarized Activation

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Abstract

Macrophages are cells from the innate branch of immune system with central roles both under physiologic and pathologic conditions. Such complex behaviour relies on extreme functional plasticity of these cells. Cellular activation and plasticity in macrophages are complex phenomena that require tight regulation. In recent years, microRNAs and lncRNAs emerged as important regulators of many cellular processes and have been proposed as key mediators of the plasticity observed in macrophages.

In this study we investigate the possible roles of different microRNAs on macrophage activation and functionality, taking advantage of animal models selectively depleted of candidate miRNA in macrophages.

We found that selective depletion of miR-125a in macrophages did not affect clinical outcome in different in vivo models of inflammation. Nonetheless it altered macrophage activation and functions, as evidenced by reduced secretion of TNFα after endotoxin challenges. Consistently, macrophages deficient in miR-125a displayed reduced killing of extracellular bacteria although they showed increased phagocytic rate as compared to miR-125a competent cells.

With a similar approach, we confirmed that miR-9 is induced in macrophages upon inflammatory stimuli. Increased expression of miR-9 mature form in mice was sustained by the synchronous activation of transcription in both pri-miR-9.1 and pri-miR-9.3 loci, in contrast to human.

Finally we identified a lncRNA interfering with the processing of a pro-inflammatory miRNA in differentially activated human macrophages, highlighting a new layer of regulation of macrophage activation.

The finding of the present project give new insights into the complex mechanism regulating macrophage activation and underscores the need for future studies to thoroughly identify the molecular mechanisms and the involvement of different miRNA in macrophage activation. Moreover, such studies could set the basis for the transition from basic research to new therapeutic options enabling precise regulation of macrophages activation in different immune pathologies.

Introduction

1 The Mononuclear Phagocytic System

1.1 Monocytes

Monocytes are cells from the innate immune system. They originate from precursors in the bone marrow, whose development is guided by the sequential effect of different factors like granulocyte/macrophage-colony stimulation factor (GM-CSF) and macrophage-colony stimulation factor (M-CSF). At the end of their differentiation, monocytes are characterised by dimensions slightly bigger than lymphocytes, with a round shape and a roughly circular or kidney-shaped nucleus. Mature monocytes mobilise from bone marrow to the blood stream where their lifespan is between one and three days for human. From there, monocytes can leave circulation and infiltrate tissues where they differentiate into macrophages. Such fate, from BM to blood and finally to tissues differentiating into macrophages, was once held as a paradigm for monocytes but now it is increasingly clear that several exceptions exist. For example, it has been shown that mature monocytes can leave the blood stream and settle in the spleen, where they may constitute a functional reservoir [2], or they can even return to the bone marrow with yet unclear functions [3]. On the one hand monocytes can leave circulation as a result of inflammatory stimuli, infiltrating affected tissues and head toward the source of inflammation where they finally differentiate into macrophages, in order to orchestrate subsequent phases of inflammatory response. On the other hand however, it is now clear that at steady state monocytes are responsible for replenishment of tissue resident macrophages only in a fraction of tissues [4]. For many other tissues, as for instance the brain, macrophages originate from cells migrating from the Yolk sack during fetal development and are maintained by local proliferation rather than recruitment of mononcytes from the blood [5]. Once considered a homogeneous population, circulating monocytes have been shown to be sub-divided in three distinct subsets based on differences in the expression levels of two surface markers. In humans, monocytes have been divided between ‘classical, inflammatory’ monocytes expressing high levels of CD14 (a coreceptor of TLR4 for LPS) but low levels of CD16 (a subunit of receptors for antibody’s invariant chains) and ‘alternative’ monocytes with low CD14 but high CD16 expression. A third group, however, has been identified with intermediate expression levels for both markers [6]. Since spontaneous conversion between two major

subtypes has been proposed for human monocytes, the ‘intermediate’ class may represent a transition class resulting from ongoing conversion process. In murine context similar subdivisions have been outlined using two different markers: Ly6C high/CD43 low monocytes that roughly correspond to inflammatory monocytes in human; Ly6C low/CD43 that highly resemble alternative monocytes [7]. Differently from human monocytes, spontaneous conversion between cellular subtypes has been proven in mice [8]. Furthermore, besides being the precursors of macrophages in tissues, monocytes also play a role in the bloodstream. Circulating monocytes already display many of the functions of the mature macrophages like phagocytosis, antigen presentation through MHC-II, cytokines production and migration along a chemotactic gradient. Thus monocytes are also involved in immune-surveillance, patrolling the blood stream. As such, they are involved in many pathological processes. The proportion of these cells in the circulation as well as the relative frequencies of different subsets may change under different pathological conditions.

1.2 Macrophages

1.2.1 Macrophage Biology

Macrophages are cells of big dimensions that dwell in tissues or differentiate there from circulating monocytes during inflammatory response. Once considered a single cell type, they are now recognised as highly plastic and heterogeneous cells. This feature was the reason for the development of the new 'Mononuclear Phagocytic System' concept [9]. The first layer of plasticity in macrophages is ontogenic. During fetal development a first wave of macrophages differentiates from yolk sack (YS) and migrates to colonise different tissues like brain, liver, lung, pancreas, skin and spleen [10]. Here, thanks to molecular signals produced locally, macrophages terminally differentiate and develop characteristics exclusive of the hosting tissue. A second wave of macrophages develops at later stages, originating from fetal liver and colonising skin and mucosa, giving birth to Langerhans Cells [11]. Finally, after birth, hematopoiesis takes place in the bone marrow. Here common myeloid precursors differentiate to monocytes that patrol the blood stream, eventually leaving circulation to infiltrate tissues and differentiate into macrophages. To add complexity to the scheme, depending on the tissue, local proliferation rather than recruitment of circulating precursors may be responsible of macrophage replenishment in tissues both physiologically or following infection [5]. Furthermore, in the same tissue may reside macrophages of different origin, exerting different functions [12]. Macrophage plasticity is particularly evident under pathological conditions. During inflammatory response many of the molecules produced have specific effects on macrophage phenotype. Bacterial moieties and type 1 inflammatory cytokines like IFNγ direct macrophages to a 'classically activated' state (M1), primed to sustain inflammation by producing pro-inflammatory cytokines like TNFα, IL12 and chemokines like CXCL10. On the contrary, type 2 cytokines like IL4 and IL13, together with anti-inflammatory cytokines like IL10 and TGFβ, induce an 'alternatively activated' state in macrophages (M2) that result in increased secretion of anti-inflammatory molecules and enhanced tissue remodelling and repair [13]. The M1/M2 dichotomy was originally based on in vitro studies but is unlikely to resemble *in vivo* complexity. In fact, a wide panel of stimuli has been proved to induce macrophage activation states intermediate between M1 and M2 [14]. Hence, these extreme polarised phenotypes are rather considered a paradigmatic reference, delimiting the spectrum of possible active macrophage state. Moreover, further blurring the boundaries between

activation states and proving once more the uncommon plasticity of these cells, macrophage polarisation is not a fixed state but rather changes accordingly with changes in the surrounding microenvironment [15]. Lastly, macrophage plasticity is strictly related to timing. In vitro, macrophages exposed to unique specific stimuli sequentially activate distinct sets of genes [16]. This dynamic process depends on different mechanisms such as epigenetic and biochemical changes or autocrine effect of secreted cytokines. Another important feature tightly linked to plasticity is the idea that these cells may display varying degrees of memory [17]. Cellular memory is the ability to adapt cellular responses according to previous experiences. In the case of macrophages it has been shown that repetitive stimulation with bacterial moieties like LPS decrease macrophage response, a condition termed endotoxin tolerance [18]. Moreover, a recent report claimed that during infections in Drosophila, macrophage ability to engulf microbes requires that the same cells previously participated in physiological phagocytosis of apoptotic cells during embryonic development [19]. The extent and duration of trained macrophage responses is still matter of debate, as well as the molecular mechanisms at the basis of innate memory. Metabolic changes have been observed in macrophages following pathogen recognition and were proposed to sustain memory in such cells [20], but are unlikely to last long. A more effective mechanism that may store memories over a longer period of time might rely on epigenetic changes.

As a reflection of their great ductility, macrophages display several cellular functions, thus playing multiple roles both under physiological and pathological conditions. The main feature of these cells, that also gained them their name, is phagocytosis. Macrophages are not the only cells in the organism with phagocytic ability, but they are by far the most specialised cells for this process, given the wide array of functional phagocitic receptors displayed [21]. Phagocytosis is a process by which particles external to the cell can be internalised by means of lipid bilayered vesicle originating from the cell membrane called ‘phagosome’. The aims of this process include removal of cellular debris or noxious agents and collection of information on the extracellulare milieu to be shared with other cells, above all lymphocytes. Phagocytosis occurs through different mechanisms always requiring molecular interactions between the particle to be engulfed with a surface receptor, remodelling of the actin cytoskeleton and the formation of an immature phagosome [22]. Immature phagosome maturates through several rounds of fusion with other intracellular vesicles like lysosoma and other endosomes, progressively dismantling phagocytised particles [23]. Phagocytosis may occur through a ‘zippering’ mechanism, in which interaction between phagocytic receptors, like FcR

and Dectin, and their ligands leads to receptor clusterization that force the surface membrane to protrude, finally embracing the target particle [24]. Other receptors, like complement receptors, upon interaction with opsonized target induce the rearrangement of actin cytoskeleton pulling in the particles, a process termed 'Sinking' phagocytosis [25]. A third mechanism that result in vesicle formation from the plasma membrane with internalisation of materials from the extracellular matrix is 'triggered' phagocytosis or 'macropinocytosis' [26]. Macropinocytosis is a common method shared by several cell types across the organism to sense the extracellular space. It occurs in a non-specific way following receptor triggering, making it difficult to strictly define what a phagocytic receptor is. In macrophages, macropinocytosis can be triggered by a wide array of receptors, including TLRs. Therefore, it has been proposed as a method by which, under inflammatory conditions, macrophages could increase the harvest of extracellular antigens to further process and expose for lymphocyte activation [27]. Phagocytic particle usually are recognised by more than one receptor. Functional redundancy in receptors and mechanisms make it difficult for a pathogen to completely avoid phagocytosis and guarantee a better profiling of the threat by macrophages. Indeed, along with internalisation, phagocytic receptors also trigger several signaling cascades, tailoring the response onto the challenged pathogen. The fate of internalised vesicles depends on a great amount of variables, including nature and dimensions of the particle and the array of receptors involved. During vesicle maturation, the degradation of the phagosome content by fusion with acidic lysosome may lead to the exposure of new binding sites, thus triggering new responses or enhancing previously activated signaling pathway [28]. Finally, the content of the matured phagosome may be exposed on cell surface, usually by means of mayor histocompatibility complex class II (MHC-II) molecules, but cross-presentation via MHC-I also has been described when pahgocytosis occurs through DNGR-1 triggering [29]. In some cases, like in the case of phagocytosis of apoptotic bodies, internalisation of target is immunologically silent, not arising any inflammatory response [30].

During infection of noxious pathogens, the internalisation alone, although limiting nutrient supply for bacteria, is insufficient to kill microbe. Indeed maturation of phagosomes containing bacteria recruits several other antimicrobial effectors. Acidification of the phagolysosome serves both the purpose of bacteriostasis and enhancement of degradative ability of lysosomal enzymes [31]. In addition, macrophages mount an effective antimicrobic response recruiting NADPH oxidase (NOX2) to the phagosome surface. NOX2 is composed of two multimeric complexes, one of which is

integral to the cell membrane (and therefore to the phagosomal membrane upon formation), while the other is cytosolic and is recruited to the surface following activation signals [32]. NOX2 oxidises cytoplasmic NADPH to NADP+ and transfers electons to molecular oxygen in phagosomal lumen to produce O-. Radical oxygen can further react to generate H2O2, subsequently dismuted to OH- by Fenton reaction. These molecules, commonly referred to as Reactive Oxygen Species (ROS), are highly toxic to living being due to their radical nature that triggers chain oxidative reactions in biological macromolecules. Along with ROS production, inflammatory stimuli also induce expression of the inducible nitric oxide synthase (iNOS) enzyme that oxidise NADPH and arginine to produce cytosolic NO and citrulline. NO can diffuse to the lumen of the phagosome and further react with ROS to generate the highly toxic peroxynitrite (ONOO-). Macrophages also exert microbicidal activities on extracellular pathogens by secreting lytic enzymes like lysozymes and members of the cathelicidins class [33]. Oxidation and degradation of phagocytised particles allow efficient loading of antigens onto MHC-II and extracellular presentation [34]. MHC-II consists of two subunits (α and β chains) that assemble in the Endoplasmic Reticulum (ER). Initially MHC-II peptide-binding groove is masked by an Invariant chain (Ii) that also stabilize the interaction of α and β chains. Immature MHC-II:Ii complexes are transported through the Golgi apparatus via transport vesicle to finally reach a specific compartment (termed MHC class II Compartment, MIIC). Here Ii is partially degraded by acidic pH and cathepsins, leaving a residual ClassII-associated Ii Peptide (CLIP) to cover the antigen-binding groove at the α:β interaction face. Vesicles from MIIC carrying matured MHC-II:CLIP complexes subsequently fuse with vesicles from the endophagocytic system, CLIP is exchanged for the antigenic peptide (of extracellular origin) thanks to the chaperone protein HLA-DM. Finally, the vesicle carrying the fully mature MHC-II:antigen complex fuse with plasma membrane exposing the processed antigen on cell surface. Extracellular antigen presentation is peculiar to specialised phagocytes, like macrophages, monocytes, DC and B-lymphocytes. Antigen presentation through MHC-II directs adaptive immune response toward specific pathogens, but alone is generally insufficient to induce T-cell activation. In order to trigger an efficient activation of T lymphocytes against common antigens, costimulatory molecules must be expressed on antigen presenting cell (APC) surface [35]. Activation of lymphocytes is further boosted by macrophages through secretion of soluble mediators of inflammations like cytokines and chemokines (extensively reviewed in Arango Duque and Descoteaux 2014 [36]). In brief, classically activated macrophages secrete a

plethora of pro-inflammatory cytokines that have pleiotropic effects such as TNFα, IL6, IL1, IL12, IL18, IL23 and IL27. The release of these molecules is accompanied by secretion of pro-inflammatory chemokines that recruit other cell types to the inflamed site. CXCL1/2, CCL2, CXCL8/9/10/11 are the inflammatory chemokines produced by M1 macrophages that sustain inflammation. On the contrary, alternatively activated macrophages and macrophages involved in resolution of inflammation switch the set of molecules secreted, preferentially releasing the anti-inflammatory molecules IL10 and TGFβ, together with CCL1, CCL17, CCL22 and CCL24 chemokines. Thanks to this wide array of soluble mediators produced, macrophages are master regulators of the inflammatory process in all of its phases. Macrophages also play an important role in angiogenesis. In fact they secrete important mediators that prime endothelial cells for new vessel formation, like WNT7 [37] and VEGF [38]; moreover macrophages can establish direct cell-to-cell contact with sprouting endothelial cells [39] and secrete matrix remodelling enzymes [40]. Macrophage control of angiogenesis is of key importance during ontogenesis [41], wound healing [42] and for cancer progression and metastasis [43].

1.2.2 Macrophage Role in Pathology

1.2.2.1 Resolution of inflammation

Inflammation is a necessary response developed by the organism to maintain integrity and fight infections. But, since persistent inflammation may be deleterious, it must be temporally limited. Initially, resolution of inflammation was considered a passive process consequent to the eradication of noxious stimuli that triggered the immune response. It is now widely accepted that resolution of inflammation is instead an active and complex phenomena that requires synergistic action of molecules produced by different immune cell types. Although resolution of inflammation is a choral effort, master regulators of the process appear to be macrophages. In fact macrophage depletion results in a prolonged influx of neutrophils in infected tissue (Chadzinska et al 2004) and increased systemic dissemination of pathogens [44]. Pro-resolving macrophages inhibits neutrophil recruitment through secretion of matrix metalloprotease 12 (MMP12). This enzyme cleaves the CXC chemokines in the ELR arm, thus impairing receptor binding, and CC chemokines like CCL7. Cleaved CCL7 is still able to bind its receptors but fails to induce signal transduction, thus acting as a pharmacological antagonist [45]. Pro-resolving macrophages not only inhibit neutrophil recruitment, but also remove dying neutrophils in a process termed ‘Efferocytosis’ [46]. Efferocytosis is a

tightly regulated process that require neutrophil release of soluble *find me* signals like lysophosphatidylcholine [47], sphingosine-1-phosphate [48], CXCL3 [49] and nucleotides [50]. Find me signals attract macrophages and the exposure of *eat me* signals like annexin-A1 [51] and phosphatidylserine (PS) [52] on dying neutrophil cell surface trigger phagocytosis. Entrance in the resolving phase of inflammation is madiated by a marked switch in macrophage activity. Intrinsic autoregulatory loops triggered by PRR activation in these cells result in the expression of the anti-inflammatory IL10 cytokine [53]. Autocrine exposure to IL10 in macrophages leads to switch off of pro-inflammatory signaling pathways and expression of genes important for resolution of inflammation [54]. In particular, IL10 in macrophages reduce the expression of COX2 and favour the expression of COX1 together with Arachidonate-15-lipoxigenase. These molecules cooperate to convert arachidonic acid into lipid mediators of resolution called lipoxins and resolvins [55]. Resolution of inflammation has been extensively studied in animal models. Intraperitoneal (IP) injection of a yeast lysate (Zymosan) results in an acute peritonitis that spontaneously resolves in 48 to 72 hours [56]. In such model, cellular dynamics have been analysed highlighting a transient increase of neutrophils in the peritoneal cavity that peaked between 4 and 12 hours post stimulation. Neutrophil influx decrease over time and monocytes take the stage. Forty eight hours after stimulation, more than 60% of leukocytes in peritoneal lavage are reported to be macrophages. Since miRNA are known to regulate complex cellular processes, it is possible that they are at least in part responsible for macrophage phenotypic switch that regulate the entrance in resolving phases of inflammation and recent data indicate that that is indeed the case [57].

