UNIVERSITY OF VERONA

DEPARTMENT OF MEDICINE, SECTION OF GENERAL PATHOLOGY

GRADUATE SCHOOL OF LIFE AND HEALTH SCIENCES

DOCTORAL PROGRAM IN INFLAMMATION, IMMUNITY AND CANCER

CYCLE XXIX/2014

GENOME-WIDE ANALYSIS OF HUMAN NEUTROPHILS MIRNOME IDENTIFIED MIR-23A AS A CRITICAL REGULATOR OF THE APOPTOTIC FAS ANTIGEN EXPRESSION

S.S.D. MED/04

Coordinator: Prof. Gabriela Constantin

Tutor: Prof. Flavia Bazzoni

Doctoral Student: Somayehsadat Ghasemi

Genome-wide analysis of human neutrophils miRnome identified mir-23a as a critical regulator of the apoptotic Fas antigen expression

Somayehsadat Ghasemi

PhD thesis

Verona, 20 February 2017

Table of Contents

Summary

Over the past decade, genome-wide studies have made it clear that the mammalian genome is pervasively transcribed and this led to the identification of non-protein coding RNA molecules. Only 2% of the mammalian genome accounts for protein-coding sequences, so that the majority is transcribed as noncoding RNA (ncRNA). ncRNAs appear to be highly conserved and are considered to be an important component for genetic regulation via epigenetic mechanisms. The regulatory ncRNAs can be classified into long non-coding RNAs (lncRNAs) and microRNAs (miRNAs).

miRNAs increasingly recognized to play a pivotal role in both physiological and pathological conditions, including mammalian development, cardiovascular, neurodegenerative and metabolic diseases, cancer and immune disorders. Despite the evidence for the importance of miRNAs in immune cell function and development, very limited information is available regarding how miRNAs regulate neutrophils development, lifespan and functions. Based on these premises, the purpose of this study was to provide a comprehensive analysis of microRNAs expression profile and to identify their role in primary human neutrophils under resting and stimulatory conditions. To achieve this goal we performed whole transcriptome analysis and characterized the pri-miRNome of resting and LPS-stimulated neutrophils. In parallel, the same analysis was performed also on autologous monocytes, in order to get insight into cell-specific miRNA profile.

The results of this study showed that TLR4 engagement triggers the transcription of 37 pri-miRNAs. Additionally, comparison of LPS-induced pri-miRNA expression profile of neutrophils with that of autologous monocytes identified subsets of pri-miRNAs modulated by LPS in both cell type in a cell-specific manner. LPS induced the expression of twelve pri-miRNAs in neutrophils, among which we further validated the expression of the mature forms of the miR23a cluster members. The members of this cluster have been described to play important roles in various biological and pathological processes, but their role has never been previously identified in resting and/or activated neutrophils. Upon LPS stimulation neutrophils upregulate only the mature forms of miR-23a-

5p and miR-27a-5p. In silico target prediction indicated Fas, a death receptor mediating cell apoptosis, among the miR-23a predicted target genes. Herein we provide several experimental proofs demonstrating that LPS reduces neutrophil Fas-induced apoptosis in a miR-23a-dependent manner. In fact, and consistent with a prolonged neutrophils' half-life, in the presence of LPS a parallel upregulation of miR-23a and down-regulation of Fas membrane protein, but not Fas mRNA, expression was detected, suggesting involvement of a post-transcriptional regulation for Fas. Both Fas mRNA and miR-23a were detected in Ago immunoprecipitates only in LPS-stimulated neutrophils, thus indicating that LPS promotes miR-mediated post-transcriptional silencing of FAS. Finally, neutrophils overexpressing miR-23a reduced Fas membrane receptor. Taken together these data demonstrated that LPS stimulation affects neutrophil apoptosis via increasing miR-23a, which in return decreases the Fas expression on neutrophil surface.

Collectively, we have described the expression profile of pri-miRNAs in resting and LPS-activated human neutrophils and their autologous monocytes, resulting in identification of cell type specific pri-miRNAs. The information from this whole transcriptome study led us to link the LPS to neutrophil apoptosis via miRNA modulation. Our data could be substantially used for further investigation on the role of miRNAs in neutrophils biology.

List of figures and tables

Abbreviations

Ab	Antibody
ARE	Adenosine-uracil (AU) Rich Elements
AUF-1	AU-rich binding Factor 1
CCL	chemokine containing Cysteine-Cysteine motif
CD	Cluster of Differentiation marker
Ct	Cycle threshold
DC	Dendritic Cells
DNA	Deoxyribonucleic acid
FACS	Fluorescence-Activated Cell Sorting
FC	Fold Change
FITC	fluorescein
GM-CSF	Granulocyte-Macrophages Colony-Stimulating Factor
GNB2L1	Guanine Nucleotide-binding protein subunit Beta-2-Like 1
HuR	Hu protein R
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IRAK	Interleukin-1 Receptor-Associated Kinase
IRF	Interferon Regulatory Factor
ISG	Interferon-Stimulated Gene
lncRNA	Long noncoding RNA
LPS	Lipopolysaccharide
MAPK	Mitogen-Activated Protein Kinase
miRNA	microRNA
MNE	Mean Normalized Expression
mRNA	messenger RNA
MyD88	Myeloid Differentiation primary response gene (88)
NET	Neutrophil Extracellular Traps
NF-κB	Nuclear Factor κappa B
NK	Natural Killer

PAO	Phenylarsine Oxide
PBMC	Peripheral Blood Mononuclear Cells
PCR	Polymerase Chain Reaction
PE	Phycoerythrin
PIPES	Piperazine-N,N′-bis(2-ethanesulfonic acid)
PLB	Polysome Lysis Buffer
PMN	Polymorphonuclear Neutrophils
PMSF	Phenylmethane Sulfonyl Fluoride
poly(I:C)	polyinosinic:polycytidylic acids
PRR	Pattern Recognition Receptor
RIP	Ribonucleoprotein Immuniprecipitation
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
RPL32	Ribosomal Protein L32
RT	Retrotranscription
RT-qPCR	real-time RT-PCR
SEM	Standard Error of the Mean
snoRNA	small nucleolar RNA
snRNA	small nuclear RNA
TAB2	TAK1-Binding protein 2
TAK1	TGF-ß-Activating Kinase 1
TF	Transcription Factor
TGF	Transforming Growth Factor
TLR	Toll-Like Receptor
TNF-a	Tumor Necrosis Factor a
TRAF	TNF Receptor Associated Factor
TRIF	TIR domain-containing adaptor Inducing interferon-ß
tRNA	transfer RNA
TTP	Tristetraprolin
UTR	Untranslated Region
VRC	Ribonucleoside-Vanadyl Complex

1. Introduction

Inflammation is the process of recruitment and activation of cells of the innate and acquired immune system in response to infection, trauma or injury [1]. The normal course of inflammation is directed toward removal of the inducing agent and repair of damage, followed by elimination of the inflammatory cells [2]. Since this process is critical to host defence and homeostasis, inflammation must be extremely tightly regulated. Indeed, it is the classical two-edge sword: too little, and one is overwhelmed by infection or injury; too much, and the disease symptoms themselves become overwhelming [3]. The inflammatory process consists of coordinated, sequential and self-limiting release of different mediators that orchestrate and control the chronological phases of leukocytes recruitment, activation and clearance [2]. Polymorphonuclear neutrophils (PMN) represent the first and the most numerous type of leukocyte recruited at the inflammatory site in the initial phases of acute inflammation, while monocytes dominate the chronic phase and set the stage for the specific immune response. These cell types play a significant role in the inflammatory reaction not only by virtue of their ability to kill pathogens, but also for their ability to produce both pro- and anti-inflammatory cytokines and chemokines [3, 4]. Neutrophils function as an essential first line of defense against invading pathogens, such as bacteria, fungi and viruses. They utilize several effector mechanisms to defeat pathogens, including phagocytosis, discharge of constitutively stored antimicrobial enzymes or toxic factors, generation of massive amounts of reactive oxygen species (ROS) and active release of nuclear material (DNA, histones and other chromatin proteins) aggregating into neutrophil extracellular traps (NETs) [5]. Accumulating in vitro and in vivo evidence indicate that, in addition to direct antimicrobial activities, neutrophils play critical functions in the regulation of innate and adaptive immune responses. These activities are exerted largely via the release of cytokines and preformed or newly synthesized mediators in response to pattern recognition receptors (PRR)-mediated sensing of danger signals generated by invading pathogens or tissue damage [6].

Some of the activities described above for neutrophils are broadly shared by monocytes. However, due to their underlying molecular peculiarities these two cell types also show functional differences. Both at steady-state and after activation, neutrophils display a unique transcriptional profile and a specific pattern of cytokine production as compared to monocytes stimulated under the same conditions [7-9]. These occurrences likely reflect fundamental differences in neutrophil and monocyte ontogeny, as well as in transcriptional and epigenomic regulation.

As described above, neutrophils and monocytes exert both over-lapping and cell-specific functions in innate immunity. Activation of the same PRR in autologous neutrophils and monocytes indeed triggers distinct gene expression programs [9-11], reflecting cell type-specific mechanisms of transcriptional and post-transcriptional regulation of gene expression. In this context, cytokines expressed by neutrophils and monocytes represent crucial mediators for the immune functions of these cells and are the focus of extensive investigations at molecular level. However, various studies have emphasized that, at least in vitro, the extent of cytokine production by human neutrophils is generally lower than monocytes or lymphocytes on a per cell basis [12]. Nonetheless, the contribution of neutrophil-derived cytokines in inflamed tissues is certainly fundamental, in view of the fact that neutrophils often outnumber mononuclear leukocytes by one to two orders of magnitude [13]. Furthermore, neutrophils and monocytes activated with the same microbial agonists produce qualitatively different cytokines, exemplified by IFNβ, IL-10 and IL-6.

Differential induction of some genes in neutrophils and monocytes can be explained by the activation of cell type-specific signaling pathways in response to the same agonist. Indeed, upon recognition of LPS, Toll-like receptor (TLR) 4 and the co-receptors CD14 and MD-2 trigger a coordinated set of biochemical events that rely on the adaptor proteins MyD88 and TRIF [14]. MyD88 signaling from the plasma membrane mediates an early wave of NF-kB and mitogen-activated protein kinase (MAPK) activation, whereas trafficking of TLR4 to endosomes is required for TRIF signaling, in turn leading to activation of interferon regulatory factor3 (IRF3) and NF-kB. The combined activity of all these transcription factors

(TFs) controls transcriptional induction of IFN, which in an autocrine manner triggers the expression of a large set of interferon-stimulated genes (ISG) with anti-viral and immunomodulatory functions. For reasons that still need to be clarified at molecular level, human neutrophils incubated with LPS fail to activate the TRIF-dependent pathway and consequently do not express IFN-β mRNA and other ISGs [15]. On the other hand, the MyD88-dependent pathway seems to be fully functional in human neutrophils [16].

Other than activation of cell type-specific signaling pathways, accumulating evidence indicates that differential chromatin organization in neutrophils and monocytes may represent a critical factor to explain transcriptional differences observed in the two cell types. Indeed, pro-inflammatory transcription factors activated by external stimuli largely act within a pre-established regulatory landscape, thus impacting on the transcriptional output of TLR stimulation [17]. In this context, for instance, recent data provide a mechanistic support to the inability of highly purified human neutrophils to produce IL-10 [10]. A comparative analysis of the histone modifications at the IL10 locus of freshly isolated human neutrophils and autologous monocytes revealed that histone modifications associated with transcriptionally permissive chromatin, (namely H3K4me3, H3K27Ac/H4Ac and H3K4me1), are detectable in monocytes, but not in neutrophils, already at steady-state. Moreover, upon stimulation with TLR ligands or acute-phase protein serum amyloid A (SAA), H3K4me3 and H3K27Ac marks were further increased in monocytes, but not in neutrophils [18].

Besides chromatin organization, other critical transcriptional regulators are involved in gene expression regulation likely include noncoding RNAs, such as long noncoding RNA (lncRNA) and microRNAs (miRNAs). In this context, our understanding of the molecular bases of gene expression in neutrophils is still poor, possibly due to the objective difficulties in isolating these cells at high purity [13] and manipulate them in culture, issues that are particularly critical in human samples.

MicroRNAs

miRNAs constitute a class of highly conserved endogenous small non-coding RNAs of approximately 22 nucleotides in length that can regulate gene expression post-transcriptionally by affecting the half-life and translation of target mRNAs [19, 20]. The discovery of these non-coding RNAs as regulators of gene activity began almost 16 years ago with the identification in the nematode worm C. elegans of lin-4, a gene that encodes short RNA transcripts able to inhibit the translation of the lin-14 messenger RNA by binding to its 3' untranslated region [21]. Since then, a large number of endogenous mature small RNAs were identified. To date, 2588 human miRNAs have been annotated and catalogued in web-based miRNA database known as miRBase (http://www.mirbase.org; Release 21), and many of them have been implicated in almost all the cellular systems and biological processes [20, 22, 23].

MicroRNAs Biogenesis

Human miRNAs are processed from primary transcripts which may be originated from introns, intergenic regions or non-coding RNAs. Mature miRNAs are produced from long primary transcripts that contain the pre-miRNA, through a series of endonucleolytic maturation steps (Figure 1). Most primary miRNA transcripts (pri-miRNAs) are transcribed by RNA polymerase II, have 5' caps and 3' poly(A) tails, and are hundreds of nucleotides long. The pri-miRNAs are processed in the nucleus by the RNase III enzyme, Drosha, and the double stranded-RNA-binding protein, DGCR8 (DiGeorge syndrome critical region gene 8), into ~70-nucleotide pre-miRNAs, which fold into imperfect stem-loop structures. The pre-miRNAs are then exported into the cytoplasm by the RanGTP-dependent transporter exportin 5 and undergo an additional processing step in which a double-stranded RNA of 19-23 nucleotides in length (referred to as the miRNA:miRNA* duplex) is excised from the pre-miRNA hairpin by Dicer, the RNAse III enzyme. Subsequently, the miRNA:miRNA* duplex is incorporated into the RNA-induced silencing complex (RISC), which uses the mature miRNA guide strand as a template to identify target mRNA. The functional miRNA strand

is preferentially retained in the miRISC complex and negatively regulates its target genes. Either strands, -5p or 3P, can be functional depending on the tissue or cell condition [24-27].