1.2.2.2 Colitis

Gastrointestinal tract (GI) is a site of direct contact between the *self* organism and the *non-self* environment. Differently from other interfaces between self/non-self like the skin, in GI tract an extensive exchange of molecules and nutrients must take place to grant survival of the organism. In vertebrates, GI tract is colonised by a myriad of microbs that are collectively referred to as ‘microbiota’ [58]. Microbiota exerts many functions that are key for the digestive process. Massive presence of microbs in the intestinal lumen poses a serious task to the host immune system. In fact, although beneficial bacteria from the microbiota are indistinguishable from pathogenic ones by innate immune cells, they must be tolerated for the sake of the organism. This goal is achieved by different mechanisms that maintain an anti-inflammatory environment and tolerance toward commensal bacteria [59]. Intestinal macrophages are key contributors for

the maintaining of anti-inflammatory homeostatic environment. Although GI tract, like other tissues, is initially colonised by macrophages during fetal development, these cells are gradually substituted by macrophages differentiated from circulating monocytes [60]. Monocytes in the intestinal microenvironment differentiate toward CX3CR1 macrophages that exhibit anti-inflammatory properties [61]. Anti-Inflammatory microenvironment in the GI tract is mainly maintained by IL10. In fact, genetic ablation of IL10 resulted in spontaneous inflammation in mice that was neutralised by IL10 administration [62]. Upon inflammatory imbalance in the gut microenvironment, increased permeability of the mucosa and infection by pathogenic bacteria species the tolerance is broken and inflammatory bowel diseases (IBD) like colitis occur. During colitis increased inflammatory cell infiltration in the lamina propria leads to severe alteration of the luminal surface of the colon with reduced mucin secretion, epithelial hyperplasia, abscess formation in the kripts. These alterations result in tissue ulceration with internal blood loss. Back in the 90s an experimental model of colitis was settled [63]. It consisted in exposing mice to freely accessible water-dissolved dextran sulfate sodium (DSS) for almost a week, followed by a recovery period in which pure water was reintroduced. DSS leads to mucosal irritation and colitis with clinical features that recall human ulcerative colitis. Taking advantage of this setting, anti-inflammatory effect of IL10 has been restricted to its role in macrophages. In fact genetic KO of the IL10 receptor α-chain resulted in worsening of the DSS induced colitis only when deletion was restricted to macrophages while it had no effect when deletion occurred in DC, B cells or T cells [64]. Furthermore, concomitant neutrophil depletion using an α-Ly6G antibody had no effect in clinical outcome, further restricting IL10 effect to macrophages. Interestingly, differences in disease severity were not accompanied by differences in macrophage number nor in surface expression levels of activatory markers. Therefore it appears that macrophages play pivotal role in intestinal homeostasis and pathology.

1.2.2.3 Sepsis

Sepsis is defined as a systemic inflammatory response to infection. Of note, septicaemia is not always a feature of sepsis, suggesting that soluble elements originating from the primary infection site may be sufficient to trigger sepsis. The term ‘severe sepsis’ is employed when systemic inflammation results in organ dysfunction or insufficiency. ‘Septic shock’ instead refers to conditions of systemic inflammation with refractory hypotension. Adding complexity, sepsis may occur as a systemic inflammatory response syndrome (SIRS) or a compensatory anti-inflammatory response syndrome (CARS) or

both syndromes may take place sequentially [65]. SIRS is characterised by high levels of pro-inflammatory cytokines in plasma of patients and haemostatic abnormalities that may result in disseminated intravasal coagulation (DIC). High TNFα levels and DIC are main actors of multi-organ failure that occurs in severe sepsis and may cause patient death within few days. Nonetheless, treatment with anti TNFα antibodies failed to increase survival in clinical studies on patients with SIRS [66]. On the contrary, CARS is characterised by an anergic state of immune cells that fail to respond to common stimuli. It often occurs after sepsis with septicaemia or extensive trauma and burn. Also CARS reactions are life threatening since pathogen superinfections may occur and result in retarded death. Features similar to SIRS, like hemodinamic shock, transient increase in TNFα accompanied by sustained increase in IL6 in plasma and rapid death have been observed in experimental animals injected intravenously (IV) or IP with LPS [67]. While repetitive stimulation with sub-lethal doses of LPS trained mice to survive to otherwise lethal doses of endotoxin [68]. This effect is called endotoxin tolerance (ET) and may be considered an experimental model mimicking CARS features. Since monocytes are the major source of TNFα in the blood and are the only circulating cell population that do not undergo massive apoptosis during CARS, they appear to be the best candidates as regulators of both edges of pathology in sepsis. Interestingly, since the deactivation observed in CARS may be considered a form of cellular memory, epigenetic mechanisms are likely to intervene. Indeed possible miRNA contributions to pathogenesis of CARS have been proposed [69].

2 The non-coding Genome

Back in 1950s a great series of discoveries in molecular biology were condensed by Francis Crick in the idea that genetic information is stored in DNA and converted to protein effector molecules by mean of an RNA intermediary [70]. According to this idea, major focus converged on identifying and annotating protein-coding genes, while the noncoding regions of DNA were considered as 'junk DNA', inert genetic material accumulated with evolution. Although ribosomal RNA and transfer-RNA were already known, they were considered exceptions necessary for information flow from DNA to proteins. The central dogma has been initially shaken by the discovery of RNA molecules with enzymatic activity in 1980s [71]. Further evidences of functional activity of RNA molecules came in the 1990s with the discovery of RNA interference, initially proven in insects and plants but later confirmed also in mammals [72]. RNA interference refers to the ability of short stretches of RNA (siRNA or miRNA) to assemble into riboproteic complexes and bind by complementarity to mRNA molecules, inhibiting their translation to protein, eventually leading to their degradation [73]. The advent of Next Generation Sequencing [74] techniques finally proved that only half of the transcriptome is protein coding, while the remaining part is composed of RNA molecules with no translational potential [75]. Many of these noncoding RNAs are currently under investigation and for many of them structural or regulatory roles have been identified.

2.1 MicroRNA

MicroRNA in their mature form are RNA molecules as short as 20-22 nucleotides that exert negative regulatory effects on mRNA translation. In the genome, microRNA can be found alone or more commonly in clusters and are localized in intergenic region or intronic regions. As a consequence, these miRNA genes can rely on independent promoters or be dependent on the host gene expression, although little is known about the regulation of miRNA expression. MicroRNA originate from longer precursors transcribed in the nucleus into primary transcript microRNA (pri-miRNA). Since pri-miRNAs often display 5'-capping and 3'polyadenilation, they are considered to be mainly transcribed by Pol II, although in some cases Pol III is responsible for pri-miRNA transcription. Pri-miRNA are characterised by hairpin structures that undergo further processing finally resulting in mature miRNA. Hairpins are recognised by a type

II endonuclease complex composed of DGCR8 and Drosha subunits that binds to a characteristic mismatch at the basis of the stem and excide it, leaving two overhanging nucleotides at 3' end. Excided stem-loops are precursors of microRNA (pre-miRNA) and are exported to the cytoplasm by exportin V that recognise the overhanging nucleotides left by Drosha. In the cytoplasm pre-miRNA are further cleaved by another endonuclease, the Dicer enzyme, that cuts the loop between 5p and 3p arms, leaving an imperfect miR-5p:miR-3p duplex. The duplex denatures and one of the arms is loaded in the RISC complex. Selection of the arm to be loaded probably relies on intrinsic thermodynamical stability and binding strength between the 5' end of miRNA strand and the protein complex. The main core of RISC complex are proteins from the Argonaute [76] or the PIWI families. These proteins are characterised by PIWI and PAZ RNA binding motives that interact with both overhanging nucleotides produced in miRNA strand by nuclease activity and target mRNA. Moreover, thanks to their structural similarity with RNAse-H enzymatic site, these domains may display ribonuclease activity. Although for most miRNAs only one of the arms is generally recruited in the RISC complex while the other undergoes degradation, for some miRNAs both strands may be loaded. The selected miRNA strand directs the riboproteic complex to its molecular targets and mediates translational silencing through different mechanisms. Perfect complementarity between miRNA and target mRNA results in target degradation (in human only Ago2 displayed nuclease ability) while incomplete binding leads to mRNA silencing either by competition with ribosome binding or by sequestering the ribonucleic complex to Processing Bodies (P-bodies) [77]. The interaction between miRNA and target mRNA is complex. The critical region for miRNA:target interaction is termed seed. It consists of a region of perfect match generally spanning 6 or 7 nucleotides (respectively referred to as 6mer or 7mer seed) from position 2 at the 5' end of RISC-loaded mature miRNA [78]. Additional regions of complementarity external to the seed may further stabilise the interaction but are not critical for target recognition [79]. Since complementarity is restricted to the short sequence of the seed region every miRNA is allowed to bind a wide array of different targets. This feature candidated miRNAs as regulatory molecules able to finely tune complex cellular processes. Indeed a wide corpus of literature has been collected linking microRNA activity to extensive multistep control of inflammation [80]. Recent data show that miRNA play pivotal role already during cellular specification in the bone marrow [81,82]. In Acute Myeloid Leukaemia (AML) treatment of immature blasts with PMA resulted in partial unlocking of differentiation toward monocytic fate through

overexpression of a panel of miRNAs (namely, miR-155, miR-222, miR-424 and miR-503; Forrest et al 2010). Interestingly, among the molecules identified as targets for these miRNAs mediating such an effect, a precursor for another miR stood out. Hence, microRNAs can both act maintaining an immature state or promoting cellular differentiation. MiRNAs also play a role during macrophage differentiation. Indeed microRNAs have been identified interfering at multiple levels with the machinery for M-CSF or GM-CSF signal transduction [83,84]. More in general miRNAs may well be responsible at least in part of different features of macrophage plasticity. In fact many of these molecules have been linked to polarized states of activation in macrophages. Several microRNAs establish complex crosstalks with transcription factors relevant for polarised macrophage response [85,86]. As an example, one of the prototypical M1 miRNA, miR-155, is induced in macrophages by NFkB p50:p65 activation and exerts a pro-inflammatory effect by directly targeting transcription factors like C/EBPb that are important for anti-inflammatory phenotypes [87].

2.1.1 miR-9

MicroRNA-9 was initially identified as one of the most highly expressed miRNA in neural development [76,88]. Mir-9 gene exists in three different isoforms, each mapping in a different chromosome. In non-neural tissues and in adult brain, miR-9 expression is inhibited by extensive methylation of CpG islands in miR-9 gene promoters. Interestingly, a lncRNA was recently reported to mediate methylation in miR-9 promoters [89], while miR-9 was shown to negatively regulate the lncRNA MALAT1 [90], further extending the known interconnections between ncRNA classes. In neural precursor cells miR-9 expression favours proliferation by inhibiting migration [91]. Therefore it is not surprising that miR-9 alterations contribute to many malignant features of different tumour types. Interestingly, high levels of miR-9 increased motility of epithelial cells in human breast cancer [92] and many types of solid tumours are able to secrete miR-9 in microvescicles, inducing motility and migration in vascular endothelial cells, that result in increased angiogenesis [93]. These results suggest that miR-9 effect on motility and migration is dependent on cell type. In fact, in other tumour types miR-9 antagonises cell migration [94]. Since miR-9 in non-neural tissues is not expressed at steady state, miR-9 expression in tumour cells is a late acquired feature that may confer pro-metastatic abilities. In the immune system, miR-9, more specifically miR-9.1 gene was shown to be induced both in monocytes and neutrophils upon stimulation with pro-

inflammatory molecules in human [95]. In the same report, NFkB was identified as a target of miR-9, suggesting that this miRNA may form a negative feedback that contrast cellular pro-inflammatory activation. More recently, ectopic expression of miR-9 in immature myeloid precursors led to altered neutrophil development [96]. Indeed overexpression of this microRNA has been observed in cases of acute myeloid leukaemia (AML). Therefore it appears that miR-9 might play multiple roles in innate immune cells, regulating differentiation process, activation and maybe migration. Further studies are needed to fully understand miR-9 role in such contexts.

2.1.2 miR-125a

MiR-125 family comprises three miRNA isoforms that share the same seed sequence. It includes miR-125a located in chromosome 19q13, miR-125b-1 in chr.11q23 and miR-125b-2 mapping in chr.21q21. The role of this miRNA family is still elusive. In fact, they have been reported to exert opposite functions in different tumour types [97] [98]. Also in autoimmune diseases these miRNA appear to have different effects in different pathologies. As an example, miR-125a levels are reduced in patients with systemic lupus erythemathosus (SLE) [99] but increased in rheumatoid arthritis [100]. In SLE reduced levels of miR-125a result in increased expression of its target KLF13 that is a transcription factor for the inflammatory cytokine RANTES. In the immune system miR-125a plays multiple important roles. It is mostly expressed in long-term hematopoietic stem cells (HSC) and is progressively lost during differentiation [1]. In HSCs apparently miR-125a is important for the maintenance of staminality although conflicting reports suggest that miR-125a overexpression in HSC leads to BM exhaustion [101]. Moreover, miR-125a may also play a role in haematopoiesis regulating myeloid differentiation. In fact miR-125a overexpression blocks erythroid differentiation and results in myelodisplastic syndrome like acute myeloid leukaemia (AML) [102]. In addition, a recent paper showed that genetic ablation of this miRNA leads to decreased number of circulating neutrophils [103]. Also in mature cells from the immune system miR-125a seems to play a role. In fact, it has been reported overexpressed in classically activated macrophages [104,105]. Its role in such context is still controversial. Indeed it has been shown to directly target TNFα and IL6 exerting anti-inflammatory functions while others showed a pro-inflammatory role for this microRNA demonstrating its targeting against TNFAIP3 that leads to increased expression and activation of NFkB [106]. Similarly, a recent report [107] showed that

artificial overexpression of miR-125a in macrophages increased M1 features like iNOS expression, secretion of TNFα and IL12, phagocytosis of tumoral cells and T-cell activation. On the contrary, Banerjee and collaborators showed that miR-125a could directly target TNFα, IL12 and iNOS, consequently its blockade led to increased expression in such molecules [108]. Consistently, in the same report it was shown that miR-125a artificial expression in macrophages led to increased expression of M2 markers upon IL4 stimulation and increased phagocytosis of apoptotic cells but reduced bacterial killing. All these conflicting reports clearly link miR-125a to immune system but the real role of this miRNA in immune cells is still elusive. Previous unpublished data from our lab support the idea that in human macrophages miR-125a plays an anti-inflammatory effect. In particular we have evidences that this microRNA is induced with a late kinetics in macrophages stimulated with LPS and its expression is further induced by IL10. Moreover, among the molecular targets of this miRNA we identified many molecules important for classical M1 functions like TLR4 itself, its co-receptor CD14, the adaptor molecule IRAK1 and the effector cytokines TNFα and IL6 together with the pro-inflammatory chemokines CCL3 and CXCL8. Indeed ectopic expression of miR-125a in the monocyte like cell line THP-1 resulted in decreased level of secreted pro-inflammatory molecules upon LPS stimulation, while miR-125a blockade led to the opposite result. Collectively these results prompted us to consider this miRNA as a molecular effector of IL10 signaling, exerting anti-inflammatory roles.

2.1.3 miR-135b

Human miR-135b gene maps in chromosome 1q32.1. It has been widely studied in oncologic context where it appears to exert opposing functions from one tumor type to the other. Some of these reports identified targets for this miRNA like, for example, STAT-6 [109] and SMAD-4/5 [110] that suggest a possible role for this miRNA in immune cells. Furthermore recent works directly linked induction of miR-135b to pollutants-induced inflammation in the lung [111-113]. Although miR-135b expression in inflamed lung was attributed to fibroblast and epithelial cells, possible contributions from innate immune cells were not ruled out. Hence, miR-135b appears to be an interesting and mostly unexplored miRNA in inflammation.

2.2 Long Non Coding RNA

Long Non Coding RNAs (lncRNAs) are tautologically defined as RNA molecules longer than 200 nucleotides with low or no coding potential. The length threshold is arbitrarily defined to distinguish members of this class from the previous defined and better known classes of small non coding RNA like rRNA (ribosomal RNA), tRNA (transfer RNA), snRNA/snoRNA (small nuclear and small nucleolar RNA) and miRNA/siRNA (micro and small interfering RNA). The definition of lncRNA is further challenged by the observation that some molecules annotated as lncRNA may display unconventional or cryptic ORF as evidenced by experiments of Mass spectrometry in murine models [114]. To further blur the boundary between RNA classes, maturation of some lncRNAs requires cleavage of primary transcript generating a mature lncRNA and a residual small noncoding RNA that may itself be functional [115]. Moreover, some pri-miRNA genes have been identified nested in host lncRNA sequences [116]. Such an ephemeral definition of lncRNA category reverberates in a complex classification. To date, many different criteria have been proposed for lncRNA classification, included subcellular location, position in the genome relative to other annotated elements, sequence and structure conservation or association with biochemical pathways [117]. Every criterion considered alone fails to fully categorise lncRNAs and different criteria show little overlap. Therefore a real classification is still elusive and will probably require contemporary use of several criteria. LncRNAs are transcribed by Pol-II and commonly share many features with mRNA, like 5' capping and 3' poly-A although lncRNA expression usually appears to be more specific with respect to tissue, condition and developmental stage [118]. LncRNA transcripts may originate from intergenic regions, pure exonic, pure intronic or mixed intronic/exotic regions of a gene, either in sense or antisense orientation, as well as being associated with promoter sequences, repeated sequences or telomeric regions. This complex genomic landscape has been referred to as 'Transcriptional Forest', highlighting that multiple transcripts may share the same genomic space, just like trees in a thick forest, and furthermore, different mature RNA species may branch from a common primary transcript [74]. As a class, lncRNAs are homogeneously distributed between nucleus and cytoplasm but many lncRNA species show a specific enrichment in subcellular locations [118]. Since lncRNAs are commonly capped and poly-adenilated, they display stability similar to mRNA, although with some important exceptions. In fact, lncRNAs originating from splicing events lack of both 5' cap and 3' poly-A and are thus degraded at higher pace than mRNA. Similarly, lncRNAs that are also host genes for miRNAs undergo microprocessor-mediated cleavage resulting in a capped but not poly-adenilated transcript, that is more sensitive

to degradation through Nonsense Mediated Decay (NMD). Vice versa, circularRNAs (ciRNAS), although originating from splicing events through back-splicing or lariat stabilisation, have and half-life longer than the average lncRNA thanks to their circular shape that hides 5' and 3' terminals. To exert their biological functions, lncRNAs take advantage of both their sequence, that allows interaction by complementarity with other nucleic acids, and their 3D structure, that allows direct interaction with proteins. Thus in the nucleus these molecules are perfect targeting and scaffolding elements, able to orchestrate recruitment and assembling of the epigenetic machinery. Similarly, in the cytoplasm these molecules may take part in biochemical pathways by promoting the assembly of macromolecular complexes as a scaffolding element or on the contrary, may compete with elements of the complexes for the binding of their molecular partners. Since most of lncRNA functions depend more on structure than on pure sequence, they are less sensitive to mutations than coding genes and are therefore less conserved evolutionarily, although more than random sequences [119]. Because of their wide array of molecular interaction with both genetic and protein elements and their proved role in directing epigenetic modifications, lncRNAs are suitable candidates as regulators of complex processes. Indeed, recent evidences progressively unveil lncRNA contribution in controlling cell proliferation, survival and differentiation in health and disease. In particular important roles for lncRNA molecules in the control of immune system development and function have been highlighted. A recent study profiled lncRNA expression in haematological precursors [120]. More than 2500 transcripts mapped to lncRNAs were shared between precursors, while 159 were overrepresented in long term Hematopoietic Stem Cells (HSC). Depletion of one of these transcripts, named lnHSC1, resulted in abnormal myeloid differentiation. Another report showed that H19 is a lncRNA that acts recruiting the methyl-CpG binding protein (MBD1) and histone methyl transferases. It is important for methylation of imprinted loci but plays a role also in maintaining pluripotency in long term HSC. In fact, upon conversion from long term HSC to short term HSC this lncRNA is drastically decreased. Moreover, genetic ablation of this gene led to decreased number in long term HSC that was paralleled by an expansion of short term HSC. H19 favours long term staminality by inducing the silencing the pro-differentiating growth factor IGF2 [121]. At later stages, lncDC is enriched during monocyte to DC differentiation [122]. Apparently it favours the conversion through a competing mechanism. In fact, lncDC interacts with C-terminus of cytoplasmic STAT3 and prevent it from binding SHP1, a cytosolic phosphatase. Increased levels of lncDC lead to increased phosphorilation and subsequent nuclear

translocation of STAT3. Beside differentiation, lncRNAs also play an important role in cell survival. Trophic signals for myeloid cells like IL3, IL5 and GM-CSF induce the expression of the Morrbid transcript that in turn acts in *cis* recruiting Polycomb complex to the promoter of the pro-apoptotic neighbouring gene Bcl2l11. As expected, lncRNAs also play a pivotal role during immune cell activation. A recent report catalogued more than 150 lncRNAs differentially expressed in monocytes/macrophages after LPS stimulation [123]. Most of these transcript were in close proximity with known inflammation-related protein-coding genes. Interestingly, lncRNAs overexpressed in cells of the innate immunity under inflammatory conditions to some extent shared conserved microdomains that may be important for lncRNA connectivity and function. One of the first lncRNA molecules observed to be overexpressed following TLR pathway triggering is lincRNA-COX2 [124]. LincRNA-COX2 induction results in perturbation of almost 500 genes, among which stand out many important regulators of the immune response like the cytokines IL6, IL23, the TLR1 receptor and the chemokines CCL-5 and CX3CL1. LincRNA-COX2 effect on gene expression is mediated by its direct association with the Heterogeneous Nuclear Ribonucleoparticle type A and B (hnRNP A/B) that affect stabilisation, splicing and cytoplasmic transport of target mRNAs. Another ncRNA with similar effect is THRIL. Similarly to lincRNA-COX2, THRIL is overexpressed downstream of TLR2 activation and affects expression of almost 300 genes included chemokines (CXCL10 and CCL-1) as well as cytokines (CSF-1, IL8 and TNFα). Also the mechanism is similar between the two, since THRIL is shown to associate with hnRNP L [125]. A third molecule that shares this mechanism of action is lncRNA-13. It is already expressed at steady state and interacts with hnRNP-D and histone deacethylase (HDAC) maintaining transcriptionally silent many genes with pro-inflammatory effect. Upon TLR2 triggering, lncRNA-13 levels decrease, releasing its suppression on MyD88, STAT1/3 and TNFα [126]. Intriguingly a SNP in the sequence of this transcript is associated with chronic inflammatory disease like celiac disease. Two other lncRNAs induced under inflammatory conditions are LETHE and PACER. These lncRNAs show similar mechanisms of action but opposed effects. In fact LETHE and PACER act through a competing endogenous mechanism sequestering respectively p65 and p50 subunits of NFkB. Differences in the outcome depend on the fact that while p65:p50 NFkB dimers have a pro-inflammatory effect, p50:p50 dimers exert anti-inflammatory roles [127,128]. Therefore binding of p50 by PACER stechiometrically favours p65:p50 dimer formation supporting inflammation [129]. On the contrary, requisition of p65 performed by LETHE favours p50:p50 interaction with overall

dampening of the inflammatory response [130]. NFkB is a nevralgic element in the control of innate immunity being the converging point of many inflammatory signaling pathways. As such it is a strategic target to broadly regulate inflammatory response. Therefore is no surprise that so many regulatory mechanism have developed to finely tune NFkB activity. Indeed in addition to the aforementioned lncRNAs affecting NFkB activation, a third molecule has lately been described with negative regulatory effect. It is called NKILA and apparently interacts with NFkB:IkB inactive complex and masks phosphorilation sites on IkB, thus preventing IkK mediated phosphorilation of IkB and its subsequent degradation that allows NFkB activation [131]. NKILA expression is increased after exposure of macrophages to pro-inflammatory cytokines like TNFα and IL1b, thus it appears to act as a molecular brake to control inflammation.

2.2.1 BLACAT1

Bladder cancer associated transcript 1 (BLACAT1 also known as linc-UBC1) has initially been identified as the most highly expressed ncRNA in bladder cancer tissues as compared with adjacent normal tissue, hence the name bladder cancer transcript-1 [132]. It is annotated as a 3kb, mono-exonic gene in chromosome 1q32.1. BLACAT1 mainly localise in the nucleus where it directly interacts with SUZ12 and EZH2. These proteins assemble together and form the core of the Polycomb Repressive Complex 2 (PRC2). Consistently, treatment with siRNAs targeting BLACAT1 in bladder cancer cell lines resulted in decreased levels of H3K27 tri-methylation of known PRC2 modulated genes and was paralleled by increased transcript levels. While PRC2 target genes were modulated after BLACAT1 depletion, genes mapping in close proximity of BLACAT1 itself were not affected. These results depose on behalf of an *in trans* effect of this lncRNA rather than an *in cis effect*. Other than in bladder cancer, BLACAT1 has been associated with many types of solid cancers like gastric cancer, colorectal tumours, lung adenocarcinomas and lung squamous carcinomas [133,134]. In all these tumours, BLACAT1 overexpression correlated with poor prognosis due to increased metastasis and reduced survival rate. Consistently, BLACAT1 silencing in cancer cells led to reduced proliferation and motility *in vitro* that correlated with reduced metastasis *in vivo*. Overall these results depict BLACAT1 as a pro-oncogenic transcript that acts *in trans* through association with PRC2. Although a direct role for this lncRNA was proved in cancer cells, a possible role in immune cells has never been assessed so far,

although it is now widely recognised that immune system is crucial for cancer progression.

2.3 A new layer of complexity: lncRNA-miRNA interactions

Discovery of functional roles for ncRNAs completely revolutionised the field of molecular biology. These molecules have been proved to be capable of tightly regulate complex macromolecolar networks and cellular processes. The ability of these transcripts to simultaneously interfere with many targets greatly expanded the complexity of regulatory networks. In fact molecules from these classes can reciprocally affect members of the others. For example, miRNAs have been mapped inside lncRNA genes and their excision is a maturation step for both molecules [116]. Furthermore, pri-miRNAs may be considered all the way lncRNA and some of them may even have proper function other then being a precursor for microRNA and the balance between the two natures may depend on context. MicroRNA have also been reported to target lncRNA, negatively regulating their functions [90]. On the contrary, a specific class of circular-lncRNA (ciRNA) are enriched in repeated miRNA responsive elements and act as a 'sponges', buffering the available cytoplasmic levels of mature miRNA [135]. Moreover a recent report convincingly proved that a lncRNA was able to interfere with processing of a specific miRNA by competing with Drosha for its binding region on pri-miRNA stem-loop structure [136]. Overall these new classes of molecules appear to be two widely interconnected systems with regulatory roles on cellular behaviour, both at steady state and after stimulation, in health and pathology. Future studies will further characterise their interactions as well as their functions, possibly yielding a functional classification for lncRNAs.

Aim of the study

The first aim of this study is to further characterise the relevance of previously identified microRNAs on macrophage activation. In particular, animals selectively deficient for miR-125a in macrophages are studied in different inflammatory pathologies. With a similar approach, murine macrophages deficient for miR-9.1 are assayed in vitro to characterise cellular response to inflammatory stimuli in the absence of this regulator.

The second aim of this work is to unveil a new layer of complexity in macrophage activation. We study the negative regulatory effect on miRNA processing exerted by a lncRNA that has never been characterised in immune cells before.

Materials and Methods

Genotyping

Similar genotyping strategies were employed to identify WT and KO animals in both miR-9.1 and miR-125a mice. Routine genotyping was performed by PCR reaction on tail biopsies genomic DNA. Primers used for genotyping are reported in Table.1. PCR with 'flox' and 'rev' primers could confirm homozygosity for the floxed allele. Discrimination between KO and WT mice was allowed by genotyping for the CRE gene. Homozygosity for the band generated by 'CRE null' and 'CRE rev' primers indicated WT animals. On the contrary, heterozygosity for the 'CRE null'/'CRE rev' and 'CRE'/'CRE rev' generated bands identified KO animals. Effective deletion of targeted genes was assessed by end point PCR on macrophage gDNA using the common reverse 'rev' primer downstream to the loxp site and forward primers specific for either floxed allele 'flox' or deleted 'del' alleles. Results were analyzed by gel electrophoresis with intercalating dyes. Gel pictures were acquired with Gel Doc EZ System (BioRad) and analysed with Image Lab™ Software (BioRad). MiR-9.1 deletion efficiency was further proved by real-time qPCR using the same primers.

Table.1

Target	Orientation	Sequence
125a flox	FW	ATTCAGTCCCAGCCCCAAAATCTA
125a del	FW	CTCTCTCTGAGGCACTGTTTTGTC
125a rev	RW	CTTCTCTAAGGGATGTGCCTGGTT
9 flox	FW	CCGAAGTTCCTATTCCGAA
9 del	FW	CCTTTCTGAGGTCTCGTCGT
9 rev	RW	GCTTGCACTTTCCCAGTACTT
CRE	FW	CCCAGAAATGCCAGATTACG
CRE null	FW	TTACAGTCGGCCAGGCTGAC
CRE rev	RW	CTTGGGCTGCCAGAATTTCTC

Bone Marrow Derived Macrophages (BMdM) preparation

Experimental mice were kept under SPF conditions. BMdM were obtained from 8-12 weeks old male C57BL/6. Femurs were obtained, muscles were removed and washed in sterile IMDM (Lonza) and then both epiphyses were removed. The bone marrows were obtained by centrifugation (5000 rpm, 2 min) in a 1.5 mL sterile eppednorf tube. Erytrocytes were lysed by 5 min incubation in ACK buffer (150mM NH_4Cl, 10mM

$KHCO_3$, 1mM Triplex-EDTA). The obtained cells were either stained for FACS analysis or plated in 100mm x 20mm not treated polystyrene plates (corning). Percursor cells were then differenntiated in complete IMDM medium (10% FBS, 1% penicillin-streptomycin, 1% glutamine) supplemented with murine mM-CFS (50ng/ml). After 7 days of culture, macrophages (BMdM) were detatched using Accutase (Sigma-Aldrich) and plated at desired concentrations. After ON adhesion, macrophages were ready for subsequent experiments.

Human Macrophage differentiation and polarization.

Human monocytes were isolated from healthy donor buffy coats by two step gradient centrifugation using Ficoll-Paque (GE Healthcare) and Percoll (Amersham). Isolated monocytes were plated in RPMI without serum for 10 min. Non-adherent cells were discarded, and purified monocytes were cultured in 6 well at $2x10^6$ cells/well in 2ml RPMI 1640 (Lonza) supplemented with 10% fetal bovine serum (FBS; Lonza), 1% penicillin–streptomycin (Lonza), and 1% glutamine (Lonza). Monocytes were differentiated in macrophages by exposure to 50 ng/ml recombinant human 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 IL4 for 24 h before being evaluated. Resting macrophages (M0) were left untreated in culture medium.

Cell Stimulation

LPS from Escherichia coli serotype 055:B5 was purchased from Sigma- Aldrich. Human IL10, IFNγ, IL4, TGFβ and M-CSF were purchased from R&D Systems as well as murin INFg, TNFα and mM-CSF. Dexamethasone was from Sigma-Aldrich. Prior to stimulation, exhausted media were removed and cells were washed in PBS. Stimuli were prepared at the desired dilutions in complete cell colture media.

RNA isolation and gene expression analysis

Stimulated cells were harvested in TRIzol reagent (Ambion). Total RNA was purified using DirectZOL™ RNA Miniprep (ZymoResearch) according to the manufacturer's

instructions. Total RNA quantity and purity were determined at Nanodrop 2000c (ThermoFisher). 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 (Table.2) and Fast SYBR® Green Master Mix 2X (Applied Biosystems). Reactions were assembled mixing cDNA (2ul of 5ng/ul dilutions from RT), Fast SYBR Green Master Mix (5 ul of 2x product), primers (0.5 ul for each 10uM primer dilutions) and water (3ul to final 10ul of reaction). For pri-miR-9 isoform quantification, Taqman probes were preferred (Table.3, ThermoFisher) and performed in similar conditions using Fast TaqMan ® Master Mix 2x (Applied Biosystems). Reactions were performed on a VIIA-7 Real-Time PCR Detection System (Applied Biosystems). The thermal cycling conditions were standard fast-cycling conditions. Relative expression values were calculated using ΔΔCT method normalized on GAPDH as housekeeping.