MicroRNAs Function

It is widely accepted that miRNA binds to its target mRNA and negatively regulates its expression with the final effect of reducing the total amount of target protein. However several lines of evidence supports the existence of two distinct silencing mechanisms, depending on the miRNA and the cell type; in some cases, miRNAs have been shown to repress target gene expression at the translational level, whereas in the other cases miRNAs trigger the degradation of target mRNAs, both ultimately leading to down-regulation of gene expression [23, 28]. So far, the detailed mechanisms of gene silencing by miRNAs remain elusive. The translational repression by miRNAs might be due to the inhibition of translation initiation [29, 30] or the inhibition of elongation [31] or the enhancement of ribosomal drop-off from translating polysomes. Endogenous miRNAs are, indeed, associated with translating polysomes, thus suggesting that miRNAs may inhibit ribosome movement along mRNAs [32].

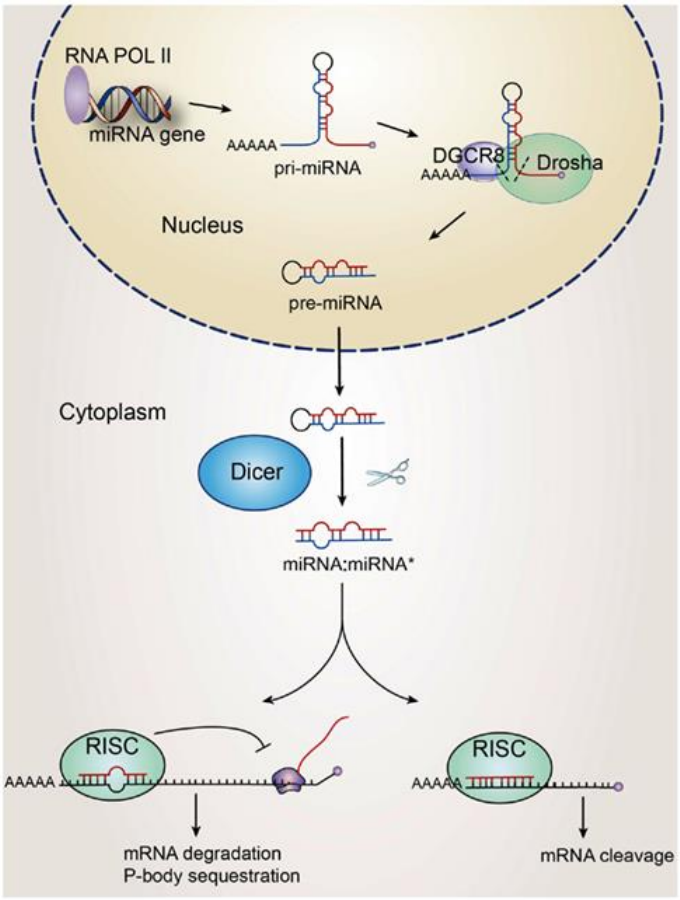


Figure 1. **Biogenesis of miRNAs.**
Refer to the text for details. From Barca-Mayo O. et al., Front Neurosci. 2012; 6: 13.

It has been demonstrated that miRNAs and their target mRNAs accumulate in cytoplasmic compartments referred to as processing bodies (P-bodies) or, more broadly, stress granules where mRNA turnover occurs. These cytoplasmic granules are major sites where cleavage and degradation of mRNA occurs. A model for miRNA function has been described in which miRNA targets associate with Argonaute proteins (AGO) via miRNA:mRNA base pairing interactions. The GW182 protein, a major component of P-bodies, interacts with AGO and recruits deadenylase and decapping enzymes leading to degradation of mRNA [33]. The evidence that the inhibition of essential structural components of P-bodies leads to both a loss of P-bodies and also an impairment of miRNA function, further supports this model [34]. P-bodies also serve as sites for storage of repressed mRNAs: indeed, the entry of an mRNA into a P-body does not inevitably lead to its degradation and repression by miRNA can be a reversible

process. An important new hypothesis is that mRNAs may return to polysomes to synthesize new proteins and that certain cellular proteins may facilitate the exit of mRNA from P-bodies. For example, the CAT-1 mRNA, negatively regulated by miR-122a, escapes both translational repression and P-body entrapment following amino acid starvation, oxidative stress or endoplasmic reticulum stress. Following depression, the CAT-1 mRNA is released from the P-bodies and is recruited by polysomes, where it is actively transcribed [34, 35].

Although miRNAs are mainly reported as negative regulators of mRNA translation there are few studies showing that under certain conditions miRNAs can also enhance the translation of target mRNAs [36, 37]. Translation upregulation by miRNAs might occur by two different mechanisms: activation by a direct action of miRNA on the target mRNA [38] or relief of a strong repression of the mRNA translation [39]. However, the physiological impact of such modes of regulation remains to be determined.

MicroRNAs Target Genes

It is estimated that the expression of at least 60% of human genes is subjected to miRNA-regulated process; it is evident that the identification of miRNA-target genes holds the key to decipher the molecular mechanisms by which miRNA exert their biological functions. Computational algorithms have been the major driving force in predicting miRNA targets. These approaches are mainly focused on programming alignment to identify complementary elements in the 3′-UTR with the seed sequence of the miRNA and the phylogenetic conservation of the complementary sequences in the 3′-UTRs of orthologous genes. However, evidence suggests that perfect seed pairing may not necessarily be a reliable predictor for miRNA interactions [40-42], which may explain why some predicted target sites are non-functional.

Based on the experimentally validated miRNA:mRNA interactions, the following general rules have been recognized: (i) miRNAs regulate the expression of target genes by forming perfect or imperfect base-pair interactions with them; (ii) multiple miRNA binding sites in the target UTRs might be required for efficient regulation; (iii) miRNAs and their target sites are evolutionarily

conserved among related species; (iv) an effective miRNA:mRNA interaction needs an open structure on the target site to begin the hybridization reaction [42, 43]. Furthermore, the ‘core sequence’ in the mRNA 3’UTR, that forms perfect or near-perfect base pairs with seven or eight bases near the 5′end of its miRNA cognate (called the ‘seed region’), seems to be crucially important for mediating miRNA inhibitory activity [44].

Once the potential physical interactions between miRNAs and mRNA targets have been predicted by bioinformatics tools, the predicted putative miRNA target(s) must be validated, usually via luciferase reporter, gain/loss of function and ribonucleoprotein immunoprecipitation (RIP) approaches [41, 45].

MicroRNAs as regulators of Toll-like receptors (TLRs) signaling

Toll-like receptors (TLRs), a family of pattern recognition receptors (PRRs), play a crucial role in early host defense against invading pathogens, given their ability to activate innate immune responses to provide effective host defense against pathogen infections. However, TLR-signaling pathways are also likely to stringently regulate tissue maintenance and homeostasis by elaborate modulatory mechanisms. Recent studies, performed mainly in monocytes/macrophages, have indicated that miRNAs play important roles in regulating the TLR-signaling pathways and innate immune responses and function as an essential part of the networks involved in regulating TLR-signaling pathways. [46]. In 2006, Baltimore Lab first documented that stimulation of human monocytes with lipopolysaccharide (LPS) upregulates the expression of miR-146a, miR-155, and miR-132 [47]. In turn, miR-146 functions as the effector arm of a negative feedback mechanism regulating TLR signaling in response to bacterial products, thus preventing the risk of excessive inflammation [48]. Subsequent studies showed an increase in expression of many other miRNAs. To date, an array of miRNAs are involved in regulating of the TLR-signaling pathways and innate immune responses by targeting multiple molecules at multiple levels, including TLRs themselves, TLR-associated signaling proteins, TLR-associated regulatory molecules, TLR-induced transcription factors, and TLR-induced functional cytokines (Figure 2) [49].

Collectively, these studies indicate that TLR signaling pathways induce multiple miRNAs, which in turn regulate the strength, location, and timing of the TLR signaling pathways, and might be involved in controlling the switch from a strong early inflammatory response to the resolution phase of the inflammatory process in a timely and orchestrated manner. The innate immune system utilizes multiple miRNAs to properly regulate its functional capacity, creating a finely tuned balance between activation and repression in TLR-signaling pathways. Nevertheless, the mechanisms regarding TLR-signaling pathways mediated miRNA expression regulation, through transcriptional repression or posttranscriptional destabilization, need to be further deeply investigated.

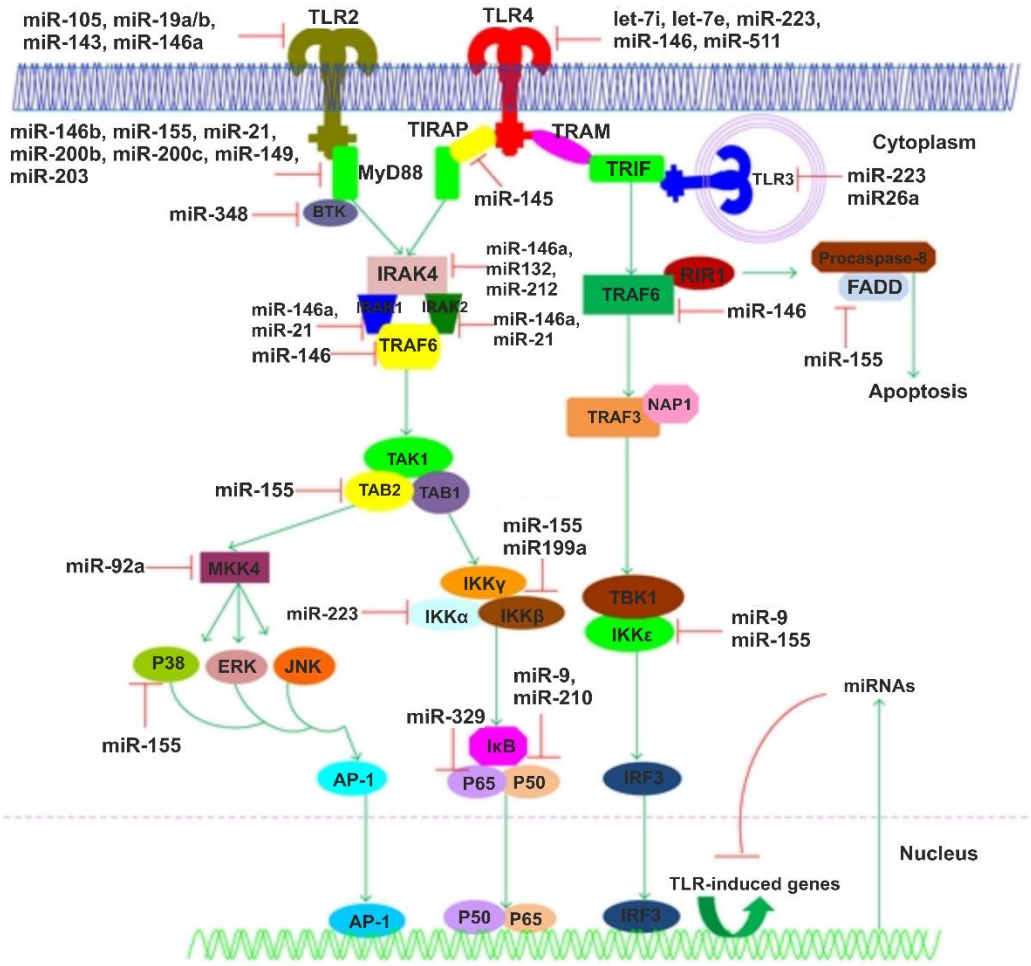


Figure 2. **The fine-tuning of the TLR-signaling pathways by miRNAs.**
Refer to the text for details. From He X. et al. Biomed Res Int. 2014; 2014: 945169.

MicroRNAs in Neutrophils

The literature addressing the roles of miRNAs in neutrophil biology is relatively scarce to date. This may be attributed to the obvious challenges associated with in vitro studies of purified primary neutrophils.

The majority of the miRNA studies on human neutrophils are focused on neutrophil development and are performed in neutrophils from mice deficient of specific miRNAs, such as miR-223-deficent mice [50], or with myeloid-specific *Cebpa-Cre Dicer1* deletion [51]. Future work is needed using neutrophil-specific Dicer knock outs to differentiate between the role of miRNAs in neutrophil development and effector function. Meanwhile, a few specific miRNAs have been implicated in neutrophil biology, although their regulatory mechanisms are not fully elucidated (Figure 3) [52, 53]. Whether the biological relevance of the findings arising from such mouse models aids our understanding of human neutrophils is an open question, given the vast differences between human and mouse neutrophils. miR-223 was originally found to be restricted to myeloid cells (Gr-1+ and Mac-1+) [54], subsequently it was shown to be one of the most abundant miRNAs expressed by human granulocytes ($CD15^{+}$) [55], and it has been shown to act as a negative modulator of neutrophil activation. In fact, neutrophils from miR-223 deficient mice are hypersensitive to activating stimuli [50] (Figure 3). Of considerable importance is the role of miR-4661 in inflammation, as Li et al. [56] demonstrated that miR-4661 overexpression in murine bone marrow neutrophils promotes the initiation of inflammation, characterized by a significant increase in leukocyte infiltration. In murine bone marrow neutrophils, miR-466l overexpression increased amount of IL- 1β, TNF-α, IL-10, accompanied with increased chemotaxis to C5a and TNF-α, as well as increased reactive oxygen species production [56]. In the model proposed in this study, miR-466l serves as a signaling molecule that is initially expressed in neutrophils to promote the initiation of inflammation, but is then upregulated in macrophages to resolve the inflammation. Interestingly, miR-466l increases target gene expression through stabilization of its mRNA targets, opposite to the role for the vast majority of other miRNAs. Finally, miR-451 has been shown to negatively regulate mouse and human neutrophils chemotaxis in vitro by

downregulating the p38 MAPK signaling pathway [57] (Figure 3). Yang et al. [53] provided some evidence that decreased miR-150 in neutrophils after traumatic injury may up-regulate the protein kinase C alpha (PRKCA) expression. This protein has several cellular functions, such as cell adhesion, secretion, proliferation, differentiation, and apoptosis, and its increased level results in neutrophil activation related to traumatic injury [53].