Table.2

Terget	Orientation	Sequqnece
pri-miR-155	FW	CAGATTTCCCCTTCCTGGTTT
	RW	AGCTTTATAACCGCATGTGCATAC
pri-miR-135b	FW	TCCACTCTGCTGTGGCCTAT
	RW	AGCCCTACATGAGTTTGGGAC
BLACAT	FW	GCATGGTTTCCCGAATCTATGC
	RW	TTTGCCGGCATGCTTTTCAG
KLF4	FW	TCTCCAATTCGCTGACCCAT
	RW	CGGATCGGATAGGTGAAGCT
cMyc-C	FW	ACAGGATACATCCTGTCCGT
	RW	GCACAAGAGTTCCGTAGCTG
GAPDH	FW	GATCATCAGCAATGCCTCCT
	RW	TGTGGTCATGAGTCCTTCCA

Table.3

Assay Name	Assay ID
mmu-pri-miR-9.1	Mm04227702_pri
mmu-pri-miR-9.2	Mm03306269_pri
mmu-pri-miR-9.3	Mm03307250_pri

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 differently 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). Expression 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 as it was found to be stably expressed across experimental condition (Fig.M1).

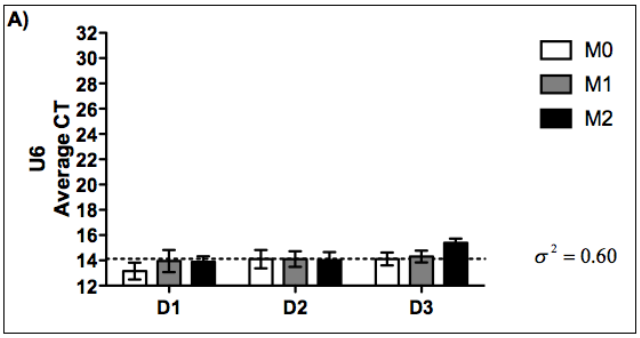

Figure.M1 Stability of U6 across donors and conditions in TLDA assays. (A) Average *Threeshold Cycle* (CT) were calculated for three donors in all experimental conditions and were found to be acceptably stable ($\sigma^2 = 0.60$)

MicroRNA isolation and Quantitative RT-PCR Analyses

Stimulated cells were harvested in TRIzol reagent (Ambion). Total RNA was purified using DirectZOL™ RNA Miniprep (ZymoResearch) according to the manufacturer's instructions. For each sample, 100 ng of total RNA were reverse transcribed with individual stem-loop RT-primers using TaqMan miRNA assay (ThermoFisher). Subsequently, 10 ng of each individually synthesized cDNA were amplified in 10μL of final qPCR reaction with specific TaqMan probes (Table.4). Real-time PCR was performed on VIIA-7 Real-Time PCR Detection System (Applied Biosystems). Relative miRNAs expressions were determined by using the ΔΔCt method, normalizing the levels with U6 snRNA (human) or snoR202 (murine). U6 was chosen to improve consistency with preliminary TLDA analisys, while SnoR202 is widely used as endogenous control for miRNA and was found to be stable across all tested experimental conditions (Data not shown).

Table.4

Assay Name	Assay ID
hsa-miR-125a-5p	002198
hsa-miR-9	000583
hsa-miR-155	002623
hsa-miR-135b	002261
U6	001973
SnoR202	001232
mmu-miR-155	002571

Macrophage Transfection

Differentiated were transfected with 100pmol of BLACAT1 LNA™ longRNA GapmeR (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 4ul of GAPmeR 50uM were mixed with 200ul of OPTIMEM medium (Gibco) and 8ul of TransIT TKO. GAPmeR containing liposomes were allowed to form for 30 min at RT. Mixes were finally added to the well drop by drop. After ON incubation, transiently transfected cell were further 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.

Sepsis

Sepsis was assessed in mice employing well established models to test either SIRS or CARS resembling conditions. SIRS conditions were modelled by IP injection of increasing doses of LPS (30mg/kg, 50mg/kg, 100mg/kg). After stimulation, survival and body weight loss were monitored for a week. Endotoxin Tolerance was adopted as a model for CARS phases in sepsis. Either 8-12 week old mice or differentiated BMdM were pretreated with LPS (in vivo: 5mg/kg, in vitro: 10ng/ml) or PBS and allowed to recover ON. Tolerant and responsive subjects were then challenged again with higher

doses of endotoxin (in vivo: 50mg/kg, in vitro: 100ng/ml). Blood samples were withdrawn from stimulated mice after 1, 4 or 8 hours. Plasma were isolated and cytokine abundance tested by ELISA assay. For in vitro BMdM, ELISA assay were performed directly on supernatants 24 hours.

Peritonitis

Self-resolving peritoneal inflammation was induced as previously described [56]. Briefly, 1mg of Zymosan (Invivogen) was IP injected in 8-12 week old mice. At indicated time points (6, 24, 48, 72 hours) mice were sacrificed and peritoneal wash were performed to collect peritoneal exudate cells (PEC). Leukocytes were collected by centrifugation, stained and analyzed by FACS.

Colitis

Inflammation in the GI tract was induced exposing 8-12 week old mice to water-dissolved, ad libitum-available dextran sulfate sodium (DSS, MP Biomedicals), according to well established protocols. After one week DSS was removed and pure H2O reintroduced allowing mice to recover over a week. Three cycles of DSS/H2O were performed, continuously monitoring disease activity index (DAI). DAI is the sum of scores attribute to body weight loss, stool consistency and presence of fecal blood. BW loss inferior to 1% scored 0, a score of 1 was assigned for BW losses comprised between 1% and 5% . A score of 2 was given to BW losses between 5-10%, 3 for 10-15% BW loss and 4 for losses higher than 15%. Fecal consistency was arbitrarily assessed with respect to normal stools. Normal feci scored 0, impaired consistencies were valued 2, while diarrhea was scored 4. Similarly, absence of blood was considered 0, presence of occult blood counted 2 while evident hemorrhage scored4. At end point, colons were isolated and measured in length. Macrophages were subsequently isolated from lamina propria and further analysed.

Macrophage Isolation from Colon lamina propria

Isolated colons were longitudinally cut and extensively washed in PBS 1x and wash solution (HBSS 1x, 5% FCS) to eliminate fecal residues. Colons were then cut in pieces 0.5 cm in length and incubated three times with warm EDTA solution at increasing concentrations (2.5 < 3.5 < 5 mM) for 20 min at 37°. After each round of incubation, colon pieces were filtered in a tea strainer. Subsequent EDTA treatment eliminated luminal mucosal epithelial layer, exposing lamina propria for enzymatic digestion. Excess EDTA was quenched by washing pieces with wash solution supplemented with 2.5mM CaCl2. Residual colon pieces were further minced and incubated at 37° with wash solution with 1mg/ml Dispase, 1mg/ml DNAse, 0.25mg/ml Collagenase and 5mM CaCl2. Dissociated cells were filtered sequentially through 100um and 70um filters. Subsequently macrophage cells were purified by centrifugation on Percoll gradient. Isolated macrophages were counted and further purified using CD11b microbeads (Milteny) according to manufacturer's instructions.

Peritoneal Wash

Peritoneal cells were collected by injecting 5ml of Physiological Saline in the peritoneum and by re-collecting the volume.

Blood withdrawal

Blood samples were collected from the retro-orbital sinus of 9-11 week-old WT and KO mice previously anesthetized with ketamine/xylazine Ketaset® CIII/Rompun®, dose 100 mg/kg ketamine and 10 mg/kg xylazine. For FACS analysis blood samples were collected in Vacutainer blood collection tubes (EDTA, sodium citrate, heparin; BD) to prevent coagulation. Erythrocyte lysis was obtained incubating samples in ACK buffer for 5-10 minutes. Leukocytes were collected by centrifugation and further prepared for FACS analysis. For cytokine quantification in plasma, blood samples were collected in 1.5ml Eppendorf tubes and kept in ice. Cellular components were subsequently pelleted by centrifugation (13000g for 10 min at 4°C) and plasma separated and stored at -80°C.

Phagocytosis

For phagocytosis assay, 5x105 macrophages were seeded in 1 ml in 24 well plates and allowed to adhere ON. Contemporarily bacteria were grown in standard conditions. From ON cultures, 5x106 E.Coli particles per well were stained with 10uM Vybrant® CFDA SE Cell Tracker Kit (Invitrogen) in 1 ml for 30 min. Stained bacteria were washed three times with PBS. Bacterial pellet were resuspended in 500ul IMDM. Two hours prior to experiment, exhausted medium was removed from macrophages, cells were washed in PBS and only 0.5ml of IMDM were reintroduced. At T0, 0.5ml of stained bacteria were added to each well. After desired time interval supernatants were removed and kept in ice. Macrophages were detached with 400ul of Accutase and stained with anti-F4/80. Stained macrophages were analyzed by FACS analysis. Supernatants containing spared bacteria were serially diluted and plated. Bacteria were allowed to grow ON, then colonies were counted.

FACS staining

Prior to staining, cells were washed with FACS Buffer (PBS 1X, 1% Bovine Serum Albumin) and centrifuged at 1500 rpm for 5 min. Pellets were resuspended and stained with antibody mix at 4 °C for 30 min in the dark. Samples were then washed again with FACS Buffer and centrifuged at 1500rpm for 5 min. Finally, stained cells were fixed in 350 µl FACS fix (PBS 1X, 1% paraformaldehyde) and incubated at RT for 30 min under chemical hood. After the fixation, samples were washed with FACS Buffer, centrifuged at 1500rpm for 5 min, resuspended in 700 µl of FACS Buffer. Fluorescent data were acquired with BD FACSCanto™ II and analyzed with FlowJo Software (v.9.3.2). The antibodies used are listed in Table.5.

Table.5

Target	Fluorophore	Brand	Clone
CD45	V450	BD	30-F11
CD11b	PerCP-Cy5.5	BD	M1/70
Ly6G	PE-Cy7	BD	1A8
Ly6C	FITC	BD	AL-21
F4/80	AF647	Biolegend	BM8
TLR4	PE	BD	MTS510
CD14	FITC	BD	rmC5-3
CD86	PE	eBioscience	Sa2-8
MHC-II (I-A/I-E)	FITC	eBioscience	M5/114.15.2

In silico prediction

Pri-miR-135b hairpin sequence (MI0000810) was retrieved from miRbase (www.mirbase.org); BLACAT1 transcript sequence (ENST00000626538.1) was downloaded from ensembl (www.ensembl.org). Prediction of possible interactions between the two sequences were obtained executing the RNAduplex program from ViennaRNA package [137] in R environment (version V.3.4.2; R Core Team 2014). Graphical rendering of the predicted structures were designed using the *RNAsmc* and *R4RNA* libraries [138,139]. In Script.1 we report a prototypical script developed for such analysis.

Script.1 R script for RNA-RNA interaction prediction and structure plotting

```
# Inizialize workspace
setwd("C:/Users/XXX/XXX")
library(RNAsmc)
library(R4RNA)

# Upload and edit sequences
fasta.seq<-read.fasta("z.txt")
seq_1<-paste(fasta.seq[[1]][1:length(fasta.seq[[1]])], sep = "", collapse= "")
seq_2<-paste(fasta.seq[[2]][1:length(fasta.seq[[2]])], sep = "", collapse= "")
seq<- c(seq_1,seq_2)

# Run RNAduplex command
RNAduplex_path<-"C:/Users/XXX/ViennaRNA/RNAduplex"
duplex<-system(RNAduplex_path, intern = TRUE, input = seq)

# Edit RNAduplex output
result<-unlist(strsplit(duplex, split=" "))
seq_1.ext<-unlist(strsplit(result[4], split=","))
seq_2.ext<-unlist(strsplit(result[7], split=","))
mirna<-paste(fasta.seq[[1]][seq_1.ext[1]:seq_1.ext[2]], sep = "", collapse= "")
lncrna<-paste(fasta.seq[[2]][seq_2.ext[1]:seq_2.ext[2]], sep = "", collapse= "")
aligned_seq<- paste(mirna, "&", lncrna, sep = "", collapse = "")
output<-rbind(aligned_seq, result[1], result[8])

# Convert from dot-bracket notation to Helix notation
wien<-unlist(strsplit(output[2], split= ""))
junction<-which(wien== "&")
wien[junction]<- "."
wien<-paste(wien, sep = "", collapse = "")
wien<-viennaToHelix[1]
lab<-unlist(strsplit(output[1], split= ""))

# Plot arc-diagrams and add annotations
plotHelix(wien, bg="gray95")
axis(1, at= 1:length(lab), labels= lab, pos= 0, cex.axis= 0.2, mgp= c(0,-0.5,0), tick =
FALSE)
lines(c(0,junction-1),c(0,0),lwd=2, col="red")
lines(c(junction+1, length(wien)),c(0,0),lwd=2, col="blue")
lines(c(16,38),c(0,0),lwd=2, col="darkorange")
lines(c(0,16),c(0,0), lwd=2,col="darkred")
text(c(length(wien[junction:0])/2,length(wien[junction:length(wien)])/2+length(wien[juncti
on:0])), c(-5,-5), labels = c("pri-miR-135b","BLACAT1"),col = c("red","blue"))
text(c(0,junction-1,junction+1,length(wien)), c(-2.5,-2.5,-2.5,-2.5), labels=
c(ext_pri[1],ext_pri[2],ext_lnc[1],ext_lnc[2]), col=c("red","red","blue","blue"), srt=45,
cex=0.5, adj=1)
text(250,100, labels= paste("Energy:", result[8], "kcal/mol"), cex = 0.75)
text(27, -2.5, labels="miR-135b",cex=0.8, col="darkorange")
text(8, -5, labels="DROSHA\nBinding Site",cex=0.8, col="darkred")
```

Results

MicroRNA-125a and macrophage functions

To generate a mouse strain lacking miR-125a selectively in macrophages, *Ozgene Pty Ltd* developed a breeding line carrying the miR-125a gene flanked by loxP regions on a C57Bl/6j background. Animals homozygous for the loxed allele were then crossed with a strain of mice (C57Bl/6j, *purchased at Charles River International Inc*) expressing the CRE-recombinase enzyme under control of the macrophage specific gene Lysozyme M gene (LyzM). Thus animals heterozygous for both genes were obtained and further crossed. MiR-125af$^{lox/flox}$/Lyzm-CRE$^{+/-}$ and miR-125af$^{flox/flox}$/Lyzm-CRE$^{-/-}$ were then selected from the progeny and bred together to generate littermates homozygous for the floxed allele but either CRE negative (miR-125a$^{lox/lox}$/Lyzm-CRE$^{-/-}$, WT) or CRE positive (miR-125a$^{lox/lox}$/Lyzm-CRE$^{-/+}$, KO).In the KO mice, CRE heterozygosity was preferred to avoid loss of function of the LyzM gene. Pups born from the latter breeding were screened for both genes at weaning and were to be born at the expected ratio between sexes and genotypes (fig.1). To validate our KO model the effective deletion of the targeted genomic region was evaluated. Genomic DNA from total splenocytes and from bone marrow derived macrophages (BMdM)of WT and KO animals was recovered and evaluated by PCR amplification with primers specific for either the WT allele (#) or for the deleted one (*). Since deletion of the microRNA was expected to occur only in macrophages, PCR amplification with primers specific for the deleted allele will generatate a specific band in KO BMdM, but not in KO splenocytes nor in WT cells (both BMdM and splenocytes). Conversely, WT primers are expected to generate a band both in KO splenocytes and in WT cells. WT primers may reveal also incomplete deletion of the gene of interest in KO BMdM. PCR reactions showed efficient deletion of the floxed allele in KO animals (fig.1). To assess whether the deletion observed at genomic level was sufficient to efficiently silence miR-125a, the expression of miR-125a mature form in BMdM was evaluated by RT-PCR after LPS stimulation. As expected, LPS induced miR-125a expression in WT cells whereas its expression was strongly reduced in KO BMdM (fig.1).