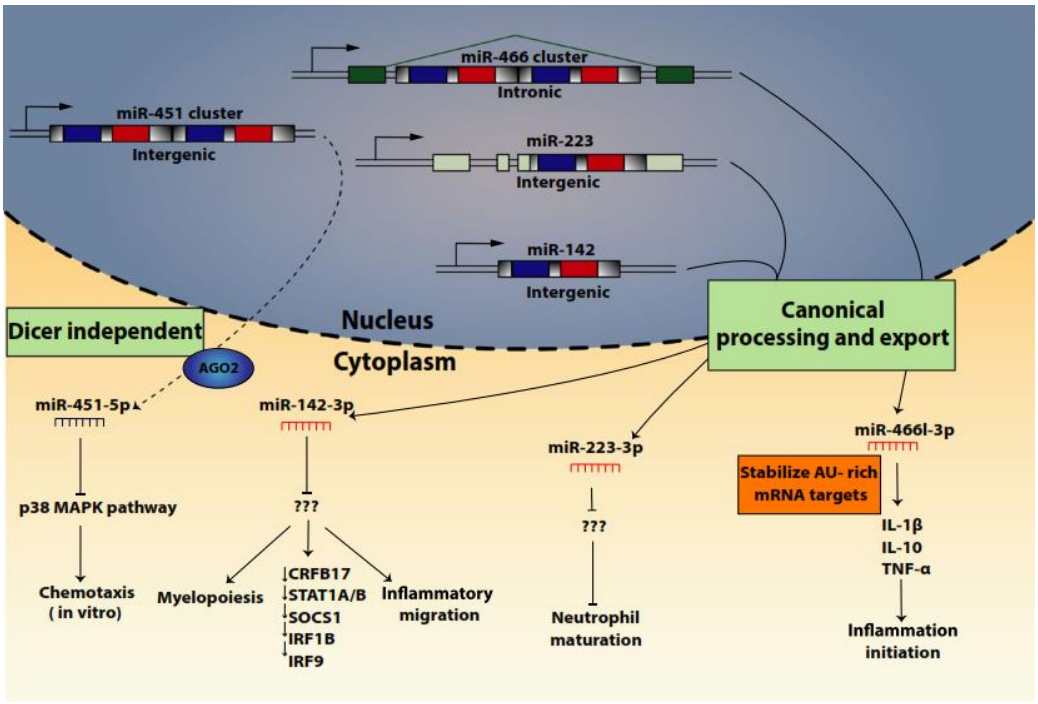


Figure 3. **Individual miRNAs that regulate neutrophil functions intrinsically.** miR-451 inhibits chemotaxis by inhibiting the p38 MAPK pathway. miR-466l performs non- canonical functions to upregulate gene expression by binding to AU- rich elements in the 3′UTR of target mRNA and prevents their degradation. miR-223 is involved in neutrophil maturation. miR-142 promotes neutrophil development and function through unknown direct targets. 5′ strand (red), 3′ strand (blue), hairpin and lower stem (gray), gene exon (dark green), non- coding exon (light green). Mature strand is indicated in cytoplasm. *From Gurol T. et al.,* Immunol Rev 2016; 273: 29-47.

To our knowledge, only one miRNA has been described so far as upregulated by LPS in human neutrophils. In fact, our group identify miR-9 as the only miRNA (among 365 analyzed using a TaqMan-based Low Density Array) up-regulated in both human neutrophils and autologous monocytes after TLR4 activation. miR-9 is also induced by TLR2 and TLR7/8 agonists and by the proinflammatory cytokines TNF-α and IL-1β. Our data showed that TLR4-

activated NF-κB rapidly increases the expression of miR-9, that operates a feedback control of the NF-κB-dependent responses by fine tuning the expression of p50, a key member of the NF-κB family [58].

2. Aim of the Study

The main purpose of this thesis has been to provide a comprehensive characterization of the miRNome of primary human neutrophils freshly purified from peripheral blood of healthy subjects and/or kept in culture in the presence or absence of LPS.

With the aim of characterizing other miRNAs involved in neutrophil function, several studies have attempted to determine the profile of miRNAs expressed in neutrophils. Three main approaches have been adopted to date to profile miRNAs in neutrophils and progenitors: miRNA microarrays, miRNA cloning/sequencing, and low-density miRNA PCR arrays. The most popular is that of miRNA microarrays, allowing for the rapid characterization of differentially expressed miRNAs between samples. It is worth noticing that none of these studies addressed the question of the role of miRNAs in TLR4-mediated activation of human neutrophils.

We have previously conducted a TaqMan-based Low Density Array to profile the miRNAs (out of the 365 mature miRNAs included in the array) constitutively expressed and induced by LPS in human neutrophils [58]. A more accurate picture of miRNAs expressed in neutrophils can be obtained by the RNA-sequencing strategy. In fact, RNAseq data over the Low Density Array that we have previously utilized provides several advantages:

1. RNAseq outperforms array platforms in several respects, notably in exon boundary detection and dynamic range of expression;
2. RNAseq can be employed in the discovery of new transcripts;
3. Another drawback of the array platforms is the limited dynamic range of detection [59]. Previous work has presented RNAseq data with a dynamic range varying over 5 orders of magnitude [60]. Consistent with this, ~40% more genes are called differentially expressed by RNAseq between two distinct subpopulations, even when using a conservative statistical test. It is also clear that the fold difference of differential expression is greater for RNAseq;

4. RNAseq allows the characterization of the whole transcriptome, thus it is informative not only of non-coding RNA but also of protein-coding gene expression. Transcriptome analysis of protein-coding genes may represent a useful tool for the subsequent identification of miRNA targets;
5. Advances in high-throughput deep sequencing of the transcriptome and the ENCODE project have led to the discovery of numerous new noncoding RNAs as compared to those deposited in the database few years ago and present in the array.

The study was performed in parallel on human neutrophils and autologous monocytes. Such a concurrent analysis was planned in order to identify shared and/or cell specific pathways regulated by miRNAs. In this context, our understanding of the molecular bases of gene expression in neutrophils as compared to monocytes is still poor, possibly due to the objective difficulties in isolating these cells at high purity and manipulate them in culture, issues that are particularly critical in human samples. For this reason, this study had two additional technical goals: (i) to develop an optimized protocol allowing us to immunoprecipitate the RISC-associated Ago proteins to integrity and to simultaneously detected RISC-associated miRNA/mRNA in neutrophils; (ii) to validate miRNAs predicted target genes directly in primary human neutrophils overexpressing the identified miRNA.

3. Materials and Methods

3.1. Materials

Ultra Pure E. coli lipopolysaccharide (LPS, 0111:B4 strain) and polyinosinic:polycytidylic acids [poly(I:C)] purchased from Invivogen (San Diego, CA, USA). Rabbit anti-Ago antibody (clone 2A8) purchased from Millipore, (Darmstadt, Germany) and rabbit anti-Actin Antibody (A2066) from Sigma-Aldrich (Saint Louis, MO, USA). Pre-miR miRNA precursor and the negative control 2 were bought from Ambion, Thermo Fisher.

3.2. Cell purification and culture

The purity of the cell populations constitutes an important concern for all gene expression studies conducted by real-time PCR approach. In fact, on one hand the development of real-time PCR-based techniques for measuring gene expression requires less RNA template than other methods of gene expression analysis and allows a reliable quantification of RT-PCR products; however, on the other hand, because of its extremely high sensitivity, it can easily amplify mRNA from a few contaminating leukocytes eventually present in neutrophil or monocyte preparations. In particular, this represents a major concern for molecular studies conducted on neutrophil, because peripheral blood mononuclear cells (PBMCs) possess 10–20 times more RNA per cell than neutrophil, and synthesize 10- to 300-fold more cytokine than neutrophil so that a contamination of only 1% PBMCs can translate into an up to 20% of contamination at the RNA level. Therefore, it is critical to work with highly purified neutrophil populations and to generate substantial evidence that mRNA expression can be directly attributed to neutrophil, as opposed to contaminating PBMC or even eosinophils [61]. For these reasons, the entire study has been performed on highly pure human neutrophil, separated by centrifugation on Ficoll-Paque Plus (GE Healthcare, Little Chalfont, Buckingamshire, UK) and enriched to reach a 99.9% purity by positively removing eventual contaminating cells (T cells, NK cells, B cells, monocytes, DCs, platelets, eosinophils, or erythrocytes) with Abs against CD3, CD56, CD19, CD36, CD49d,

and Gly-A using a custom-made EasySep kit (StemCell Technologies, Vancouver, Canada). Monocytes were isolated from PBMC after centrifugation over Percoll gradients and then purified at the 99.8% by negative magnetic selection with MACS Human Monocyte Isolation Kit II (Miltenyi Biotec, Bergisch Gladbach, Germany). The purity of both leukocyte populations was assessed by flow cytometry using Abs against CD3 for the detection of lymphocytes, anti-CD14 Abs for monocytes, anti-CCR3 Abs for eosinophils and anti-CD16 or anti-CD66b for neutrophil. Neutrophil ($6x10^6$/ml) and monocytes ($3x10^6$/ml) were then resuspended in RPMI 1640 medium (BioWhittaker, Walkersville, MD, USA) supplemented with 10% low endotoxin FCS (BioWhittaker; <0.06 EU/ml by Limulus amebocyte lysate assay) and 2 mM L-glutammine (Cambrex). Cultured cells were treated with 100ng/mL LPS or 50 μg/mL poly(I:C), according to the conditions and times indicated in the Results.

At the indicated times after stimulation, ice-cold PBS was added, cells were then collected, centrifuged twice at 500 x g for 5 min at 4°C and then manipulated as described in the sections below. All reagents were of the highest available grade and buffers were prepared with pyrogen-free water for clinical use.

3.3. Purification and quantification of total RNA

Total RNA was extracted from monocytes using the RNeasy Mini Kit (QIAGEN, Crawley, UK), according to the manufacturer's protocols. Total RNA was quantified by Nanodrop 2000c spectrophotometer (Thermo Fisher) and checked for the integrity by Agilent 6000 Nano (cat. no. 5067–1511) kit in an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

3.4. Real-time RT-PCR (RT-qPCR) of mRNAs and pri-miRNAs

The expression of NFKB1, TNFα, GNB2L1, RPL32, Fas mRNA, and the precursors of selected microRNAs was quantified by RT-qPCR using gene-specific primer pairs reported in Table 1 for mRNAs and supplementary Table S1 for the pri-miRNAs.

Table 1. **Primers used in RT-qPCR analysis for mRNAs.**

Target	Sense Oligonucleotide	Antisense oligonucleotide
NFKB1 (57bp)	GGGCAGGAAGGACCTCTAG	ACGGTGTGGGAAATTGTCAG
NFKB1 (325bp)	CCTGAGACAAATGGGCTACAC	TTTAGGGCTTTGGTTTACACGG
TNFα (70bp)	TTGACACAAGTGGACCTTAGG	CTGGGCTCCGTGTCTCAAG
TNFα (243bp)	GAGCACTGAAAGCATGATCC	CGAGAAGATGATCTGACTGCC
GNB2L1 (61bp)	GACAACCTGGTGCGAGTGTG	GCTCTGCCATAAACTTCTAGC
GNB2L1 (225bp)	TAGTTTCTCTAAGCCATCCAGTG	ATTCCATAGTTGGTCTCATCCC
FAS (73bp)	CCTACTTCTTTCTCAGGCATC	AAATGGAGAGGTGGCAAAGC
RPL32 (183bp)	AGGGTTCGTAGAAGATTCAAGG	GGAAACATTGTGAGCGATCTC

Total RNA samples were reverse transcribed using 5ng/µL random primers, 0,5 mM dNTPs, 10 mM DTT, 1X reverse-transcription buffer, 1 U/µL RNase inhibitor (RNAse Out, Invitrogen) and 5 U/µL reverse transcriptase (SuperScript III, Invitrogen) at 50°C for 60 min.

Gene expression was evaluated in duplicate from 10 ng of cDNA using the FastSYBR Green Master Mix (Applied Biosystem, ThermoScientific) in the presence of 200 nM specific primer pairs, using the ViiA7 qPCR system (Applied Biosystem, Thermo Fisher). Data were calculated with LinReg PCR 7.0 and Q-Gene software (www.biotechniques.com) and then expressed as mean normalized expression (MNE) units after GAPDH normalization. Briefly, LinReg was used for the calculation of the amplification efficiency of each gene; subsequently the MNE with standard deviations (SD) for each sample and corrected for amplification efficiencies were calculated using the Q-Gene software.

3.5. RT-qPCR analysis of miRNA expression

Single miRNA expression analysis were performed by TaqMan® miRNA Human Assay (Applied Biosystems). cDNA was synthesized from 100 ng of total RNA using TaqMan® microRNA reverse transcription kit (Applied Biosystems, Thermo Fisher) and individual miRNA-specific RT primers contained in the

TaqMan® microRNA Human Assays according to the manufacturer's protocol. The reaction was incubated for 30 min at 16ºC, 30 min at 42ºC and 5 min at 85ºC. Each generated cDNA was amplified in duplicate by qPCR with sequence-specific primers from the TaqMan® microRNA Human Assays, according to the manufacturer instructions, on the ViiA7 qPCR system (Applied Biosystems, Thermo Fisher). The reaction was incubated at 50°C for 2 min, followed by 95°C for 20 s, and then by 50 cycles of 95°C for 1 s and 60°C for 20 s. The relative miRNAs expression value were calculated according to the "comparative Ct" method using RNU44 as endogenous control [62]. Briefly, ΔCt represents the threshold cycle (Ct) of the target miRNA minus that of reference small RNA and ΔΔCt represents the ΔCt of the treated sample minus that of the control sample. Relative miRNA expression has been expressed as fold change, $FC = 2^{-\Delta\Delta Ct}$.

3.6. Cell lysis

Cells were lysed with different lysis methods: using Polysome Lysis Buffer (PLB) or Nitrogen Cavitaion. PLB was used to lyse the cells as previously described [63], briefly, monocytes (15×10^6) and neutrophils (30×10^6) were lysed in polysome lysis buffer (100 mM KCl, 5 mM MgCl2, 10 mM Hepes, 0.5% Nonidet P-40) supplemented with 1,000 U/mL RNase Out (Invitrogen), 5 mM Vanadyl ribonucleoside complexes (VCR), 5 µg/mL leupeptin, 5 µg/mL pepstatin, 1 mM phenylmethanesulphonylfluoride (PMSF), 1 mM Na3VO4, 20 µM phenylarsine oxide (PAO), 1mM DTT, and 50 mM NaF.

Nitrogen cavitation was used to lyse the cells as described by P. Patrick et al. [64] with minor modifications**.** Briefly, cells (10^8 cells/mL) were suspended in ice-cold relaxation buffer (10 mM PIPES pH 7.30, 30 mM KCl, 3 mM NaCl, 3.5 mM $MgCl_2$, 1.25 mM EGTA) containing the same cocktail of antiproteases and antiRNases mentioned above for PLB. Then cells were pressurized under a N_2 atmosphere (350 psi, 20 minutes at 4 °C) with constant stirring in a nitrogen bomb (Parr Instrument Co, Moline, IL). Cavitate was spun for 10 min, 4 °C, at max speed; the granule-free cytoplasmic fraction was collected from the supernatant layer for further analysis.

3.7. Western blotting

Lysates of cells from different lysis method, described above, were electrophoresed on 7.5% SDS-PAGE and transferred to nitrocellulose (Hybond, GE Healthcare, Little Chalfont, Buckinghamshire, UK). The blots were incubated with 1:1000 rabbit anti-Ago Abs and 1:1000 rabbit anti-Actin Abs. Detection was carried out with Alexa Fluor^{R} 680 goat anti-rabbit secondary Abs (Molecular Probes^{TM}, Invitrogen, Carlsbad, CA). Blotted proteins were detected and quantified using the Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, Nebraska). Quantification was performed with the analysis software provided by the manufacturer.