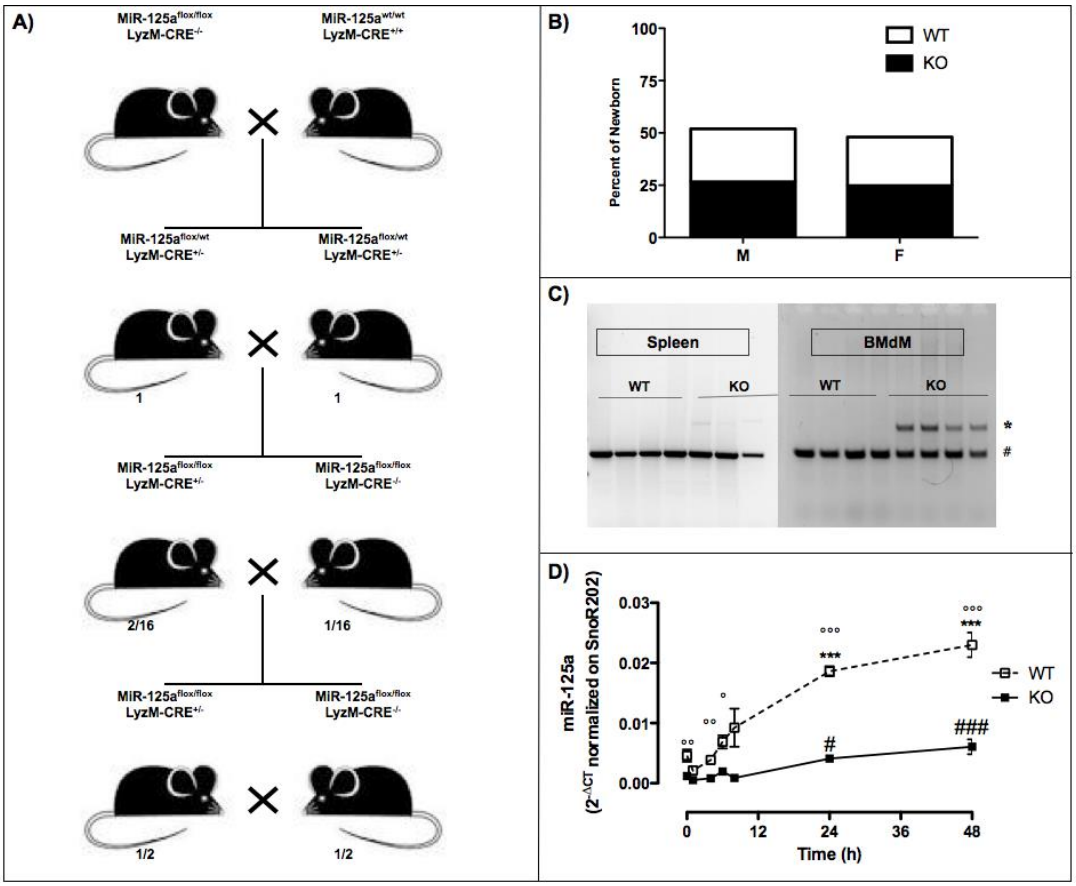

Figure.1 (A) Mice deficient for miR-125a selectively in macrophages were obtained from miR-125aflox/flox/LyzM-CRE-/- and miR-125a wt/wt/LyzM+/+ founders. (B) Newborn were monitored for sex and genotype at wheening. (C) Selective genomic recombination was confirmed in macrophages at genomic level. (D) Kinetics of expression of mature form miR-125a expression levels in WT and KO BMdM in vitro stimulated with LPS (100ng/ml. Raw data are listed in Supplementary Table1). Two-way ANOVA displayed significant interaction between factors. Therefore, genotype-restricted one-way ANOVA were performed, followed by post-hoc Dunnet's test for significant expression over time (WT: * p<0.05, ** p<0.01, *** p<0.001; KO: # p<0.05, ## p<0.01, ### p<0.001). Differences in the expression between WT and KO were determined by T-test between condition for each time point (° p<0.05, °° p<0.01, °°° p<0.001)

Increasing evidences describe miR-125a as a key player in the regulation of hematopoietic stem cell self-renewal [1] and lineage differentiation[140]. To exclude possible alterations in cellular ratio between immune population at steady state following miR-125a knockdown, we evaluated the frequency of neutrophils, eosinophils and monocytes of KO and WT mice in peripheral blood and in bone marrow at steady state (fig.2). The frequency of neutrophils, expressed as percentage of CD45+ cells, did not differ between WT and miR-125a KO animals both in bone marrow (WT=33.5%, sd=5.7; KO=40.0%, sd=6.1) and in peripheral blood (WT=11.2% , sd=3.9; KO=17.4% , sd=6.7). The frequency of monocytes displayed the same trend in both compartments with higher abundance in bone marrow (WT=6.7%, sd=2.3; KO=8.1%, sd=1.7) as compared to the blood (WT=3.9%, sd=1.9; KO=2.7, sd=1.4). No differences between

the groups were observed also in circulating eosinophils (WT=2.2%, sd=0.8; KO=2.6%, sd=1.1).

Our data demonstrate normal frequencies for all the analyzed cell populations in both WT and miR-125a KO animals at steady state in agreement with O'Connel and coworkers [141]. These results suggest that the critical role of mir125a in hematopoiesis and fate determination precedes acquisition of LyzM expression in precursor cells. Therefore, our next observations on this animal model are not biased by uneven basal level in cellular abundances.

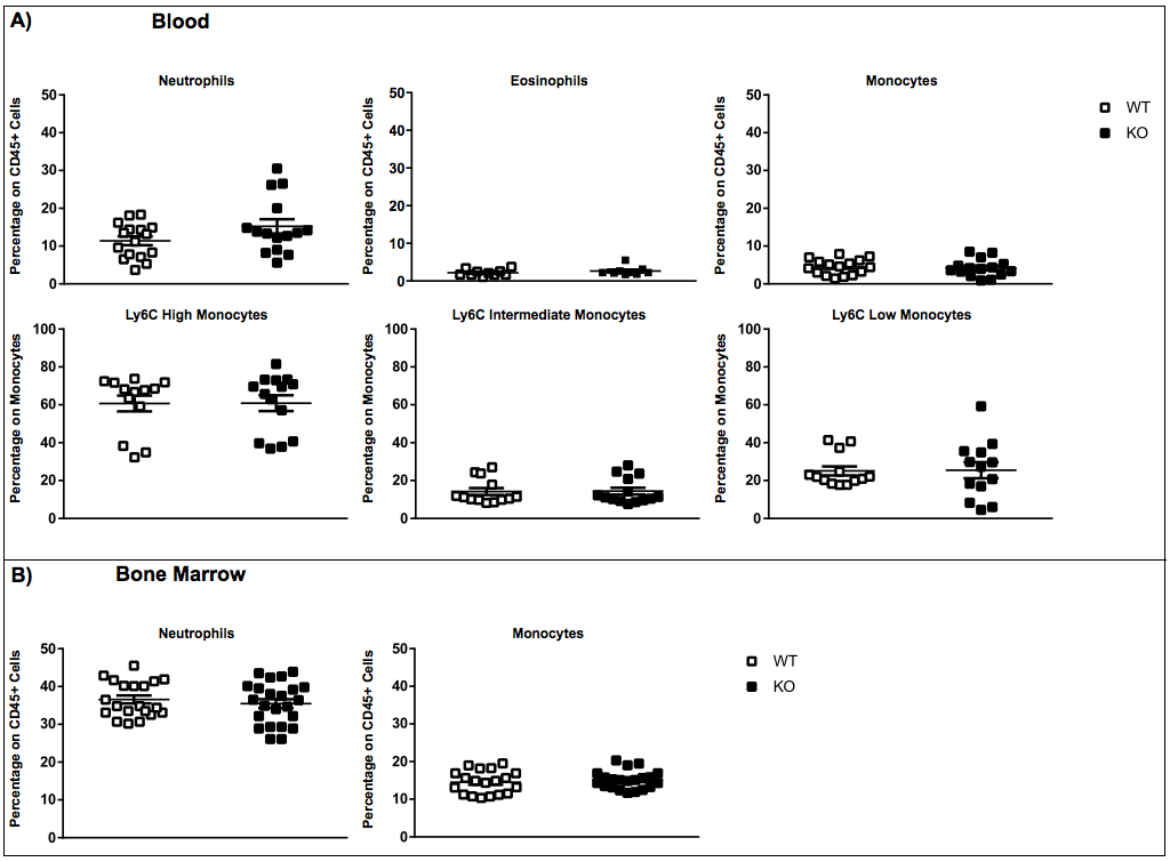

Figure.2 Innate immune cell popuations were analyzed in (A) peripheral blood and (B) bone marrow compartments of WT and miR-125a KO. T-test was not significant for any of the cell type analyzed.

Considering the sensitivity of miR-125a to LPS stimulation in macrophages and its negative regulatory effect on TLR4 pathway itself, this microRNA appears to act as a molecular brake against excessive inflammation. The first context in which we attempted to test this hypothesis is sepsis. In case of sepsis, death occurs either in early phases or at later time points. Early death is due to the cytokine storm that takes place during SIRS [65]. Genetic ablation of a negative regulator of TLR4 pathway, like miR-125a, may lead to increased inflammation and exacerbation of death rate at early time

point of septic shock. To evaluate whether this was indeed the case, WT and KO animals were intraperitoneally injected (IP) with increasing doses of LPS and survival and body weight (BW) loss were monitored for a week (fig.3). Although we observed a correlation between administered dose and death rate (fig.3A), no differences in survival or BW could be detected between WT and KO animals at any of the tested concentrations (fig.3B). Although no difference in the survival rate was observed between the groups, blood sampling at early time points after stimulation with LPS at lethal dose 50 (LD50, 50mg/kg in our experimental setting) and subsequent ELISA quantification of TNFα, a mayor mediator of septic shock, displayed greatly reduced levels in KO animals (fig.3C). Taken together these results suggest that the lack of miR-125a is impairing TLR4-dependent TNFα production, without any effects on survival rate. These results are in line with the observation that experimental anti-TNFα treatments in sepsis alone are not sufficient to increase survival [142,143].

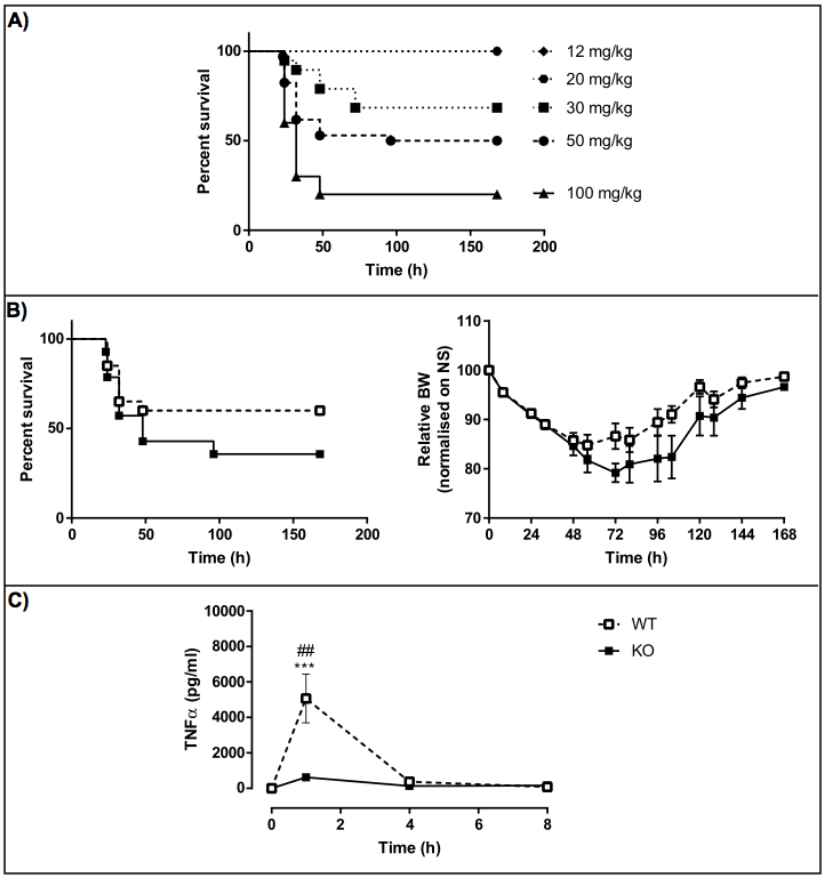

Figure.3 (A) WT mice exposed to increasing doses of LP show lethal dose 50 (LD50) at 50 mg/kg. (B) Surivival and BW loss in WT and KO mice esposed to LPS at LD50. (C) TNFα levels in plasma of mice exposed to LPS at LD50. Two-way ANOVA displayed significant interaction between factors. Therefore, genotype-restricted one-way ANOVA were performed, followed by post-hoc Dunnet's test for significant expression over time (WT: *** p<0.001). Differences in the expression between WT and KO were determined by T-test between condition for each time point (## p<0.01)

Septic patients that survived early phases of SIRS can still develop CARS [144]. The Immunosuppression observed in CARS may favour pathogen super-infection leading to patient death at later time points. miR-125a can be considered as a good candidate for mediating the immunosuppressing phase in sepsis. Therefore, we investigated the role of miR-125a in Endotoxin Tolerance, a suitable experimental model for CARS [145]. ET is a condition characterised by macrophages hypo-responsiveness to noxious stimuli upon repetitive challenge. To evaluate a possible involvement of miR-125a in ET, WT and KO animals were primed with an IP injection of low dose of LPS (5mg/kg), followed by a LPS bolus at higher dose (50mg/kg). Blood samples were collected at different time points after second exposure and plasma levels of TNFα were assessed by ELISA (fig.4). As expected, repetitive stimulation with LPS decreased circulating TNFα in WT animals. On the contrary, but consistently with previous results, KO animals displayed reduced TNFα levels in plasma already after first stimulation but did not display any degree of tolerization.

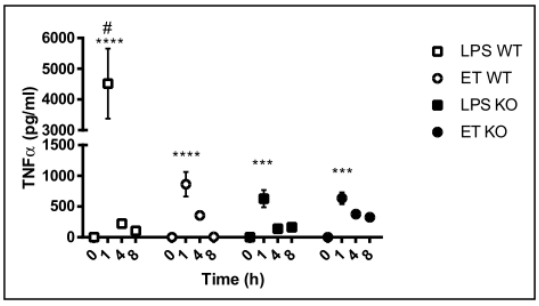

Figure.4 Plasmatic levels of TNFα in response to single (squares) or repeated (circles) IP stimulations with LPS in WT (white symbols) or miR-125a KO (black symbols). Two way ANOVA displayed significant interaction between factors. One-way ANOVA evaluated single treatment effect over time relative to T0 (*** p<0.001, **** p<0.0001). One-way ANOVA compared different conditions at every time point (# p<0.05)

Alongside with systemic administration, we also tested repeated LPS stimuli in vitro on BMdM (fig.5). In these cells, repetitive stimulation with LPS led to reduced level of secreted TNFα but at the same extent both in WT and KO cells (fig.5A). In WT BMdM, ET induction correlates with increased levels of miR-125a , whereas KO cells displayed significantly lower miRNA expression in all conditions (fig.5B). FACS analysis showed that LPS tolerant macrophages and, to a lesser extent, LPS stimulated cells, increased the expression of analysed markers (fig.5C). The observed increase was generally more pronounced in the absence of miR-125, although this common trend reached statistical significance only for CD14 that notably is a direct target of this miRNA.

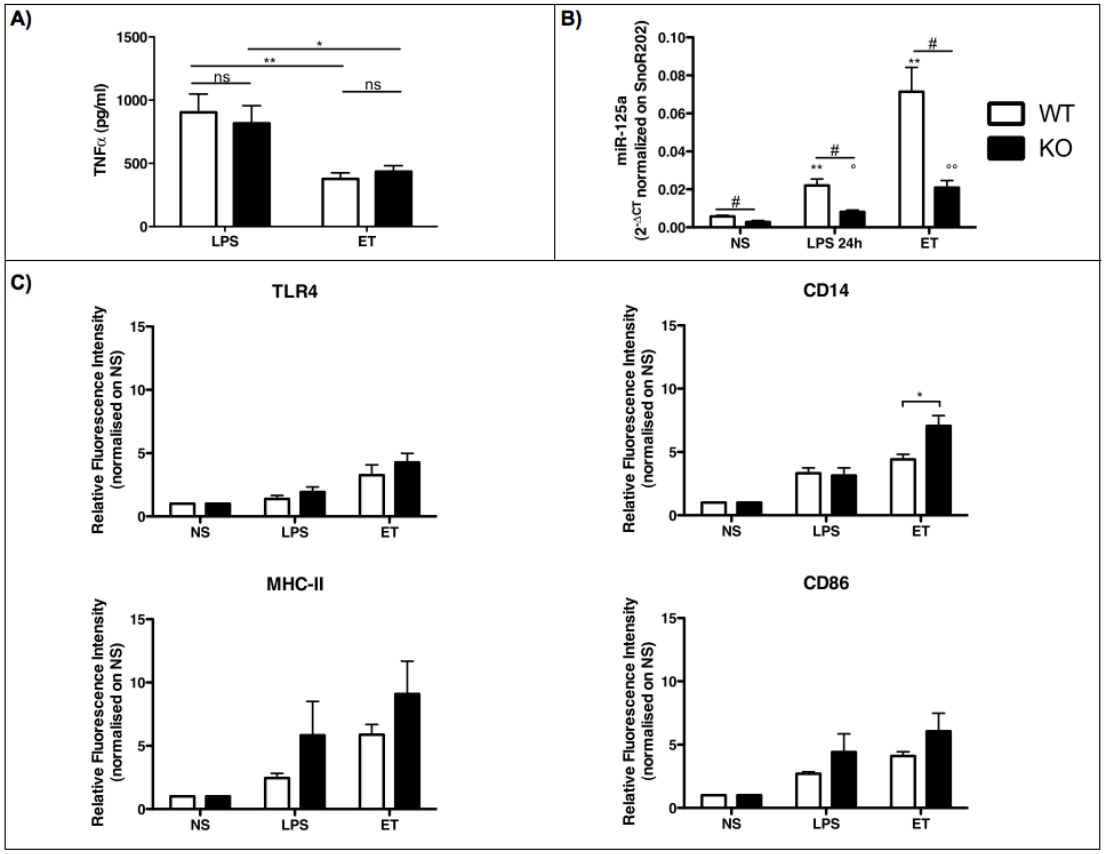

Figure.5 (A) TNFα production by LPS treated or tolerized (ET) BMdM from WT (white bars) or KO (black bars) animals. T-test was performed between conditions (* p<0.05). (B) miR-125a expression in WT or KO BMdM exposed to a single dose of LPS or tolerized. Two-way ANOVA displayed significant interaction between factors. Therefore, genotype-restricted one-way ANOVA were performed, followed by post-hoc Bonferroni's test for significant expression over time (WT: ** p<0.01, *** p<0.001; ° p<0.05, °° p<0.01). Differences in the expression between WT and KO were determined by T-test between condition for each time point (# p<0.05). (C) Surface expression of inflammatory markers in treated (LPS), tolerized (ET) or untreated (NS) BMdM from WT (white bars) or KO (black bars) mice. T-test compared genotype effect across conditions (* p<0.05)

An interesting set of observation links miR-125a expression to TLR pathway activation with a late kinetics [104,146], suggesting that miR-125a is involved in late inflammatory responses. Moreover, miR-125a is enhanced in the presence of IL10, a master regulator of resolving phases of inflammation. A third important observation is that miR-125a directly targets molecules important for signal transduction via the TLR pathways. Thus the emerging evidences suggest that this microRNA may be an important effector of IL10 anti-inflammatory properties, counteracting inflammation by reducing availability of important inflammatory signal transducers. To dissect in vivo the involvement of miR-125a in resolution of inflammation *in vivo* we adopted a model of self-resolving inflammation [56]. We injected IP Zymosan, an inactive yeast lysate, and we monitored cellular dynamics in peritoneal cavity, blood circulation and bone marrow over time

(Fig.6). 6 hours after stimulus peritoneal macrophages were reduced and were replaced by neutrophils and, to a lesser extent by eosinophils. Over time, the frequencies of granulocytes in the peritoneum were reduced whereas the frequencies of recruited monocytes and newly differentiated macrophages were increased (Fig.6A). In the blood, neutrophils and monocytes peaked at 6 hours after Zymosan injection and decreased over time, probably due to recruitment in the peritoneum. Instead, after an initial decrease, eosinophil levels in the blood remained constant (fig.6B). Neutrophil, eosinophil and monocyte frequencies in the bone marrow were reduced after 6 hours upon Zymosan treatment. It suggests an egress of these cells from the bone marrow toward the blood stream. Initial depletion of granulocytes and monocytes in the bone marrow was followed by a reactive increased production of these cells, as expected during inflammatory response (Fig.6C). Although cellular frequencies were in line with an ongoing local inflammatory status prone to resolution, no differences could be observed between WT and KO mice.

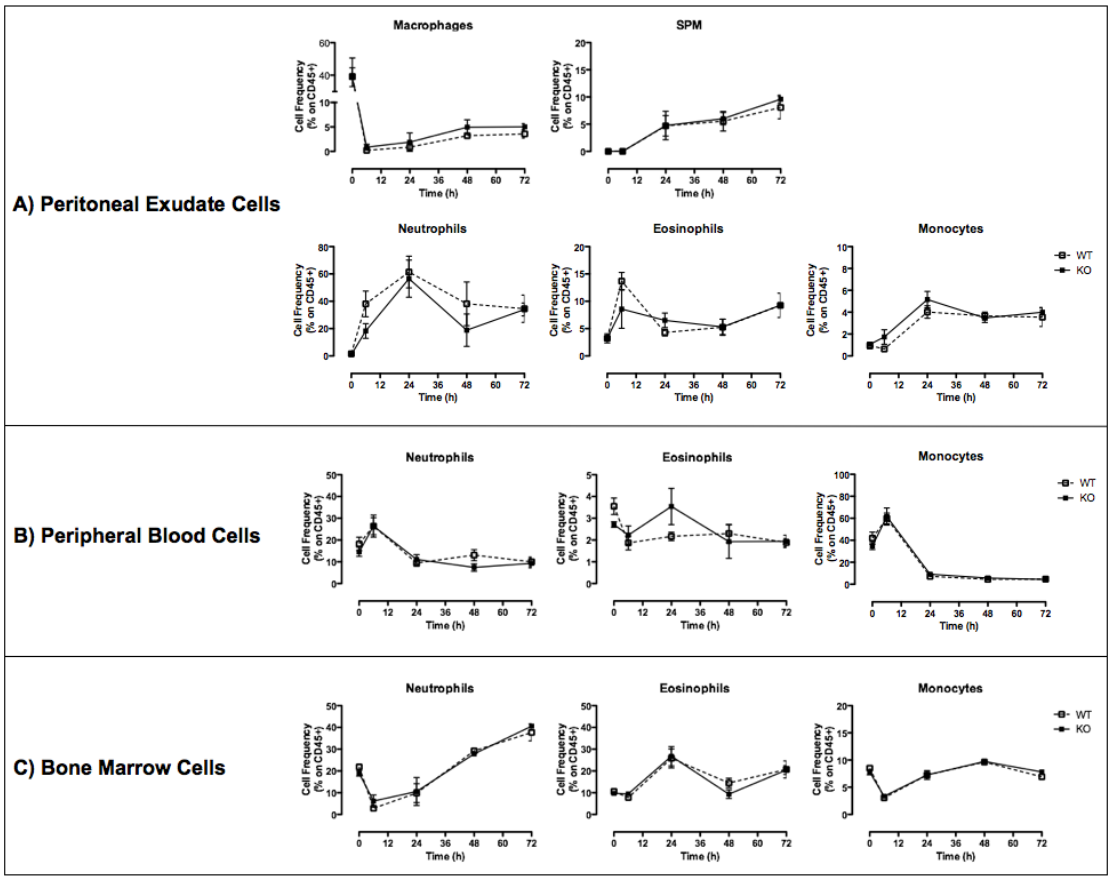

Figure.6 Innate immune cell popuations were analyzed in (A) peritoneal cavity, (B) peripheral blood and (C) bone marrow compartments of WT (white squares) and miR-125a KO (black squares) at indicated time points after IP injection of 1mg of Zymosan.

MiR-125a appears to act as molecular mediator of IL10 anti-inflammatory effect. Therefore miR-125a depletion may resemble phenotypes observed in IL10 KO in experimental colitis [62,147]. We applied a well-established protocol for *in vivo* development of chronic colonic inflammation [148]. WT or KO mice were exposed to water-dissolved DSS, a chemical reagent that induces inflammation in colonic mucosa, for one week and then they were allowed to recover for two weeks. This stimulus-recovery cycle was repeated three times and progression of disease was periodically monitored evaluating both body weight and disease activity index. The disease activity index is an arbitrary score that takes into account pathological signs like body weight loss, stool consistency and presence of faecal blood (Fig.7). Each DSS administration was associated to a sharp increase of the disease activity (Fig.7A) accompanied by BW loss (Fig.7B). At the end of last cycle, animals were sacrificed and the colon was isolated for further analysis. Post mortem evaluation of colon length showed no differences between the groups (Fig.7C). Although no differences were detected in any of the analysed parameters, macrophages isolated from lamina propria indeed displayed reduced miR-125a levels in KO mice (Fig.7D) confirming that miR-125a depletion in macrophages do not increase DSS sensitivity.

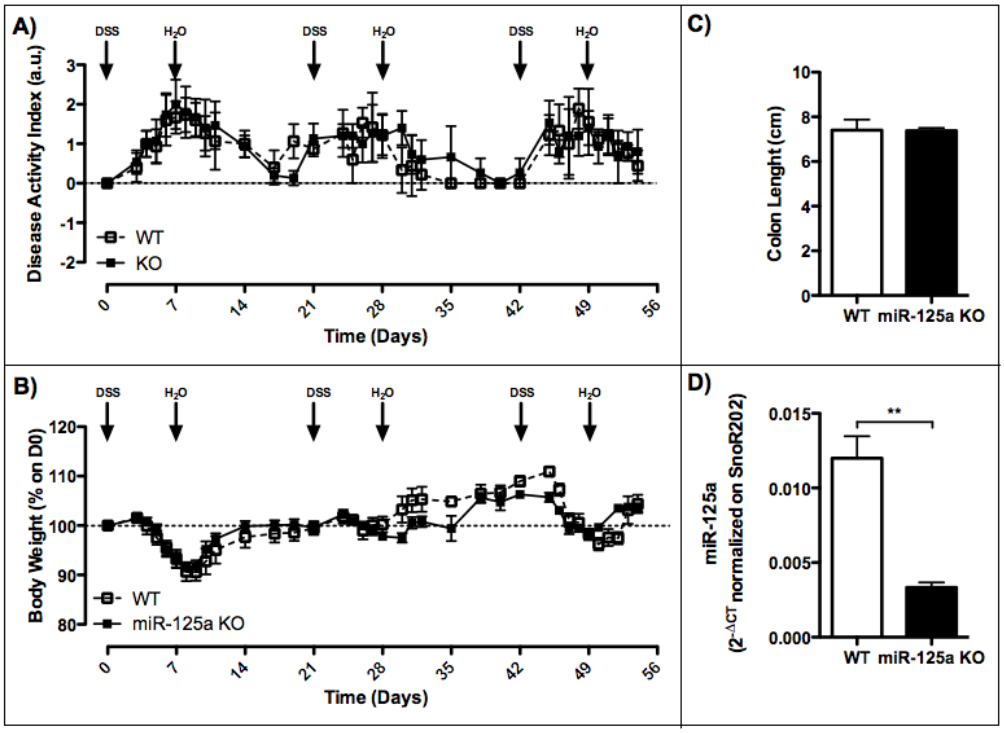

Figure.7 WT (white symbols) and miR-125a KO (black symbols) repetitively exposed to DSS monitored for DAI (A) and BW loss (B). End point evaluation of colon length (C) and miR-125a expression in lamina propria macophages (D). T-test compared genotype effect across conditions (** p<0.01)

The role of miR-125a in macrophages is still under debate [107,108]. Banerjee and colleagues showed that ectopic expression of miR-125a in macrophages leads to increased phagocytosis of thymocytes, that is considered as a feature typical of M2 macrophages, counterbalanced by a reduced microbicidal activity, that is considered an M1 feature [108]. Consistently, the artificial blockade of miR-125a leads to reduced phagocytosis (no data on the microbicidal activity in the same context were shown). Conversely, Zhao and colleagues showed that increased miR-125a levels correlate to increased phagocytosis of tumoral cells and with enhanced T-cell activation (both considered as M1 features) [107]. To determine the role of mir125a in macrophages, we evaluated the phagocytosis of living E.Coli by WT or mir125a KO macrophages (Fig.8). WT and KO BMdM were challenged with CSFE-labelled E.Coli for different time points. The macrophage supernatant containing spared bacteria was removed, serially diluted and cultured over night (ON). On the other hand, treated macrophages were analysed by FACS for F4/80 expression (that reveal their differentiation state) and for acquired CFSE positivity, that is proportional to the number of bacteria engulfed. Almost all macrophages were expressing F4/80 at high levels (Fig.8A, panel3) and

almost 100% of them were also CFSE positive (Fig.8A, panel4). It indicates that the phagocytosis assay efficiently worked. Moreover, monitoring mean fluorescent intensity for CSFE in macrophages, the phagocytic index increased over time in both WT and KO macrophages but the lack of mir125a did not affect the phagocytic index of KO macrophages. Indeed, the phagocytic ability of KO macrophages is comparable to the one of WT macrophages (Fig.8B). Nevertheless, the supernatant of KO macrophages generated higher number of colonies compared to the supernatant of WT macrophages, indicating higher bacterial survival. It appears that although miR-125a is not playing a role in the ability of macrophages to phagocyte bacteria, macrophages lacking mir125a may be defective in extracellular killing mechanisms (Fig.8C).

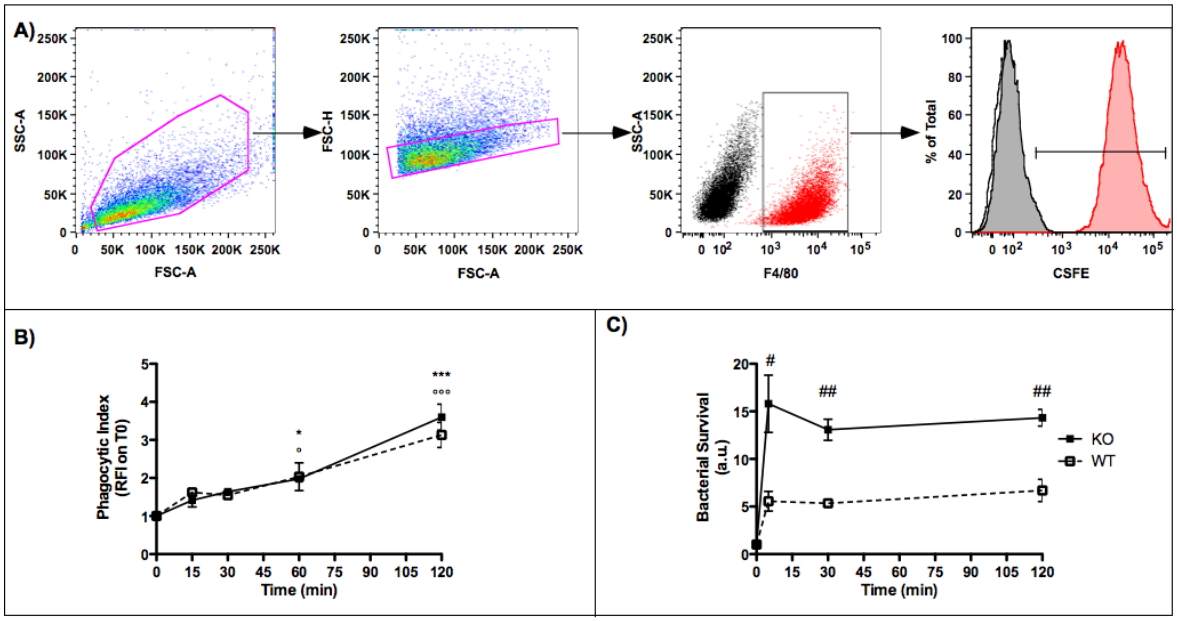

Figure.8 Phagocytic capacity of WT or KO miR-125a exposed to stained bacteria. (A) Gating strategy defining positive phagocytic events. Black dots indicate unstained samples, black histogram indicate macrophages exposed to unstained bacteria, red histogram indicate phagocytitc macrophages. (B) Extent of phagocytosis in WT (white symbols) and miR-125a KO (black symbols) over 2 hours. Two-way ANOVA displayed significant interaction between factors. Therefore, genotype-restricted one-way ANOVA were performed, followed by post-hoc Bonferroni's test (WT: * $p<0.05$, *** $p<0.001$; ° $p<0.05$, °°° $p<0.001$). (C) Colony formation assay of bacteria spared from phagocytosis. T-test compared genotype effect across conditions (# $p<0.05$, ##$p<0.01$)

MicroRNA-9 and inter-species variability

Another microRNA that may play a role in macrophages during inflammation is miR-9 [95]. To determine miR-9 contribution to inflammatory process, we developed a strain of mice deficient in microRNA-9.1 gene exclusively in myelomonocytic lineage. The breeding strategy was similar to the one employed to generate KO mice for miR-125a in macrophages in order to have miR-9$^{flox/flox}$/LyzM-CRE$^{-/-}$ (WT) and miR-9$^{flox/flox}$/LyzM-CRE$^{-/+}$ (KO) animals. An equal ratios between WT and KO littermates was observed, ruling out that miR-9 deletion results in a lethal phenotype (Fig.9A). Effective deletion of mir-9 in KO BMdM (as compared to WT) was assessed (Fig.9B) by PCR amplification of genomic DNA using primer specific for either the floxed allele (*) or the deleted one (#). As expected, primers specific for the deleted allele produced a detectable band only in KO animals. To further confirm efficient recombination, we evaluated allelic abundance by real time PCR on genomic DNA with different sets of primers specific for each allele (Fig.9C). We observed that floxed allele was significantly decreased in BMdM from KO animals as compared to WT. Coherently KO macrophages also displayed increased levels in deleted allele, although not statistically significant. Sensitivity of this assay was hampered by high background signal during deleted allele quantification in WT animals. In fact, deletion-specific primers still find complementary regions in the floxed allele, although at a way too great distance to be properly amplified by real time PCR, resulting in nonspecific amplification. Collectively, these results show effective deletion of miR-9.1 gene in target cell population.