3.8. Ribonucleoprotein Immunoprecipitation and Ago-Associated RNA Analysis

Cells were lysed with PLB buffer or Nitrogen cavitation as described above and Ribonucleoprotein immunoprecipitation (RIP) experiments were performed as described elsewhere [65]. Briefly, immunoprecipitations were carried out for 3 h at 4 °C using protein G Sepharose magnetic beads (GE Healthcare) conjugated with anti-Ago (clone 2A8; Millipore) or isotype IgG1k control antibodies. An aliquot of IP supernatants, corresponding to 10% of cell equivalents, was removed after immunoprecipitation and used as input. Immune complexes were washed four times with NT2 buffer (50 mM Tris·HCl, 150 mM NaCl, 1 mM MgCl2, 0.05% Nonidet P-40 supplemented with 20 mM EDTA, 1 mM DTT, 400 μM VRC, and 500 U/mL RNase Out) and were detached from beads with guanidinium-based lysis buffer (RLT buffer, RNeasy kit; Qiagen) supplemented with β-mercaptoethanol. RNA then was extracted the input or immunoprecipitated samples with the RNeasy kit according to the manufacturer's protocol.

Detected RNA molecules (mRNA or miRNA) by RT-qPCR then were analyzed as follow: Ct values for replicate samples were first averaged, then each RIP RNA fractions' Ct value was normalized to the Input RNA fraction Ct value

for the same qPCR Assay (ΔCt) to account for RNA sample preparation differences.

ΔCt [normalized RIP] = (Ct [RIP] - (Ct [Input] - Log2 (Input Dilution Factor)))

Where, Input Dilution Factor = (fraction of the input RNA saved) and the default Input fraction is 10% which is a dilution factor of 10.

The normalized RIP fraction Ct value was then adjusted for the normalized background [non-specific (NS) isotype control Ab] fraction Ct value (ΔΔCt).

ΔΔCt [RIP/NS] = ΔCt [normalized RIP] – ΔCt [normalized NS]

Finally, the Assay Site IP Fold Enrichment was calculated above the sample specific background (linear conversion of the ΔΔCt).

Fold Enrichment = $2^{(-\Delta\Delta Ct\ [RIP/NS])}$

3.9. Flow cytometry

For phenotypic studies neutrophils ($1x10^5$) were initially incubated in PBS containing 5% complement-inactivated human serum (for Fc receptor blocking). Neutrophils were then stained for 20 min at room tempreture with FITC anti-CD66b, PE anti-CD16 (BioLegend, San Diego, California, USA), APC-Cy7 anti-CD95 (Miltenyi Biotec, Bergisch Gladbach, Germany), and their related isotype controls. Sample fluorescence was then measured by using an eight-color MACSQuant Analyzer (Miltenyi), data analysis performed by FlowJo software Version 8.8.6 (TreeStar).

Cell viability was analyzed using Vybrant™ DyeCycle™ Violet-negative (Life Technologies, Carlsbad, CA, USA) [66]. Phenotypic analysis was performed on live cells, identified as Vybrant-negative cells.

3.10. Neutrophils transfection

Freshly purified neutrophils ($12x10^6$) were transfected with the indicated amount of Pre-miR miRNA precursor or the negative control (pre-miR-Ctr), using the Amaxa Nucleofector (Amaxa Biosystems GmbH) and the Human Monocyte Nucleofector kit (Lonza), according to the manufacturer's protocol. Transfected

cells were plated in recovery medium (Amaxa) supplemented with 2 mM glutamine and 10% FCS and cultured for 6 h later as indicated.

3.11. RNA sequencing

Total RNA was extracted from monocytes and autologous neutrophils as described before and libraries were constructed following the manufacturer's protocol of the TruSeq RNA sample preparation kit (Illumina). Briefly, after oligo-dT selection, mRNA was fragmented, double-stranded cDNA generated from fragmented RNA and index-containing adapters were then ligated to the ends. Libraries were evaluated using Agilent Bioanalyzer, quantified with the Qubit fluorometer using the quant-it HS dsDNA reagent kit (Invitrogen), and diluted according to Illumina's standard sequencing protocol. High-throughput sequencing was done on an Illumina HiSeq 2000 platform in order to obtain an average of $50x10^6$ 51-base-pair (bp) paired-end reads.

After quality-filtering according to the Illumina pipeline, short reads were aligned to the GrCh38 reference human genome (Genome Reference consortium) and the *H. sapiens* transcriptome (Ensembl, version 78) using TopHat [67] with the following parameters: *-r 250 –p 8 –G*. Summed exon reads count per gene were estimated using HTSeq-count [68] with the following parameters: *-r name – s no – m union –t exon –i gene*. Differential expression analysis was performed using the negative binomial distribution-based method implemented in DESeq2 on the summed exon reads count per gene [69]. Genes with adjusted p-value<0.05 and $|\log_2 FC|>1$ were considered as differentially expressed. Gene expression levels were expressed as fragments per kilobase of exon per million fragments mapped (FPKM), calculated by using DESeq2 functions.

3.12. Gene ontology (GO) analysis

PANTHER database (Version 11.1) was used for Gene Onthology (GO) analysis of the potential targets of the differentially expressed miRNAs, in order to identify the GO terms related to Biological Processes (BP).

3.13. Statistical analysis

Data were expressed as mean ± SEM, unless otherwise indicated. Statistical evaluation was determined using the Student t-test or two-way analysis of variances (ANOVA) followed by Bonferroni post-test. P-values lower than 0.05 were considered significant. Statistical analysis was performed by using Prism, version 6.0, software (GraphPad Software, La Jolla, CA, USA).

4. Results

4.1. Characterization of the pri-miRNome of human neutrophils under resting and LPS-activated conditions.

Neutrophils (99.9% pure), purified from six independent healthy donors were immediately lysed or cultured in the presence or absence of 100 ng/mL LPS for 90 min and 4h. In order to obtain additional information helping us to better characterize the neutrophil miRNome, autologous CD14+ monocytes were purified, immediately lysed or stimulated with LPS (100 ng/mL) for 90 min and 4h. Cells were collected at the selected time points and processed for RNA purification. Purified RNA from the six donors was subsequently merged in two pools of three independent samples each. Two cDNA pair-end libraries, representing the poly-A-positive RNA molecules, were generated and sequenced. Reads were quality filtered and aligned to the reference human genome (GrCh38 – Ensamble77) as described in Materials and Methods. All data were subjected to differential gene expression (DGE) analysis by using DeSeq2. Expression profile of the pri-miRNAs annotated in miRBase sequence database was extracted from RNAseq data (Figure 4).

This analysis allowed us to characterize the pri-miRNome of human neutrophils and autologous monocytes under resting and LPS-activated conditions. Under resting conditions 381 pri-miRNAs were detected (FPKM>0) in neutrophils (supplementary Table S2). Among these, 56 pri-miRNAs in neutrophils were found modulated (up-regulated FC>2, $p<0.05$; down-regulated FC<0.5, $p<0.05$) by LPS at either time point (90 min and/or 4h) as compared to freshly purified cells (Figure 5). Additionally, de novo expression of 3 pri-miRNAs in neutrophils that were not detected in freshly purified cells (namely MIR9-1, MIR193A and MIR6772) was triggered by TLR4 activation (Figure 5).

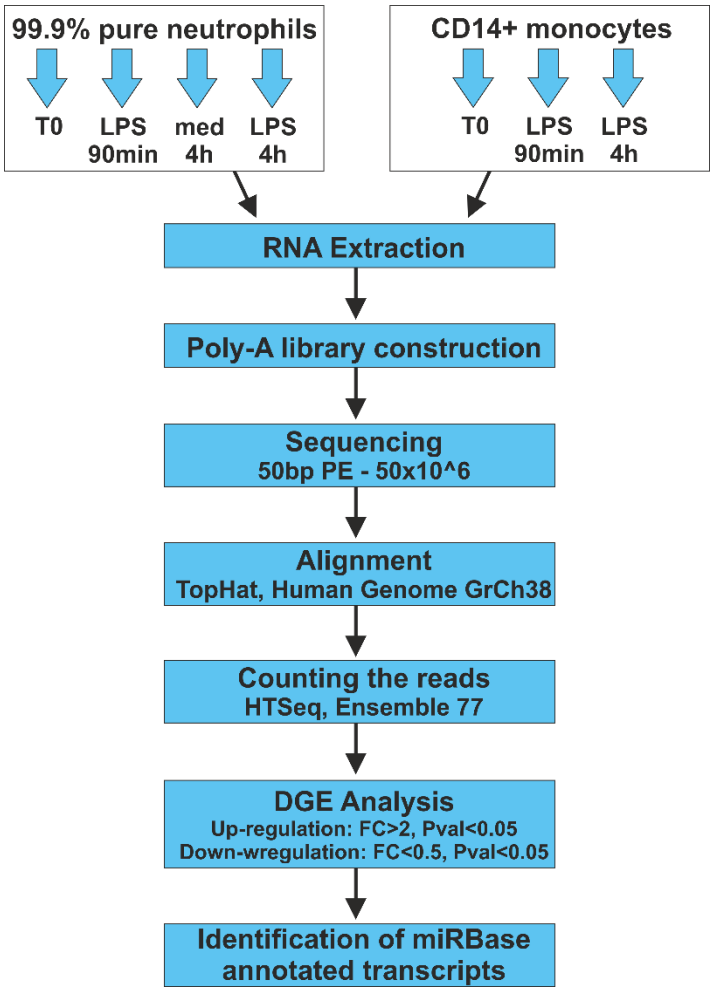


Figure 4. **RNA sequencing workflow. Identification of miRBase annotated transcript**

CD14+ monocytes and autologous neutrophils were cultured for 90 min or 4h with LPS (100 ng/mL) or left untreated. After RNA purification, two pools of three donors for each condition were used to create polyA library for RNA-sequencing. Good quality reads ($50x10^6$/condition) were aligned on the human reference genome (GrCh38) and used for the differential gene expression analysis as described in Materials and Methods.

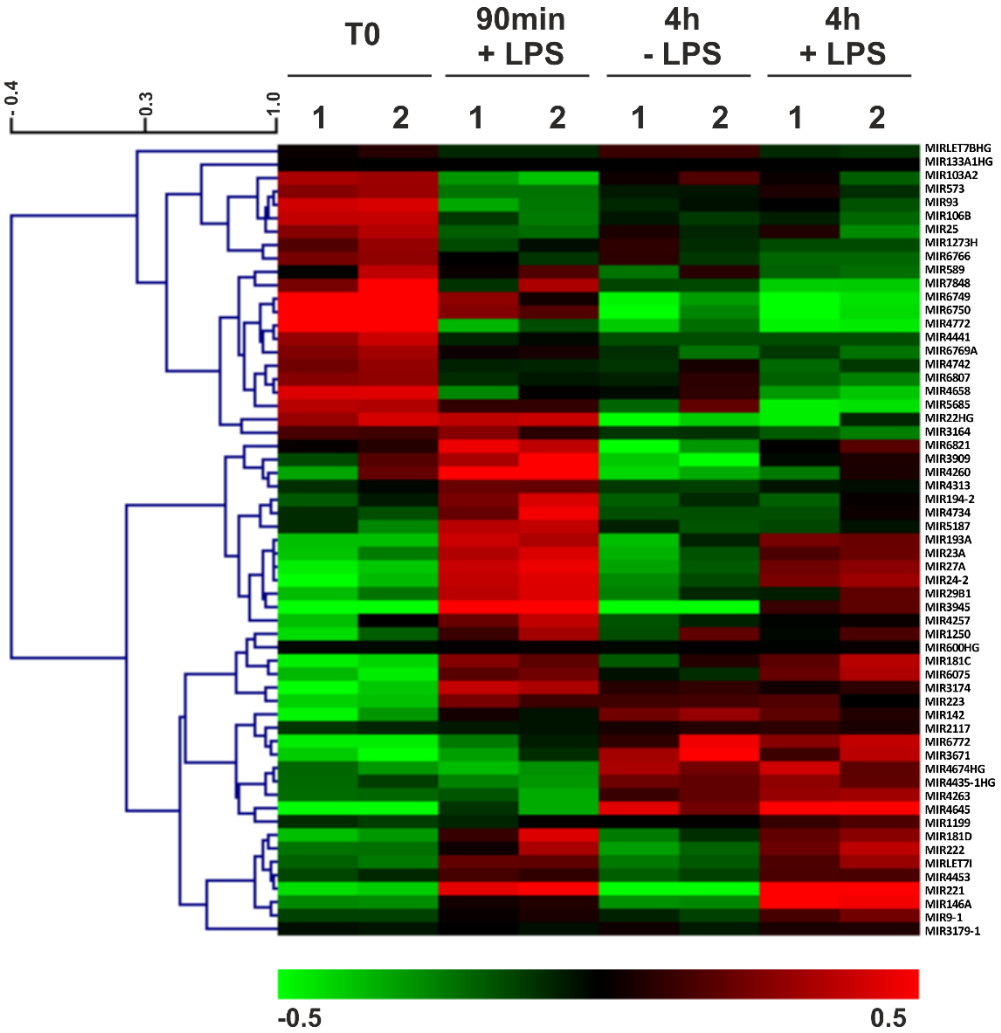


Figure 5. **LPS stimulation modulates pri-miRNA expression in neutrophils.** Hierarchical Clustering Analysis of pri-miRNA expression in neutrophils freshly purified or cultured for 90 min and 4h in the presence or absence of LPS. The expression levels of LPS-modulated pri-miRNAs ($|Log_2FC|>1$, $Pval<0.05$) in neutrophils are shown as $Log_{10}(FPKM+1)$, centered to the mean across the genes in row.

LPS-modulated pri-miRNAs were clustered based on their kinetic of expression (Figure 6):

1. Primary and transient: pri-miRNAs whose expression is rapidly modulated by LPS at 90 min, and returns to the basal level within 4h;
2. Primary: pri-miRNAs whose expression is modulated by LPS at 90 min and keeps the trend of modulation at later time point (4h);
3. Secondary: pri-miRNAs whose modulated expression is detectable 4h post LPS stimulation.

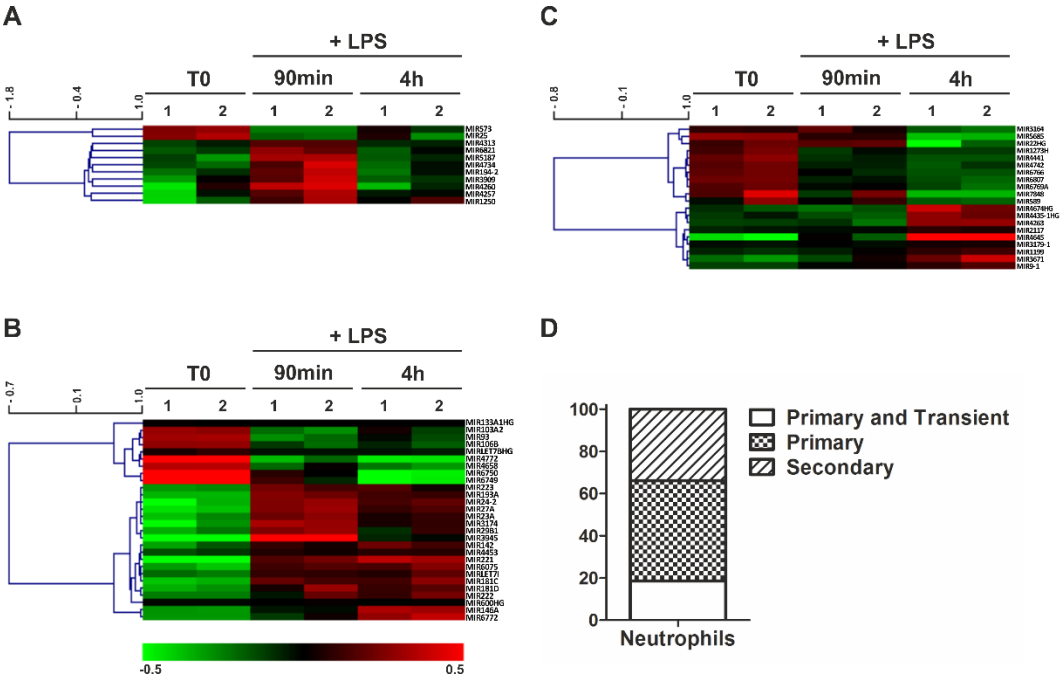


Figure 6. **LPS modulates the expression of pri-miRNAs with three different patterns: primary and transient, primary and secondary.**
LPS-modulated pri-miRNAs identified in neutrophils were clustered into primary and transient (A), primary (B) and secondary (C) according to their kinetic of expression. The expression levels of LPS-modulated pri-miRNAs belonging to each category are shown as Log_{10}(FPKM+1), centered to the mean across the genes in row. The percentage of pri-miRNAs belonging to each category is shown in panel D.

4.2. MiRNA transcripts selectively expressed in resting and LPS-activated neutrophils

Determining the specificity of miRNA expression profiles in neutrophils is clearly important for understanding the biology of this innate immune cells, that are characterized by unique functions and, correspondingly, distinct gene expression profiles. In order to identify neutrophil-specific pri-miRNAs modulated by LPS, we took advantage of the RNAseq performed in autologous CD14+ monocytes. RNAseq data were processed according to the same strategy employed for neutrophils (Figure 4). Differential expression of pri-miRNA in resting vs activated monocytes was performed with DESeq2, and showed that 70 pri-miRNAs are modulated (up-regulated FC>2, $p<0.05$; down-regulated FC<0.5, $p<0.05$) in LPS-stimulated monocytes at either time point (90 min and/or 4h) as

compared to freshly purified cells (Supplementary Figure S1). Additionally, de novo expression of 14 pri-miRNAs in monocytes (namely MIR146B, MIR4726, MIR3065, MIR4637, MIR181C, MIR181D, MIR4470, MIR4524A, MIR4773-2, MIR5696, MIR6772, MIR760, MIR7976, MIRLET7G) was triggered by TLR4 activation (Supplementary Figure S1).

Comparison between LPS-modulated pri-miRNA in neutrophils and monocytes allowed us to identify the following categories (Figure 7):

- 31 pri-miRNAs specifically modulated by LPS only in neutrophils. Precisely, 13 pri-miRNAs increased and 18 pri-miRNAs decreased in LPS-stimulated as compared to freshly purified neutrophils.
- 56 pri-miRNAs specifically modulated by LPS only in monocytes: 39 pri-miRNAs increased and 17 pri-miRNAs decreased in LPS-stimulated as compared to freshly purified monocytes.
- 21 pri-miRNAs modulated by LPS in a shared manner in both neutrophils and monocytes (i.e. up-regulated or down-regulated by LPS in both cell types);
- 7 pri-miRNAs with opposite type of LPS-dependent modulation in neutrophils and monocytes (up regulated in one cell type and down regulated in the other).

The pri-miRNAs belonging to each above-mentioned categories are displayed in figure 7 as the Log_2Foldchange of their expression in LPS condition compared to resting cells. To model the heatmap each pri-miRNAs with $|Log_2FC|>1$ and $Pval<0.05$ was considered modulated and other than this condition was defined as pri-miRNA with no modulation ($Log_2FC=0$).

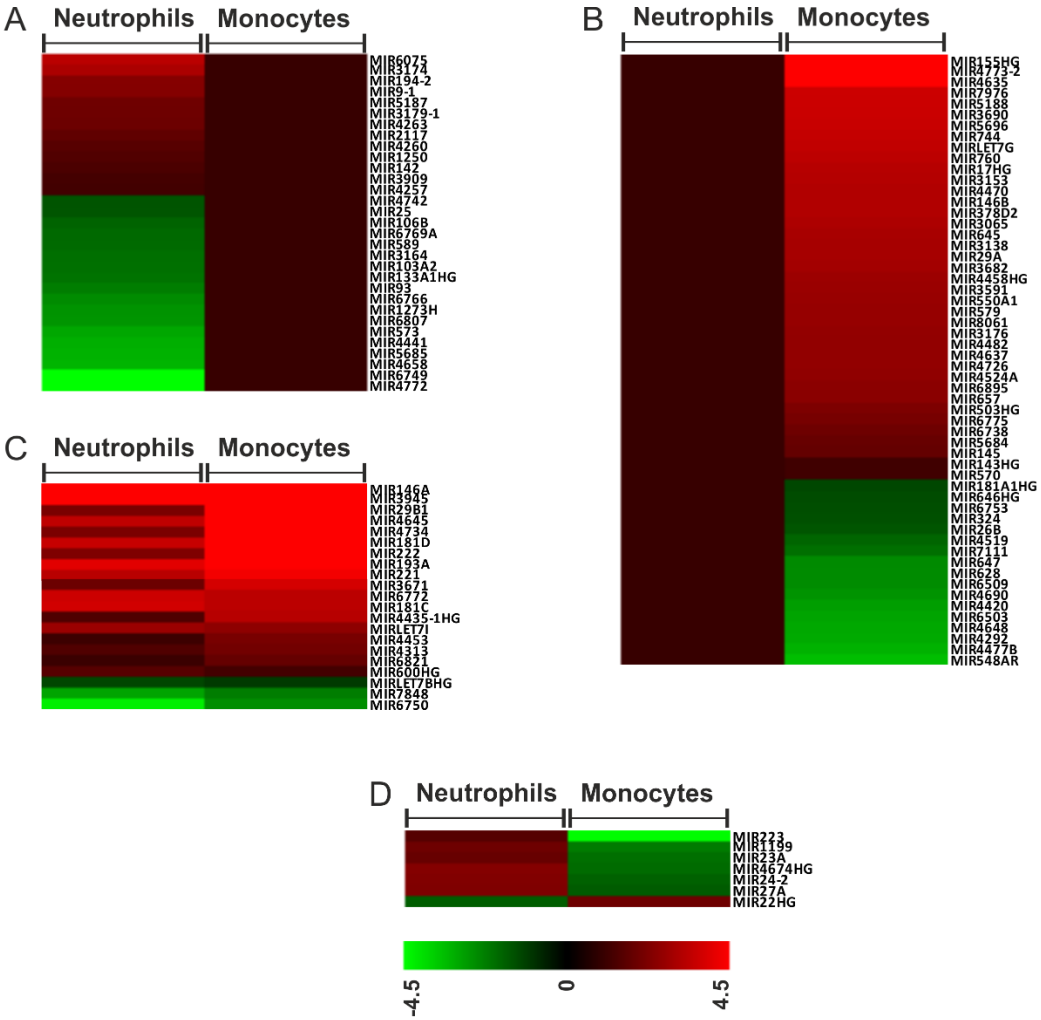


Figure 7. **Shared and cell type-specific pri-miRNAs modulated by LPS**.
Pri-miRNAs differentially regulated by LPS in neutrophils and monocytes were identified by DESeq2 and their regulation was compared between the two cell types. 31 pri-miRs were selectively modulated in neutrophils (A) and 56 selectively in monocytes (B). 21 pri-miRs were found to be modulated with the same pattern (C) and 7 with the opposite pattern (D) among neutrophils and monocytes. Cell type-specific and shared LPS-modulated pri-miRNAs are presented as Log_2FC (LPSvsT0).

4.3. Identification of pri-miRNAs upregulated by LPS in a TRIF-dependent manner.

LPS triggers different responses in neutrophils and monocytes, partly because of the selective activation of the different, MyD88- and/or TRIF-dependent, signaling pathways downstream of the pattern-recognition receptor TLR4 [70]. In neutrophils only the MyD88-dependent signaling is active, while in monocytes both these pathways are simultaneously activated downstream TLR4 [71]. It is conceivable that the monocyte-specific pri-miRNAs are not upregulated in response to LPS in neutrophils because they are transcriptionally activated downstream MyD88-independent pathway, which is not active in neutrophils. To test this hypothesis, we initially validated in monocytes the pri-miRNAs identified in RNAseq analysis as upregulated by LPS. Monocytes were purified and immediately lysed or cultured in the presence or absence of LPS for 4h, and the expression level of the pri-miRNAs was assessed by RT-qPCR. Sixteen out of the thirty-nine LPS-induced pri-miRNAs were undetectable in RT-qPCR (Ct > 35 in all the conditions), mostly consistent with their very low level of expression in RNAseq (FPKM <2). Induction of seventeen pri-miRNAs was successfully validated (Figure 8A). Furthermore, the expression of seven pri-miRNA (namely MIR3065, MIR378D2, MIR6895, MIR4482, MIR29A, MIR657 and MIR4726) increased in monocytes cultured for 4h in medium alone, and the presence of LPS did not further enhance their expression. This observation suggests that the transcription of pri-miRNAs identified as LPS-induced (4h LPS-stimulated vs freshly purified cells) might be triggered by events other than TLR4 activation, and occurring during 4h culture (Figure 8B, C).

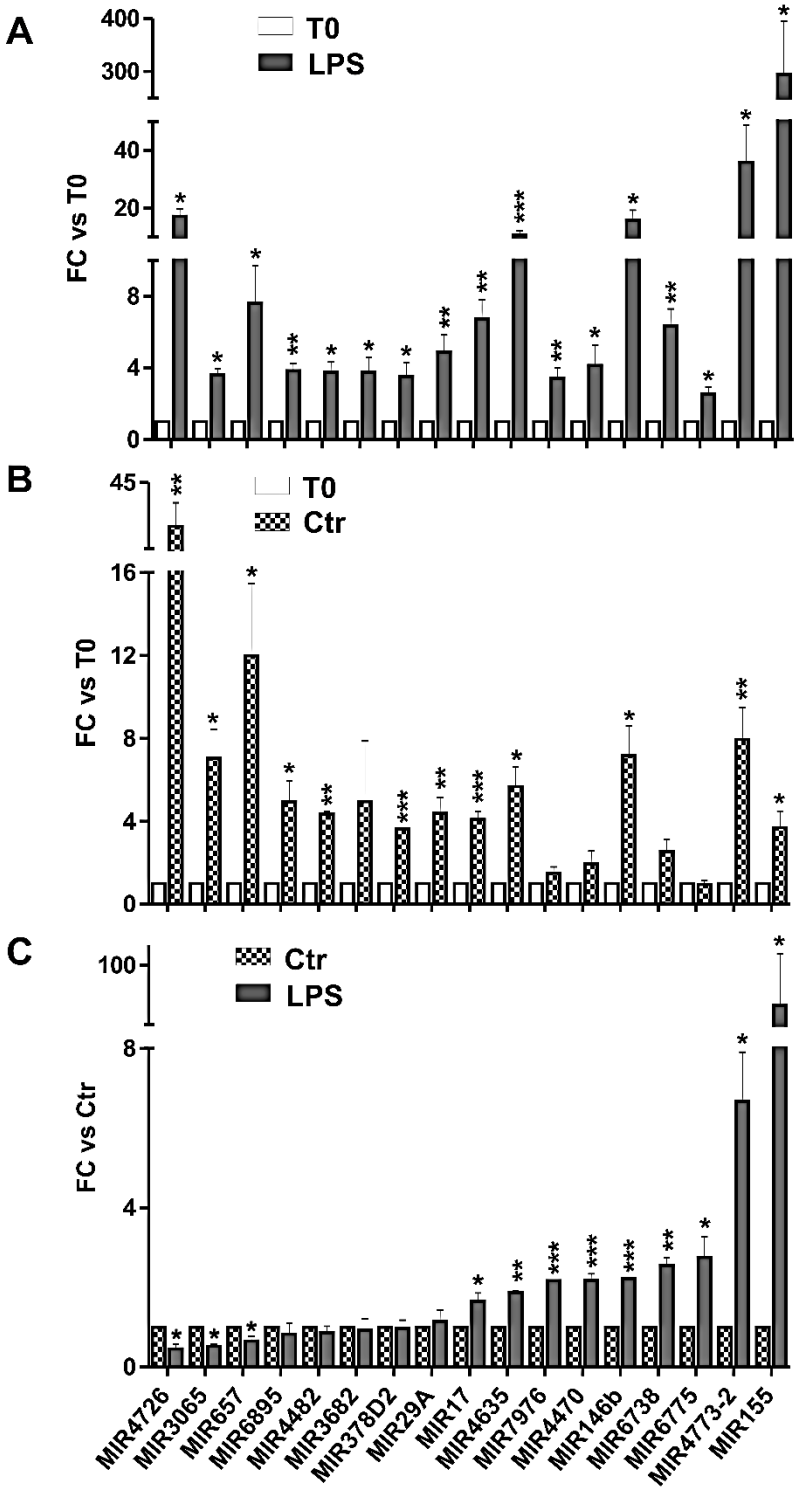


Figure 8. **Validation of LPS-induced pri-miRNAs in monocytes.**
RNA was purified from freshly purified monocytes or from monocytes cultured for 4h in the presence or absence of LPS (100 ng/mL). LPS-induced pri-miRNAs expression was determined by RT-qPCR and normalized to RPL32 as described in Materials and Methods. The results are expressed as fold change (FC) of the pri-miRNA expression in LPS-stimulated (A) or control (B) condition versus freshly purified cells, or in LPS-stimulated condition versus control cells (C). Data are shown as mean ±SEM of three independent experiments (ns P> 0.05, * P< 0.05, ** P< 0.01, *** P< 0.001).