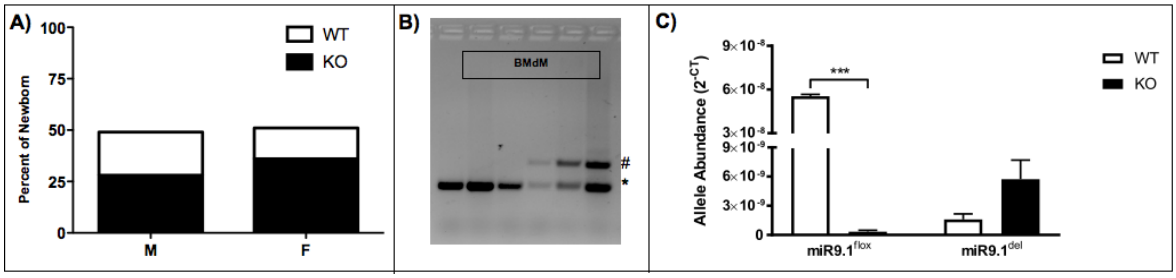

Figure.9 (A) Newborn from mice deficient for miR-9 selectively in macrophages were monitored for sex and genotype at wheening. (B) Selective genomic recombination was confirmed in macrophages at genomic level. (C) Efficient recombination was confirmed in macrophages by quantitative PCR. T-test compared genotype effect across conditions (*** p<0.001)

Several inflammatory stimuli have been shown to induce miR-9 expression in macrophages. Consistently with previous reports, we found that miR-9 steadily

increased over 24 hours following stimulation with LPS and, to a lesser extent, IFNγ (Fig.10A-B). Combined treatment with both LPS and IFNγ further increased miR-9 expression (Fig.10C). Conversely, no effect could be observed for TNFα treatment (Fig.10D). Although our data are in line with previous evidences [95] and we observed an efficient deletion of mir9 in our conditional murine model, we didn't observe significant differences between WT and KO mice.

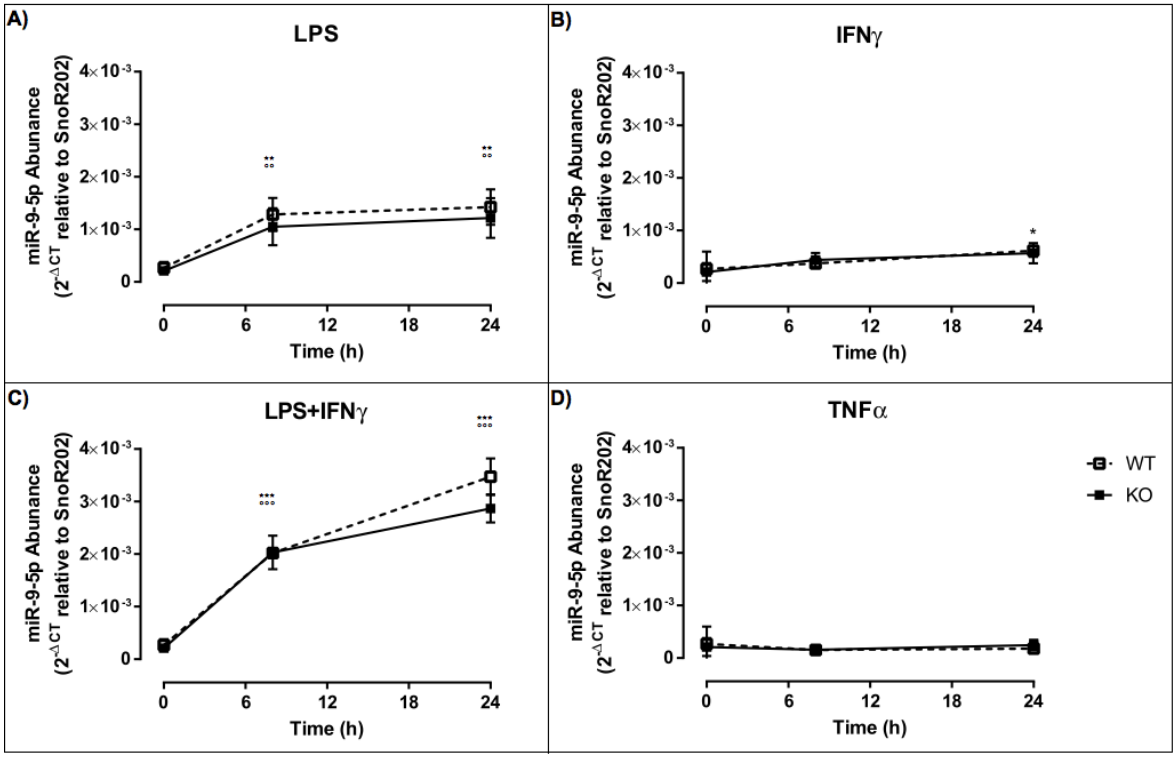

Figure.10 MiR-9 expression monitored in WT (white symbols) or miR-9 KO (black symbols) BMdM after pro-inflammatory stimulation with (A) LPS, (B) IFNγ, (C) LPS+IFNγ and (D) TNFα. Two-way anova with post-hoc Sidak's test (WT: ** p<0.01, *** p<0.001; KO °° p<0.01, °°° p<0.001)

Trying to justify this unexpected observation we treated BMdM with a combination of LPS and IFNγ for 24 hours (Fig.11). First, we confirmed the significant induction of mature form of miR-9 already 6 hours after stimulation (Fig.11A). Then, We focused on the three different isoforms of miR-9 precursors. We observed that the increase in mature form of miR-9 was not only driven by pri-miR-9.1 (as already reported in humans [95]), but also by pri-miR-9.3 (Fig.11B). It suggests that miR-9 biology is not entirely conserved between mouse and human, excluding any further use of this animal model, at least in the immediate.

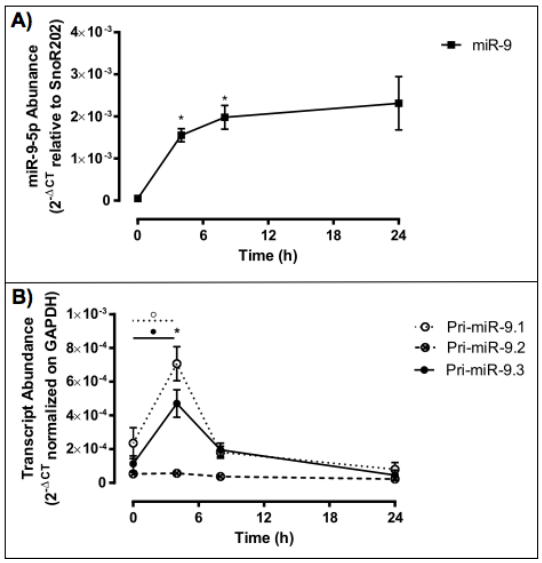

Figure.11 Kinetics of mature form (A) and pri-miR-9 isoforms (B) in WT BMdM exposed to LPS+IFNγ. One-way ANOVA followed by post-hoc Dunnet's test for significant expression over time (miR-9: * $p<0.05$; pri-miR-9.1: ° $p<0.05$; pri-miR-9.3: $p<0.05$

MicroRNA-135b and BLACAT1: waltz on a DEX tune

MiR-135b is a microRNA largely studied in oncological contexts but little is known about his function in immune system. Surprisingly, this microRNA stood out as the most enriched miRNAs in M1 macrophages in our TLDA experiments (Fig.12A), suggesting that its expression may contribute to regulate the dramatic changes that these cell experience upon activation. In order to better define mir135b role in macrophage biology, we evaluated mir135b expression in different human polarized macrophages and we observed that LPS alone was sufficient to induce miR-135b levels to the same extent of the combined treatment, as already observed for other pro-inflammatory miRNA such as miR-155 (Fig.12B). On the contrary, M2-typical stimuli did not induce neither miR-135b nor miR-155 (Fig.12C-D).

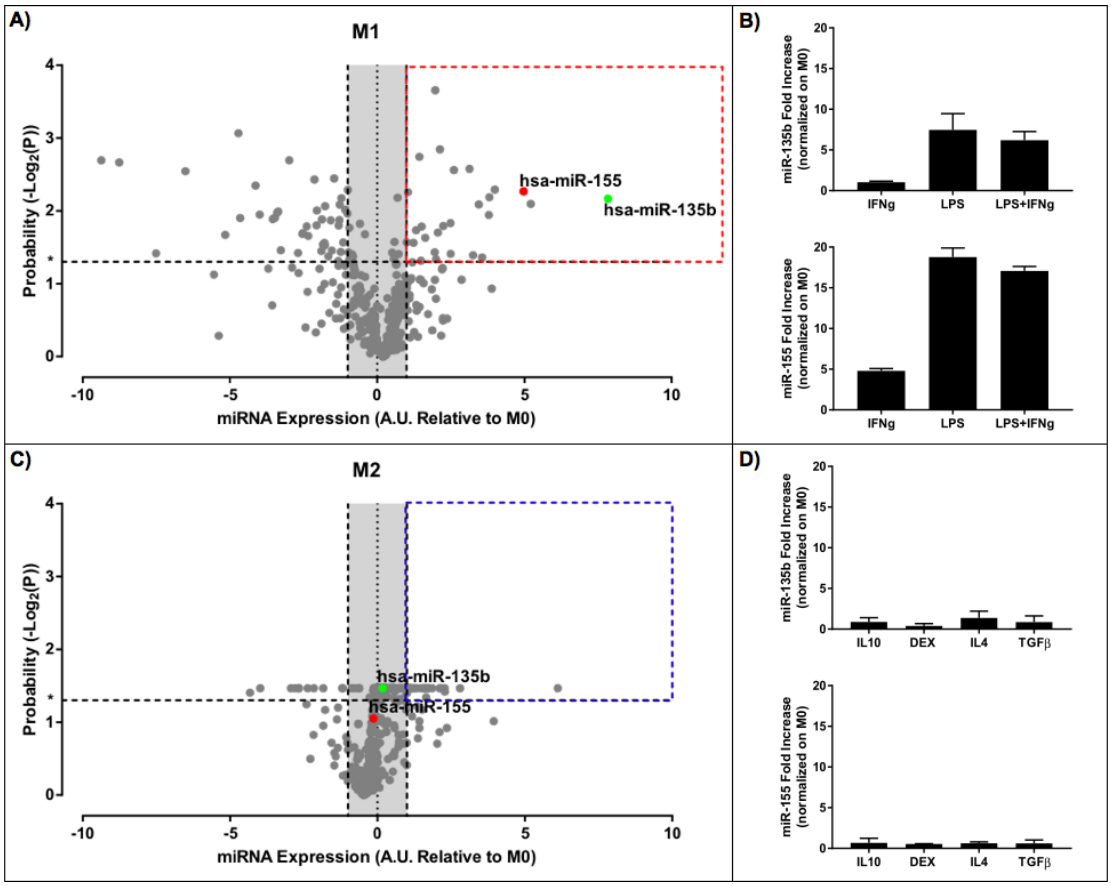

Figure.12 TLDA miRNA profiling from human M1 (A) or M2 (C) macrophages. Volcano plots showing fold change on M0 vs negative logaritm of p-values. Horizontal dashed line indicate threeshold p-value=0.05. Vertical dashed line represent cutoff of fold increase=2. Hsa-miR-155 (red dot) and hsa-miR-135b (green dot) are highly expressed following pro-inflammatory stimuli (B) but not following anti-inflammatory stimuli (D)

Interestingly, when we monitored the levels of the mature form and the precursor of miR135b following stimulation, we observed that LPS stimulation resulted in similar kinetics for both miR-155 and miR-135b. In fact, macrophages responded to LPS with induction of both pri-miRNAs already 3 hours post stimulation. Pri-miRNAs then decreased over time, converted into mature miRNAs that accumulated in the cytoplasm over 24 hours (Fig.13A). Unexpectedly, the stimulation of human macrophages with the synthetic glucocorticoid Dexamethasone (DEX) impacts differently on the expression levels of the two genes. Indeed, DEX mediated inhibition of miR-155 occurred already at pri-miRNA level, suggesting a conventional transcriptional silencing. Conversely, DEX treatment induced the up-regulation of pri-miR-135b expression, suggesting that the observed inhibition of the mature form occurs through an unconventional, post-transcriptional mechanism. (Fig.13B)

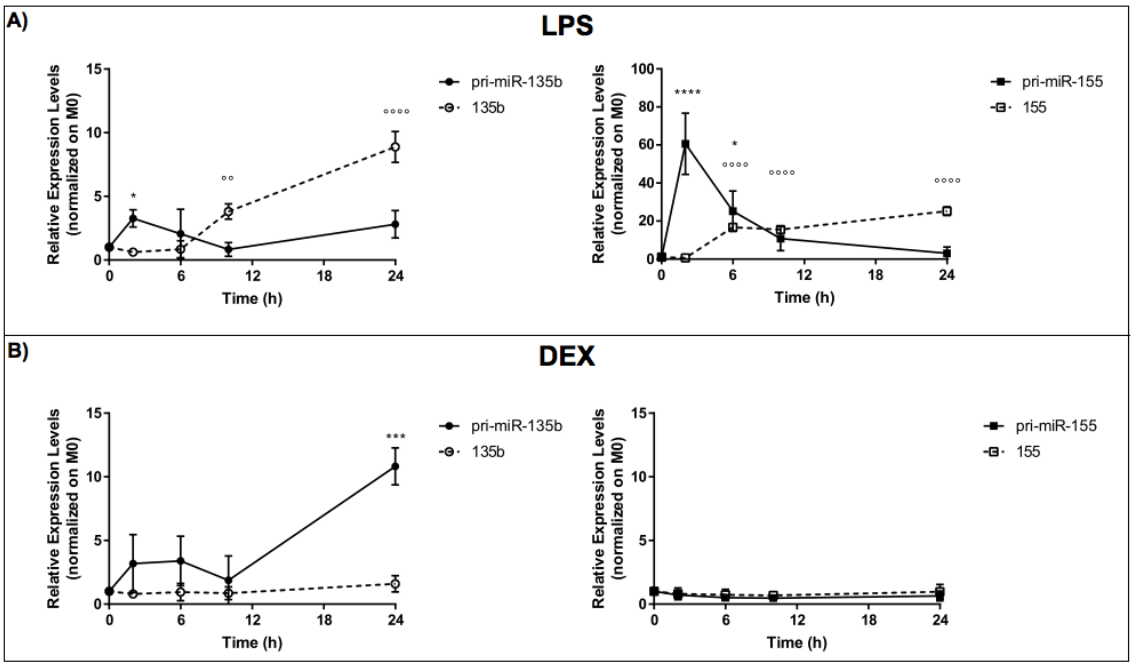

Figure.13 Mature form (white symbols) and pri-miRNA (black symbols) kinetics for miR-135b (left panels, circles) and miR-155 (right panels, squares) in human macrophages exposed to LPS (A) or dexamethasone (B). One-way ANOVA followed by post-hoc Dunnet's test for significant expression over time (pri-miRNAs: * p<0.05, *** p<0.001, **** p<0.0001; mature-miRNAs: °° p<0.01, °°°° p<0.0001)

To better understand the DEX effect on miR-135b maturation, we analysed the genomic region of the mapped miR-135b gene and we found that a recently annotated lncRNA (BLACAT1) was mapping in close proximity (<20 kb, Fig.14A). Since lncRNA have

been reported to regulate expression and maturation of proximally produced transcript, we evaluated BLACAT1 expression after stimulation of human resting Macrophages with DEX or LPS. If LPS had minor or no effects on BLACAT1 expression, DEX induced BLACAT1 production over 72 hours, resembling the pri-miR-135b kinetics we observed before (Fig.14B-C). Interestingly, BLACAT1 expression levels at 24 hours correlates with pri-miR-135b abundance (r^2=0.9597, Fig.14D), suggesting that these molecules are not only linked but they may actually be part of a single transcript. This observation is supported by the fact that both these genes map in the same intron and therefore may be expressed by the same host gene (LEMD1, Fig.12A). On the other side, the annotated distribution of chromatin accessibility markers (H3K27Ac, Fig.12A) and Polymerase II binding sites (POLR2A, Fig.12A) in this genomic region is compatible with the idea that these genes are regulated by independent promoters, although much experimental work is needed to ascertain which of the two models is real.