Based on these observations, seven pri-miRNAs were then selected as LPS-induced (FC LPS vs medium >2): pri-miR-7976 (FC=2.2), pri-miR-6775 (FC=2.8), pri-miR-4773-2 (FC=6.7), pri-miR-155 (FC=79.8), pri-miRNA-4470 (FC=2.2), pri-miRNA-146b (FC=2.2) and pri-miRNA-6738 (FC=2.5) (Figure 8C). We then investigated the requirement of MyD88 and/or TRIF adaptors in the induction of the miRNAs primary transcripts expression by LPS. Monocytes were cultured in the absence or presence of LPS, or polyinosinic:polycytidylic acids [poly(I:C)] (50 μg/ml), a synthetic mimetic of viral double-stranded RNA (dsRNA) that interacts with endosomal TLR3 and utilizes TRIF-mediated signaling [70]. Poly(I:C) increased the expression of only MIR4773-2, indicating this pri-miRNA is transcriptionally induced downstream the TRIF pathway (Figure 9). Conversely, MIR155, MIR4470, MIR146b, MIR6775, MIR7976 and MIR6738, which were not induced by poly(I:C), are likely induced by LPS in a MyD88-dependent manner (Figure 9).

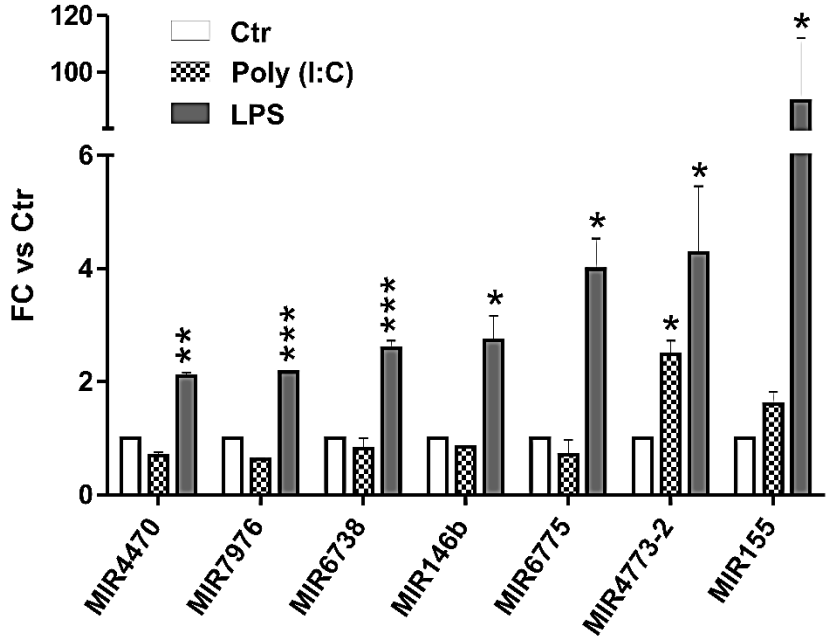


Figure 9. **pri-miRNA induction by TRIF-activating TLR agonist.**
Monocytes were cultured for 4h in the presence or absence of either LPS (100ng/ml) or Poly I:C (50ug/ml). RNA was extracted and the selected pri-miRNAs expression was determined by RT-qPCR. Pri-miRNA expression is depicted as fold change units after RPL32 normalization. Data are shown as mean ±SEM of three independent experiments (ns P> 0.05, * P< 0.05, ** P< 0.01, *** P< 0.001).

The identification of putative MyD88-dependent pri-miRNAs triggers the question of why these pri-miRNAs are not induced following LPS stimulation in neutrophils that can mobilize MyD88. To gain insights into the mechanisms responsible for the failure of LPS to upregulate the expression of MyD88-dependent pri-miNAs in neutrophils, we analyzed the accessibility state of chromatin in the pri-miRNAs promoter regions. To this aim, we took advantage of H3K4me3-ChIP-seq (histone 3 Lysine 4 trimetylated chromatin immunoprecipitation-coupled with deep sequencing) data available in the laboratory, which was performed in neutrophils and monocytes incubated with/without LPS for 4 hours (Tamassia N. et al. unpublished). H3K4me3 is a very well-studied marker of active/poised promoter [72-74]. Promoter region for intragenic miRNAs was considered as the promoter of their host genes. The promoter region of MIR146b was predicted based on its transcription start site obtained from miRStrat (http://mirstart.mbc.nctu.edu.tw/), a database of miRNA transcription start site. As the promoter region of MIR4470 has not been yet studied, we analyzed the state of H3K4me3 upstream the miRNA sequence.

The H3K4me3 state was analyzed in the region of the predicted promoters for MIR155, MIR4470, MIR6775, MIR7976, MIR6738 and MIR1446B (Figure 10). H3K4me3 signals were not detected at the MIR155 putative promoter in resting and/or LPS-activated neutrophils, whereas monocytes activation triggered a strong increase of MIR155 promoter-bound H3K4me3 (Figure 10A). Histone 3 embedded in the promoter region of MIR4470 was constitutively trimethylated and it significantly increased upon LPS-stimulation only in monocytes (Figure 10B). Taken together, these results indicate that the chromatin at the promoters of MIR155 and MIR4470 is not accessible in neutrophils, thus explaining the failure of LPS to induce these MyD88-dependent pri-miRNA

On the other hand, in the predicted promoter region of the other four MyD88-dependent pri-miRNAs we detected the same pattern of H3K4me3 in monocytes and neutrophils upon LPS-stimulation. Promoter region of MIR6775 was enriched in H3K4me3 after LPS-stimulation in both cell types (Figure 10C). H3K4me3 was constitutively detected in the promoter of MIR7976, MIR6738 and MIR146B in both cell types and LPS did not change the pattern in these regions

(Figure 10D, E and F). A possible explanation for the failure of LPS to upregulate these pri-miRNAs in neutrophils may rely on the need for cell specific chromatin modifications which are required for transcriptional activation.

Taken together these results indicate that TLR4 activation modulates microRNA expression profile in monocytes, and this modulation is precisely regulated for each microRNA via specific signaling pathways.

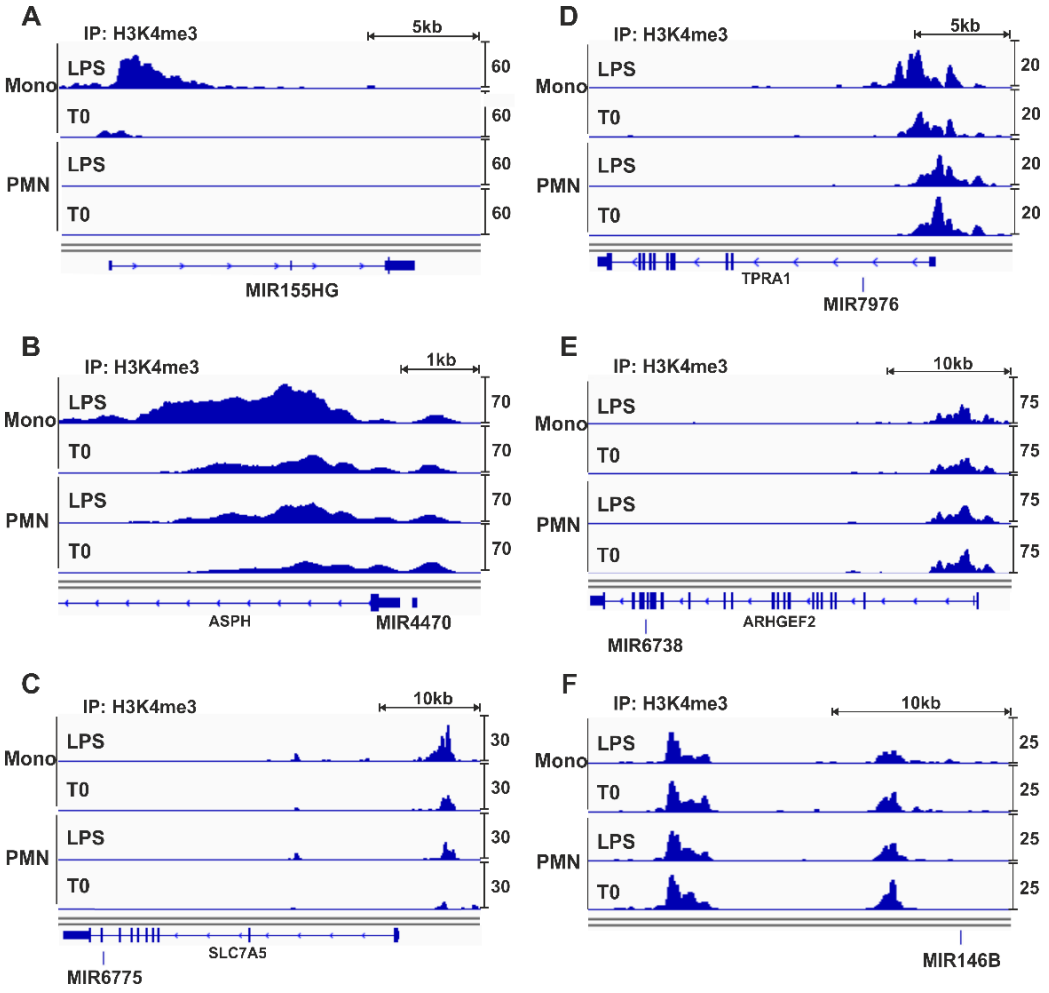


Figure 10. **H3K4me3 level at the MyD88-dependet miRNAs promoter region.** Genome browser view of promoter locus for MIR155 (A), MIR4470 (B), MIR6775 (C), MIR7976 (D), MIR6738 (E) and MIR146B (F) are presented illustrating H3K4me3 profile in monocytes and neutrophils freshly isolated or treated with 100ng/ml LPS for 4h.

4.4. LPS-induced pri-miRNAs in neutrophils

DGE analysis between neutrophils cultured for 4h in the presence or absence of LPS allowed us to identify LPS-modulated pri-miRNA in neutrophils. The primary transcript of let-7b was decreased (FC<0.5, p<0,05) and the primary transcripts for miR-146a, miR-221, miR-3945, miR-222, let-7i, miR-6821, miR-3909, miR-4453, miR-27A, miR-24-2, miR-4645 and miR-23A were significantly increased (FC>2, p<0,05) upon LPS stimulation for 4 hours (Figure 11A). The increased expression of all LPS-induced pri-miRNAs was validated by RT-qPCR (Figure 11B).

4.5. Mature forms of miR-23a cluster are increased by LPS

Given the difficulties in working with low-level transcripts such as pri-miRNAs and mature miRNA in neutrophils, we focused our subsequent analysis on those pri-miRNA that were detected at the highest levels in LPS-stimulated neutrophils. Among the twelve LPS-induced pri-miRNAs in neutrophils, expression of the miR-23a cluster primary transcript was detected at 189.5 FPKM in LPS-stimulated cells, as compared to the other pri-miRNAs whose expression didn't exceed 35.5 FPKM. Two different mature and functional microRNAs, named "3p" or "5p", can generate from processing of a pri-miRNA Time-course analysis was performed to identify the mature forms of each of the miR23 cluster (miR-23a, miR-27a and miR-24-2) in neutrophils stimulated with LPS. Both 5p and 3p mature forms of each miRNA was detected in unstimulated neutrophils. Interestingly, the constitutive expression of these mature miRNAs decreased over time in unstimulated cultured cells (Figure 12). In the presence of LPS only the mature miR-23a-5p and miR-27a-5p were significantly upregulated, whereas none of the other miRNAs showed significant induction by LPS (Figure 12A-F). Consistent with previous data, these results indicate that each miRNA of the cluster is subjected to a specific individual maturation process [25, 26, 75, 76].

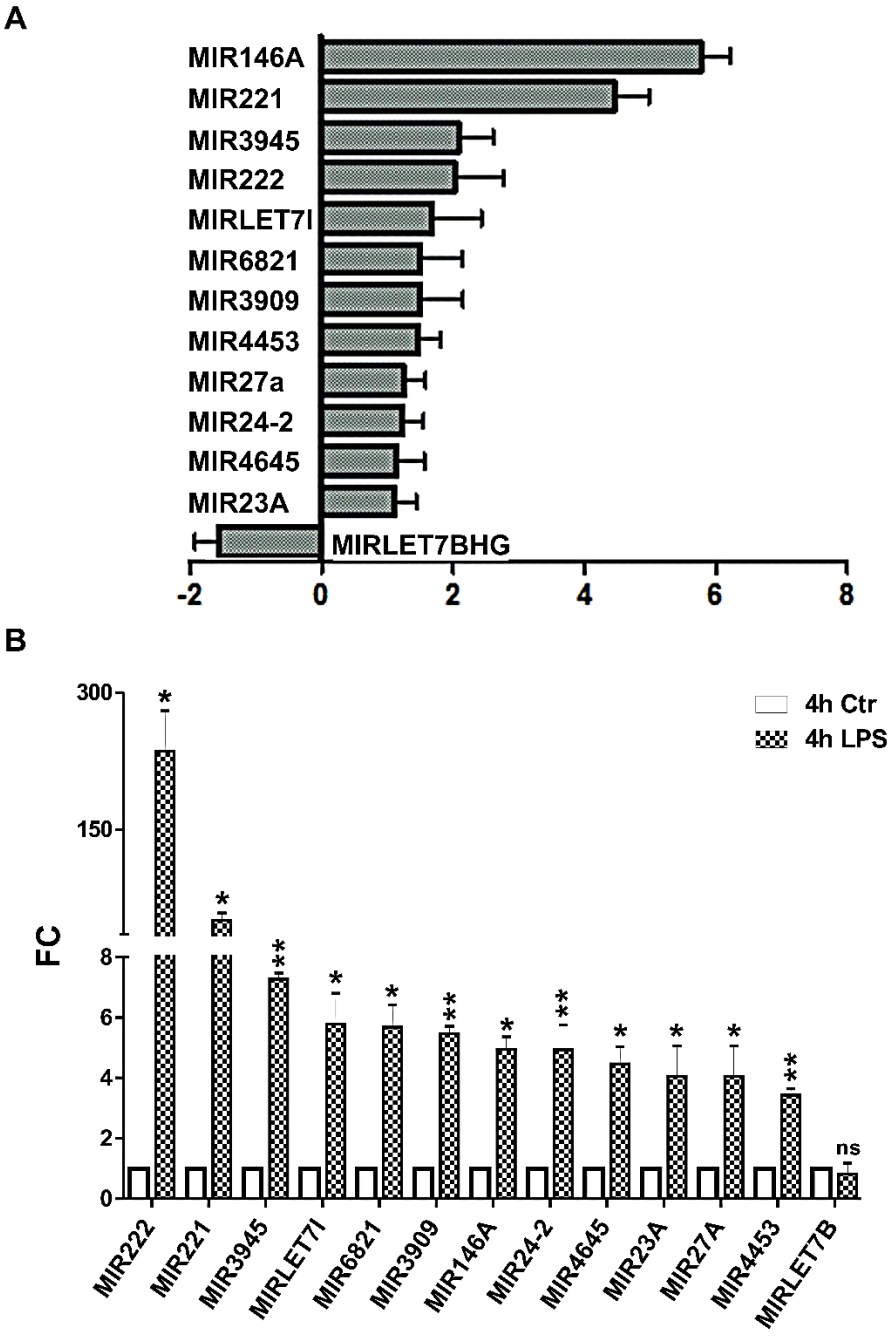


Figure 11. **LPS modulated pri-miRNAs in neutrophils.**
RNAseq data were analyzed in order to identify pri-miRNAs differentially modulated between neutrophils 4h in culture and 4h treated with LPS ($|Log_2FC|>1$, $p<0.05$). Significantly modulated pri-miRNAs are presented by the Log_2FC (4h LPS vs 4h Ctr) (A). Neutrophils were cultured for 4h in the presence or absence of 100ng/ml LPS, RNA was extracted and the expression of 13 selected pri-miRNAs were assessed by RT-qPCR and normalized to RPL32 expression. Fold change of expression is represented for each pri-miRNA in LPS treated neutrophil versus resting cells. Data are shown as mean ±SEM of three independent experiments (ns $P> 0.05$, * $P< 0.05$, ** $P< 0.01$) (B).

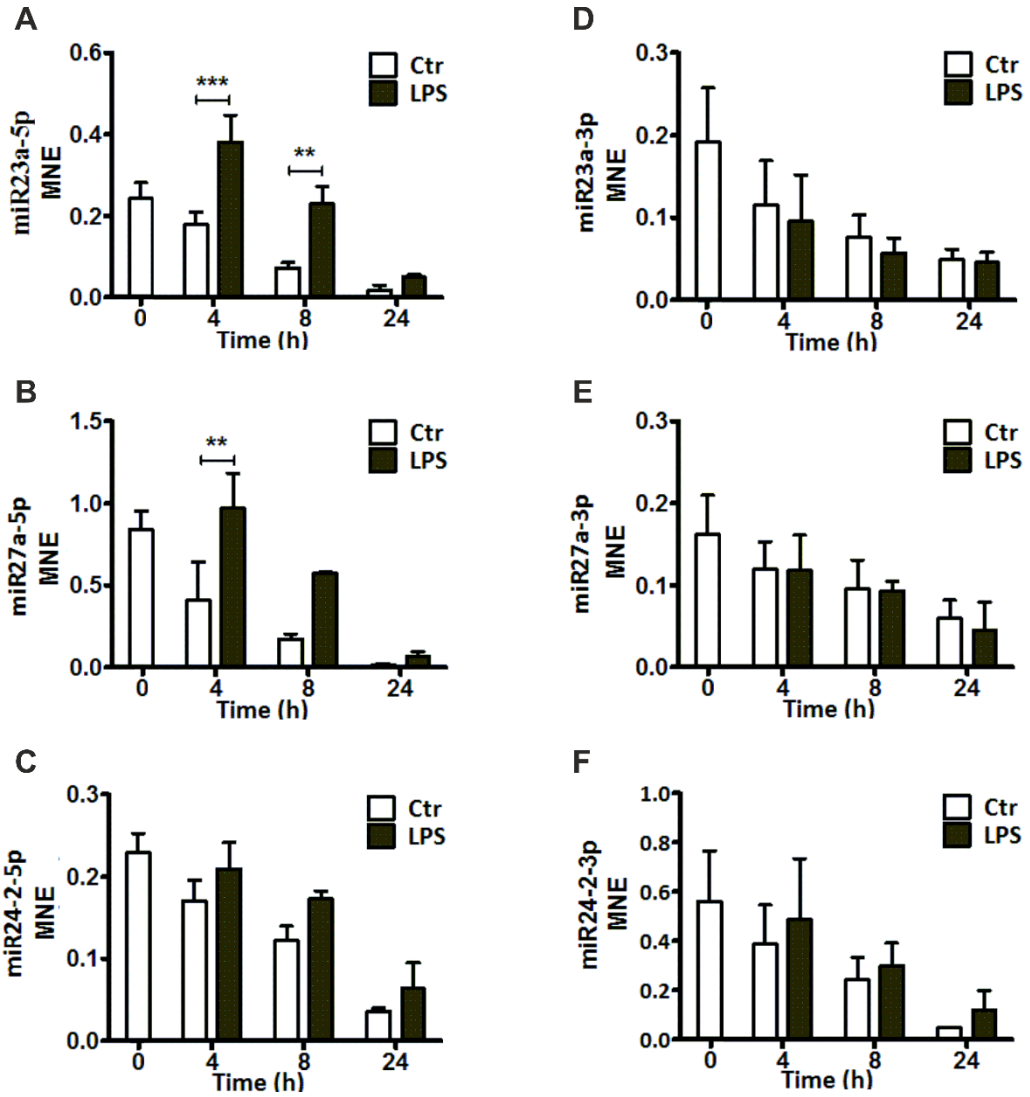


Figure 12. **Detection of the mature miRNAs of the miR23a cluster.**
Neutrophils were cultured in the presence or absence of 100ng/ml LPS for indicated time points. RNA was extracted and the expression of mature forms of miR-23a cluster members was assessed by RT-qPCR. Expression was normalized to RNU44 as described in Materials and Methods. Mean normalized expression (MNE) is presented at each time in LPS treated (black bars) and resting neutrophils (white bars). Data are the shown as mean ±SEM of three independent experiments (** P<0.01, *** P<0.001).

4.6. LPS modulates FAS expression in neutrophils via increasing miR-23a

Putative targets of miR-23a cluster

To gain insight into the biological relevance of miR-23a-5p and miR-27a-5p in LPS-stimulated neutrophils, microRNA target prediction databases, PicTar [77] and MiRanda [78], were interrogated. Putative targets commonly listed in both databases were identified and selected: 115 were predicted by both PicTar and MiRanda as common putative targets of miR-23a-5p and 231 of miR-27a-5p. The putative targets for each miRNA were then subjected to Gene Ontology (GO) terms association using PANTHER database (http://www.pantherdb.org/), and clustered according to the biological process (Figure 13). Genes in the GO subcategory 'immune system process' were then selected.

The immune related targets for each miRNA in the GO subcategory ‘immune system process’ are listed in the supplementary Table S3. Among all the immune related targets Fas was particularly interesting in the context of LPS-stimulated neutrophils s. Fas is a death receptor from the tumor necrosis factor superfamily. Fas receptor ligation by FasL mediates cell apoptosis and a role for Fas in controlling neutrophil lifespan has been described [79].

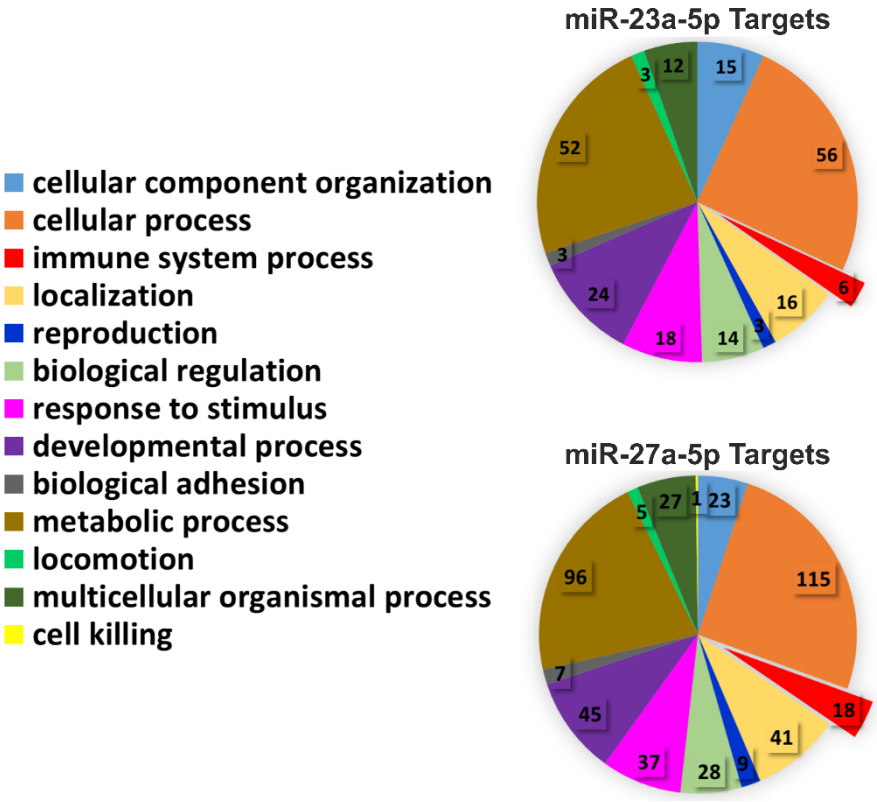


Figure 13. **GO term analysis of miR-23a-5p and miR-27a-5p putative targets.** Targets of miR-23a-5p and miR-27a-5p commonly predicted by PicTar and MiRanda, were analyzed for GO biological process terms using PANTHER database. Identified GO terms are plotted based on the number of genes in each of the terms among the targets of indicated miRNAs. The red highlighted slices are related to the term 'immune system process' which are considered as immune related genes.

LPS modulates Fas protein expression in neutrophils

In order to verify in silico miR-23a-5p and miR-27a-5p prediction, Fas protein and mRNA expression was initially analyzed in resting and LPS-stimulated neutrophils (Figure 14). Neutrophils were freshly purified or cultured in the presence or absence of 100ng/ml LPS. Cells were harvested at 4, 8 and 24 hours after stimulation and processed for Fas cytofluorimetric analysis and RNA purification . Cytofluorimetric analysis of membrane Fas showed a significant decrease in Fas protein expression in LPS-stimulated neutrophils (Figure 14A and B). Conversely, LPS significantly increased Fas mRNA expression over unstimulated cells at each time point (Figure 14C). Remarkably, the rate of Fas mRNA and protein expression were inversely correlated (Figure 14D), thus suggesting that a post-transcriptional regulatory mechanism is likely in place. Most importantly, the viability of neutrophils cultured for 8h in the presence or absence of LPS was assessed by Vybrant DyeCycle Violet staining as described in Material and Methods. A significant decrease in the percentage of the apoptotic neutrophils was observed in the presence of LPS (Figure 15), describing the pro-survival effect of LPS on neutrophils.

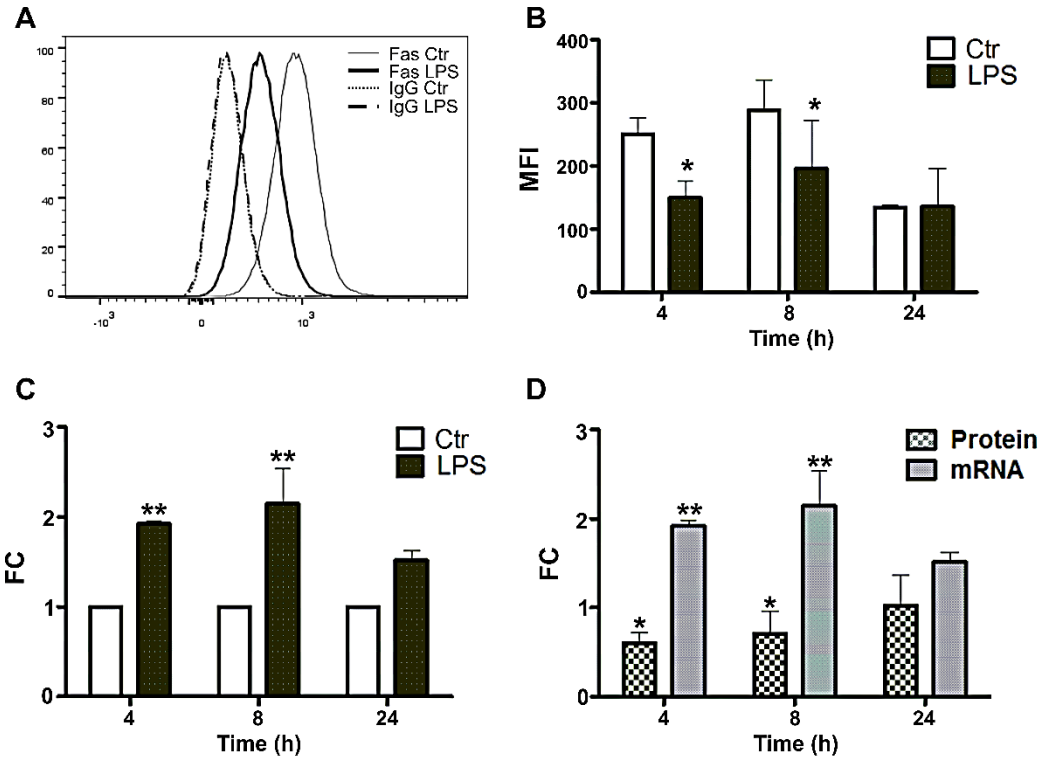


Figure 14. **Fas mRNA induction by LPS is not paralleled by the protein induction.**

Neutrophils were cultured in the presence or absence of 100ng/ml LPS for indicated time points. Cells were stained with anti-Fas mAbs or negative control mAbs. Fas expression assessed by flow cytometry is presented as the peak intensity at 4h in resting and LPS-stimulated neutrophils (A) or as the Mean Fluorescence Intensity (MFI) of Fas in indicated time points (B). RNA was extracted from the cells and the expression of Fas mRNA was assessed by RT-qPCR. Fas expression was normalized to RPL32 and is plotted as fold change (FC) of the level in LPS treated versus control cells in indicated time point (C). Fold change expression of Fas protein and Fas mRNA, in each time point, is reported for LPS treated versus resting cells (D). Data are the shown as ±SEM of three independent experiments (* $P< 0.05$, ** $P< 0.01$).