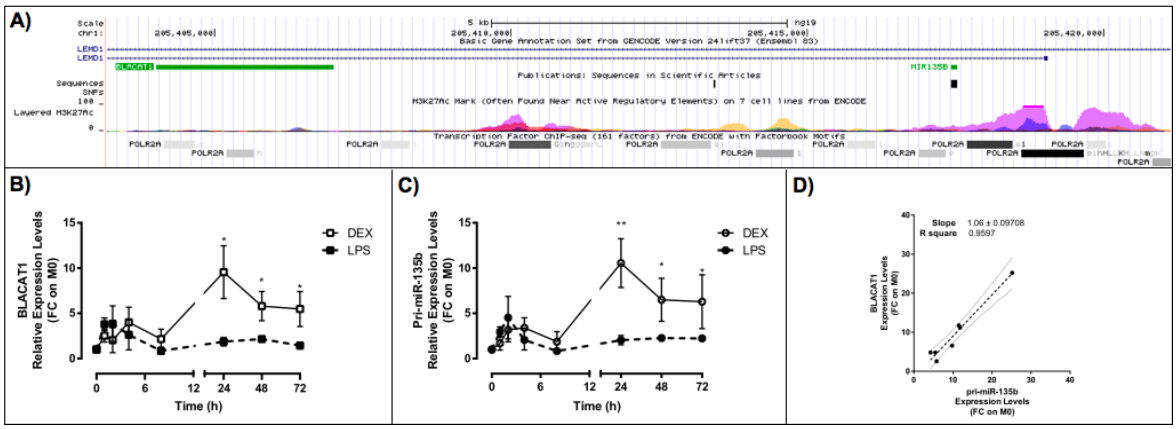

Figure.14 (A) Close genomic proximity between miR-135b and BLACAT1 genes. (B) BLACAT1 expression kinetics in human macrophages following stimulation with LPS or dexamethasone. (C) Pri-miR-135b expression kinetics in human macrophages following stimulation with LPS or dexamethasone. (D) Strict correlation between pri-miR-135b and BLACAT1 expression levels after 24h DEX treatmet (Slope= 1.06; Rsquare=0.9597)

A recent report demonstrated that a lncRNA can interfere with pri-miRNA processing [136]. It was shown that a direct interaction of 11 nucleotides between a lncRNA (Uc.283+A) and pri-miR-195 could take place in close proximity to the Drosha binding site, preventing pri-miRNA processing by that enzyme. This mechanism might justify our observation that DEX treatment on macrophages induces pri-miR-135b accumulation alongside BLACAT1 induction. In silico prediction of RNA-RNA interaction highlighted a high complementarity region between BLACAT1 and Drosha

binding site on pri-miR-135b (Fig.15A). Surprisingly, homology between BLACAT1 and pri-miR-135b was not restricted to the Drosha binding side, instead it appeared to spread across a wider region of the lncRNA, involving pri-miR-135b in almost its full length (Fig.15B) and forming a duplex characterised by a free energy (-85.0 kcal/mol) lower than the one predicted for pri-miR-135b hairpin structure (-65.0 kcal/mol). If this interaction effectively occurs, it may lead to destruction of the stem-loop structure necessary for Drosha binding. That being the case, overexpression of BLACAT1 may lead to pri-miR-135b accumulation by preventing Drosha binding through a previously undescribed mechanism.

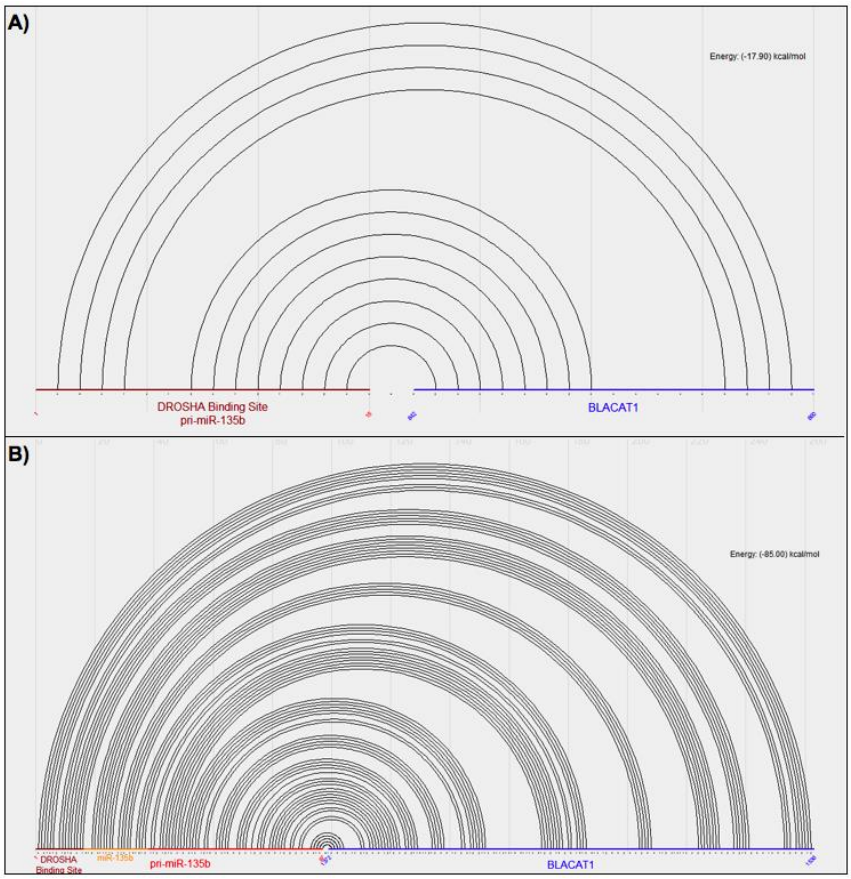

Figure.15 In silico prediction of BLACAT1/pri-miR-135b interactions. Each point on the x-axis stands for a nucleotide; arcs indicate predicted interactions between nucleotides. (A) DROSHA binding site on pri-miR-135b (dark red) shows high similiararity (12/16 nucleotide match) with a region comprised between nucleotide 842-860 of the BLACAT1 transcript (blue). (B) High homology is predicted between the whole pri-miR-135b hairpin structure (red) and nucleotides 1372-1536 of BLACAT1 (blue); DROSHA binding site and mature miRNA sequence are shown (dark red and orange, respectively.

To demonstrate that BLACAT1 induction by DEX treatment correlates with pri-miRNA accumulation by inhibition of conversion to pre-miRNA, human macrophages were transfected with LNA-GAPmeRs targeting BLACAT1 and subsequently stimulated with DEX (Fig.16). As expected, BLACAT1-specific GAPmeRs were effective in

diminishing BLACAT1 levels upon DEX stimulation (Fig.16A). The reduction of BLACAT1 levels was associated with a decrease of the pri-miR-135b abundance (Fig.16B) and it was consistent with the release of the mechanism converting pri-miR-153b to pre-miR-135b. According with that, miR-135b was more expressed in treated macrophages upon BLACAT1 inhibition (Fig.16C). Importantly KLF4, that is one of the most important targets for miR-135b in macrophages, was reduced (Fig.16D-E), indirectly confirming the increase of miR-135b levels. Altogether these results identify BLACAT1 as a previously unrecognised lncRNA mediating anti-inflammatory effects of glucocorticoids. Moreover we propose interference with pri-miR-135b processing as a general mechanism of action for this lncRNA in macrophages. However, the molecular interactions that lead to the observed results need further detailed studies.

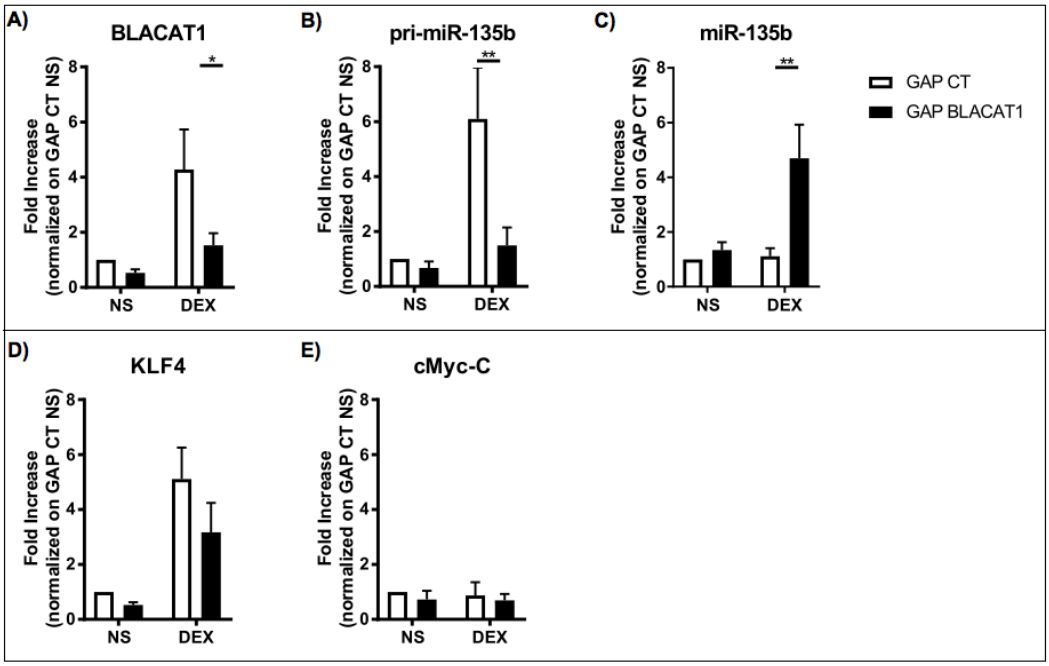

Figure.16 BLACAT1-specific GAPmeRs efficiently inhibit DEX induced BLACAT1 expression (B). BLACAT1 inhibition results in reduced pri-miR-135b levels (B) but increased mature form miR-135b (C). Increased expression of miR-135b interferes with KLF4 (D) but not cMyc-C (E) transcript abundance. Two-way ANOVA followed by post-hoc Bonferroni's test evaluate GAPmeR effect in treated samples (* $p<0.05$, ** $p<0.01$)

Discussion

In the last years the complexity of the mononuclear phagocytic system has been progressively unveiled [9]. Macrophage biology appeared to be far more complex than initially thought. Plasticity is now accepted as a fundamental feature of macrophages. Through plasticity, macrophages can adapt their behaviour to different phases of a pathologic state, allowing them to coordinate adequate responses [13,149,150]. Macrophage plasticity relies on modifications in the epigenetic program. MicroRNAs and lncRNAs, thanks to their pleiotropic effect are involved in the regulation of complex cellular processes and therefore could participate in the regulation of macrophage plasticity. We and others identified miR-125a as an important miRNA that negatively regulate TLR4 signaling pathway and therefore macrophage responses, favouring the resolution of inflammation [108]. Here we described the generation of an animal model in which miR-125a is selectively depleted in macrophages. Recent reports suggest that miR-125a may play important roles in haematopoiesis and myeloid differentiation sustaining the neutrophil fate [151]. We found that miR-125a depletion did not alter frequencies of circulating mature immune cells, suggesting that LYZM expression, with concomitant miR deletion, occur after the critical period for miR-125a action on haematopoiesis. Genetic ablation of miR-125a in monocyte/macrophages had no effect on survival during SIRS even if mir125a resulted in decreased secretion of TNFα. Reduced TNFα levels in miR-125a KO mice as compared to WT were observed also during CARS response. A positive effect for miR-125a on TNFα release and the expression of pro-inflammatory markers has already been reported [106,107]. These results are in partial conflict with our previous observation and data available in the literature [108]. Nevertheless, the debate is still open and up to date the only explanation for that is the use of different experimental settings. Moreover, we found that depletion of miR-125a in macrophages did not alter the clinical course neither of acute peritoneal inflammation nor of chronic colitis. Although they displayed reduced killing capacity, macrophages deficient for miR-125a maintained phagocytic abilities when challenged with bacteria. This result is consistent with increased iNOS expression in miR-125a transfected macrophages as reported by Zhao and collaborators, but exacerbates the conflict with previous data from Banerjee and collaborators [107,108]. Overall we were not able to identify a clear phenotype in macrophages deficient for miR-125a. Rather than excluding possible roles for this miRNA in inflammatory processes, it suggests that a sort of compensatory mechanism takes place. This compensatory mechanism may

involve the production of miR-125a by other cell types other than macrophages. In fact, many cells, also not belonging to the immune system, are sensible to pro-inflammatory stimuli like LPS and IFNγ [152]. Since in our model miR-125a deletion is restricted to macrophages, these other cells may still be capable of producing miR-125a. Moreover, it has been demonstrated that miR-125a could be transferred from a cell type to another through the secretion of microvescicles [153]. If a similar mechanism would occur during inflammation, macrophages may receive miR-125a containing vescicles from surrounding cells. Although interesting, this model is unlikely to be responsible for our results. Indeed, we didn't see any differences between WT and KO in vitro differentiated macrophages, in the absence of any surrounding cell, and when miR-125a was quantified in macrophages its expression levels were always reduced in KO cells. Another possibility is that, since the efficiency of the deletion never reached 100%, residual expression of miR-125a in KO macrophages is sufficient to mediate its physiological functions. A miR-125a full KO model may help to demonstrate this hypothesis. Nevertheless, under inflammatory conditions miR-125a levels drastically increased, suggesting that high levels of miR-125a are necessary to exert its functions. Therefore, the miR-125a left over in KO macrophages can unlikely be sufficient to restore its functions. Finally, miR-125a functions may be compensated by other miRNAs. Indeed, it has been shown that a cluster of miRNAs can be induced upon pro-inflammatory stimulation and it negatively regulates inflammation related pathways [69,154]. This hypothesis is intriguing but requires additional efforts to be properly addressed, due to several technical problems.

Our studies on miR-9 were hampered by the unexpected discovery that, differently from human, murine macrophages increased the expression of miR-9 by inducing transcription of two different pri-miRNA gene isoforms. This result may be the consequence of poor conservation in the regulatory regions between these two species. This striking difference between two mammals enlightens how important the phylogenetic distance is and how experimental results valid for one of the two models needs to be carefully proven in the other.

The last part of our work focused on the possible interaction between the lncRNA BLACAT1 and the pri-miR-135b. We found that in macrophages pro-inflammatory stimuli led to increased expression of pri-miR-135b with subsequent conversion into the mature form miR-135b. On the contrary, anti-inflammatory stimuli like dexamethasone induced the expression of pri-miR-135b even if it was not converted to its mature form. Furthermore, dexamethasone treatment induced the expression of the proximal lncRNA

BLACAT1. A clear linear correlation was observed between pri-miR-135b and BLACAT1 expression levels, raising the possibility that these two elements are transcribed as a single molecule. Analysis of the genomic region supports this possibility. In fact the lncRNA BLACAT1 and the pri-miR-135b map very close together and are nested in the intron of a gene coding for an orphan protein. Although very interesting, further experiments are needed to fully prove that lncRNA BLACAT1 and the pri-miR-135b are transcribed as a single molecule. If this hypothesis will be proven, it will shed new light on the regulation of pro and anti-inflammatory responses in macrophages. Indeed, the pro-inflammatory signals may induce a weak activation of the promoter, resulting in shorter stretches of transcript that include the pri-miRNA alone, whereas the anti-inflammatory signals may result in a more stable transcription favoring the formation of a longer transcript which functions are still unknown. In silico predictions suggested a possible interaction between BLACAT1 and pri-miR-135b in a region that has been identified as crucial for drosha binding and thus for the processing of the pri-miRNA [136]. At the same time, the in silico data predicted a second possible interaction that involves the pri-miR-135b with almost all its length. Both predictions may explain the accumulation of pri-miR-135b observed in the presence of BLACAT1. Nevertheless, both predictions are not sufficient to demonstrate whether the lncRNA BLACAT1 and the pri-miR-135b are two distinct molecules or are a single polyfunctional lncRNA. BLACAT1 cleavage mediated by GAPmeRs resulted in decreased amount of pri-miR-135b and a significant increase of the mature form miR-13b. These results suggest that indeed BLACAT1 exerts a block on pri-miR135b maturation and that it can be released by targeting BLACAT1. If these elements are part of the same transcript and the block by BLACAT1 is operated through extensive pairing, the cleavage of one of the two elements may allow refolding of the second and thus its maturation. If BLACAT1 is a separated molecule and inhibits pri-miRNA processing by specific binding to a small region, its cleavage could lead to the release of this blocking mechanism. Considering our current knowledge, both models can be reliable. Therefore, further experiments are needed to demonstrate the separate or the common identity of the lncRNA BLACAT1 and the pri-miR-135b and the type and extent of their interactions. By addressing these questions, new light can be shed not only on the regulation of miRNA expression and lncRNA functions, but also on unknown features of macrophage biology regulated by epigenetic elements.

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Supplementary Material

LPS (100ng/ml)	Raw CT						Summary				T-Test
Time (h)	KO1	KO2	KO3	WT1	WT2	WT3	KO Avg	KO SD	WT Avg	WT SD	P Value
0	29.05	28.98	29.71	26.51	27.66	27.77	29.25	0.33	27.32	0.57	0.022
1	29.66	28.49	29.59	27.18	27.68	27.32	29.25	0.54	27.39	0.21	0.027
4	29.22	28.13	30.86	26.78	27.85	26.43	29.40	1.12	27.02	0.60	0.075
8	29.17	28.22	29.41	25.74	26.21	26.57	28.93	0.51	26.17	0.34	0.005
24	27.16	26.41	28.35	24.41	23.96	24.85	27.31	0.80	24.41	0.36	0.022
48	27.98	26.55	28.51	24.81	24.71	24.51	27.68	0.83	24.68	0.12	0.033

Supplementary Table.1 Raw data for miR-125a response to LPS stimulation in KO and WT BMdM. Two-tails unpaired T-test highlights significant increase in CT values in miR-125a KO cells (i.e reduced availability of transcript).