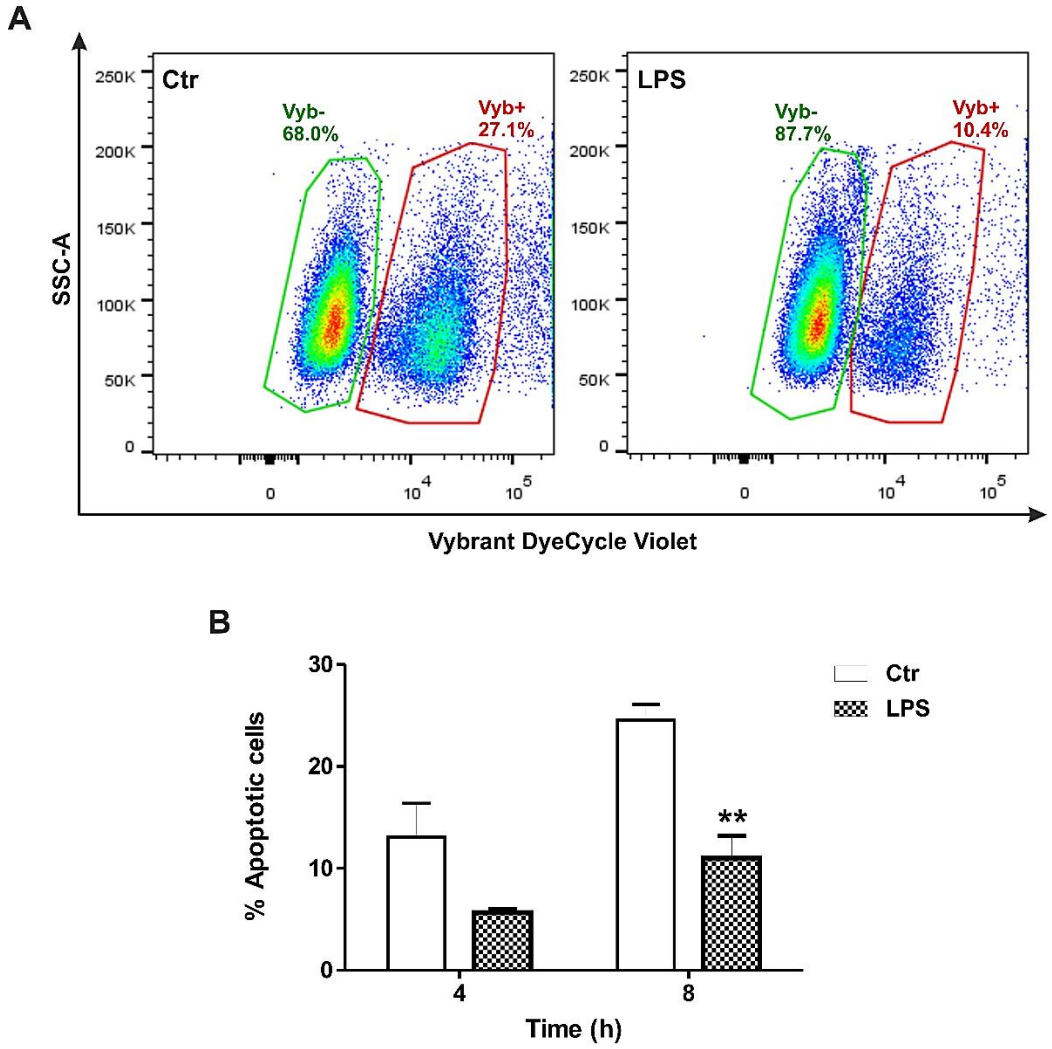


***Figure 15*. LPS protects neutrophils from apoptosis.**
Neutrophils ($1x10^5$) were cultured in the presence or absence of 100ng/ml LPS for 4 and 8h. Cells were harvested at mentioned time and were stained with Vybrant DyeCycle Violet to assess their viability by flow cytometry analysis. Gating strategy was used to identify neutrophils. Viable neutrophils were gated as Vybrant DyeCycle negative (green) and apoptotic neutrophils were gated as Vybrant DyeCycle positive (red) in 8h control or LPS-stimulated condition. Data are shown as one representative of three independent experiments. SSC-A, side-scatter-area (A). The percentage of apoptotic cells in each time point in control or LPS condition are presented as the Mean ± SEM of three independent experiments (B) (** P< 0.01).

Optimization of the RIP assay in primary human neutrophils

To proof a direct cause-effect link between the LPS-induced increase in miR-23a and miR-27a and the parallel decrease in Fas protein expression, we set up to perform a RIP assay in neutrophils. RIP is an assay which uses the stable physical association between the miRNA and the target mRNA to identify the miR–target interactions that occur in vivo. This assay has been successfully performed in many types of cell lines and primary cells. Nevertheless, performing RIP in neutrophils presents additional difficulties. In fact, the RIP protocol described in our previous work [65] in monocytes failed to produce the expected result in neutrophils since Ago proteins are degraded during the standard lysis procedure by the release of proteolytic activities that are normally stored in intracellular granules [16] (Figure 16A). In order to overcome this obstacle, neutrophils were lysed by nitrogen (N_2) cavitation, which has been already shown to preserve protein integrity [16] (Figure 16A). Under these lysis conditions, protein integrity is preserved. We next checked the quality of RNA obtained from N_2 neutrophil lysates. Microcapillary electrophoresis was performed using Agilent 6000 Nano kit in an Agilent 2100 Bioanalyzer following the manufacturer's directions. We used RNA purified with the RNeasy kit (Qiagen) as control for RNA integrity. Using nitrogen cavitation significantly decreased the RNA Integrity Number (RIN) to 3 (Figure 16C) from 8.7 of obtained by RLT buffer (Figure 16B), where a RIN of 1 represents almost fragmented and degraded RNA and a RIN of 10 represents intact and non-fragmented RNA [80]. In parallel, we show that RNA integrity remains quite the same for monocytes lysed with the two different methods (Figure 16D and E).

Given that neutrophil proteins and mRNA cannot be purified to integrity simultaneously with a single lysis method, we planned to modify the condition for miRNA and mRNA detection in degraded samples. JN Rusell et al reported that amplicon with shorter length are better detected among degraded RNA [81]. On these bases primer sets able to generate amplicons of different length were tested. We reasoned that mRNAs recruited to the RISC complex might be protected from degradation. Therefore, primers were designed to target mRNA regions most likely to be included into the RISC, such as the 3'UTR region and regions

surrounding the predicted miRNA seed region (Table 1). Neutrophils stimulated with LPS were lysed by N_2 cavitation. TNF-α, NFKB1 and GNB2L1 mRNA were analyzed in RT-qPCR using the two different primer sets able to generate long or short amplicons (Figure 17A). As shown in Figure 17B, using these specifically designed primer sets we succeeded in detecting mRNA in N_2 neutrophil lysates.

Our group previously reported that LPS induced miR-9 regulates the expression of NFKB1/p105 by directly targeting NFKB1 mRNA [58], meaning that NFKB1 mRNA should be recruited into the RISC in neutrophils after LPS stimulation. Based on these evidence, we checked the recruitment of miR-9 and NFKB1 into the RISC under the optimized condition for RIP assay in neutrophils. Neutrophils were purified and cultured for 4h in the absence or presence of LPS. Cells were lysed by N_2 cavitation, and immunoprecipitation with anti-Ago or IgG isotype control was performed as described in materials and methods. RNA was extracted by RNeasy Mini Kit (QIAGEN) and miR-9 and NFKB1 mRNA enrichment was analyzed by RT-qPCR. Figure 18 shows the successful detection of RIP-associated miR-9 and NFKB1, which are enriched only in neutrophils stimulated with LPS.

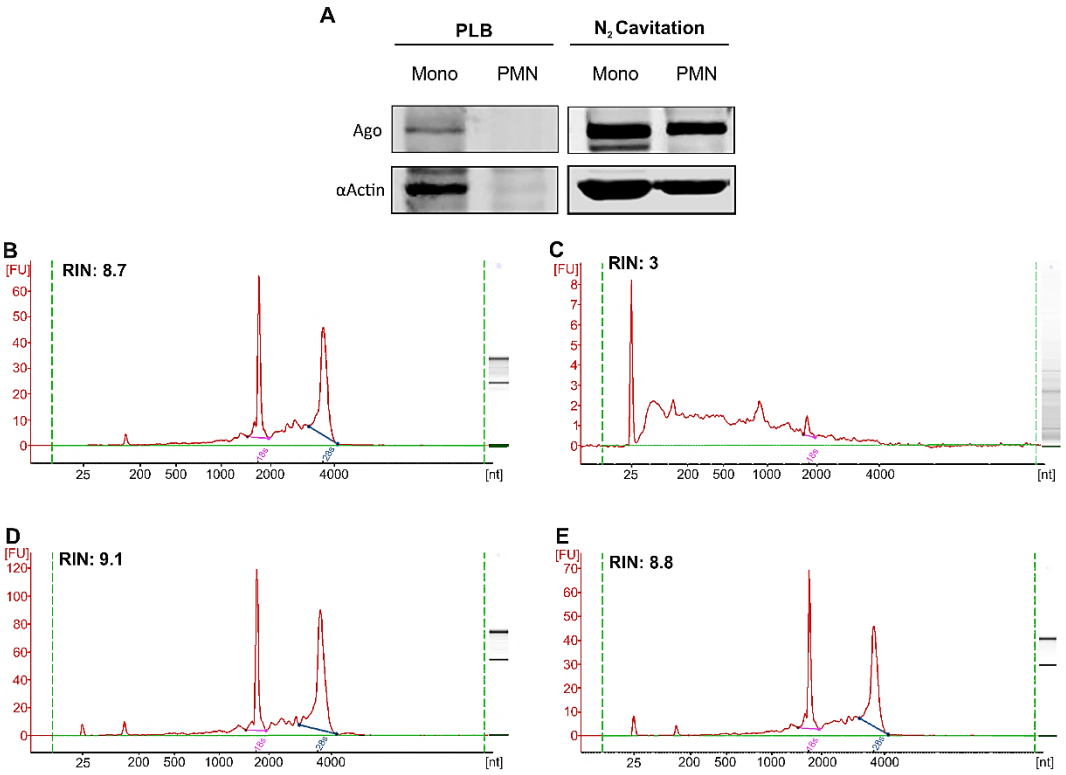


Figure 16. **Nitrogen Cavitation: the best substitution for PLB to lyse neutrophils.** Monocytes and neutrophils ($50x10^6$ cells) were lysed with PLB or by N_2 cavitation. An aliquot of lysates corresponding to $3x10^6$ cells were loaded on the gel and immunoblots were performed by Abs specific for Ago and αActin, followed by incubation with Alexa Fluor^R 680 goat anti-rabbit Abs. Shown is one experiment representative of three. The blots were scanned on the Odyssey Infrared Imaging System at 700 and 800 nm wavelengths (A). Neutrophil and monocytes were lysed by N_2 cavitation or RLT buffer (RNeasy kit, GIAGEN) as the positive control. RNA was isolated from both lysates by RNeasy Mini Kit (QIAGEN). RNA quality was checked using Agilent 6000 Nano kit in an Agilent 2100 Bioanalyzer. RNA Integrity Number is presented in each plot as RIN for neutrophil lysed with RLT (B) or N_2 cavitation (C) and monocytes lysed with RLT (D) or N_2 cavitation (E).

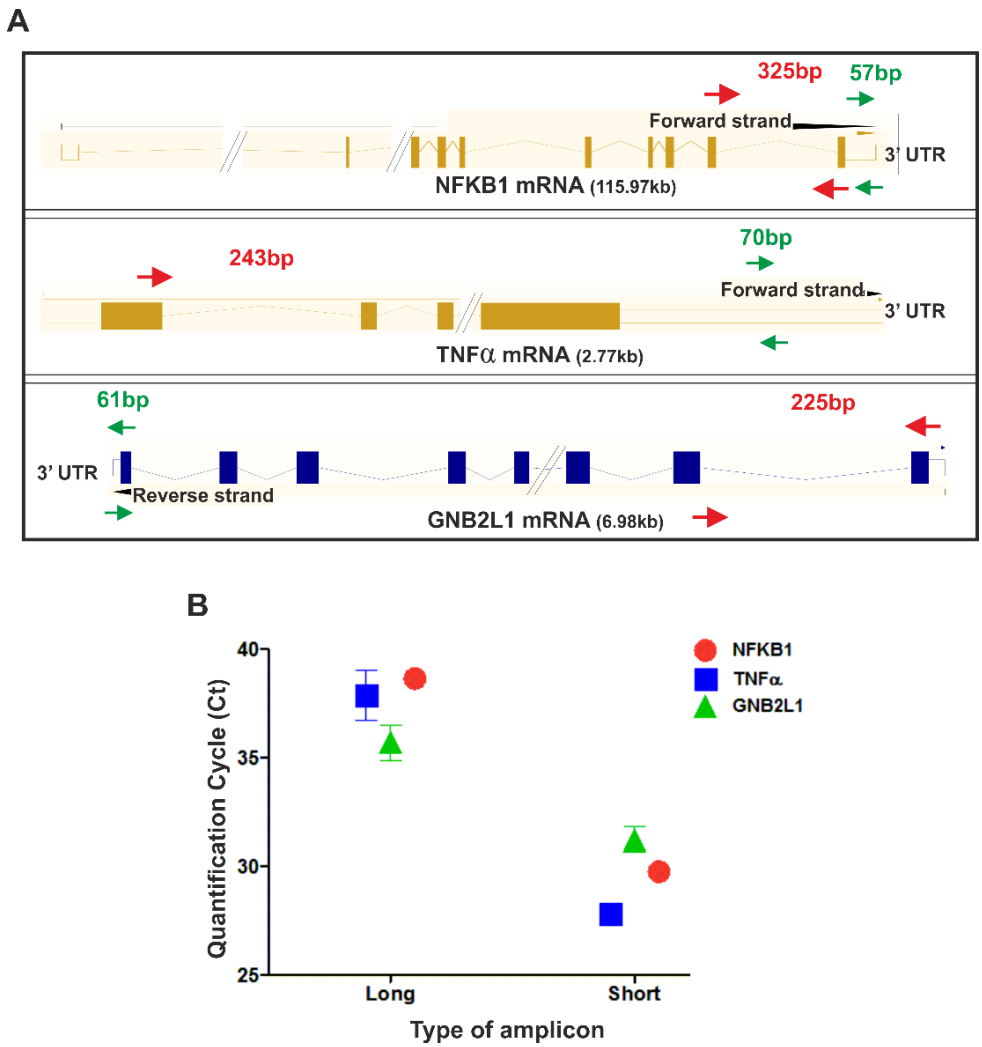


Figure 17. **Primer sets amplifying short amplicons, successfully detect mRNAs in N_2 neutrophil lysates.**

Two set of primers were designed to amplify short or long amplicons in selected genes. Schematic primer location and amplicon size are shown for each mRNA, primers with large amplicon size are shown in red and with short amplicon are shown in green (A). Neutrophils ($50x10^6$) were cultured in the presence of 100ng/ml LPS for 4h. Cells were lysed by N_2 cavitation and RNA was purified. The expression level of NFKB1, TNFα and GNB2L1 was assessed by RT-qPCR using both primer sets. The level of each mRNA is shown as Quantification Cycle (Ct) (B). Data are shown as mean ±SEM of three independent experiments.

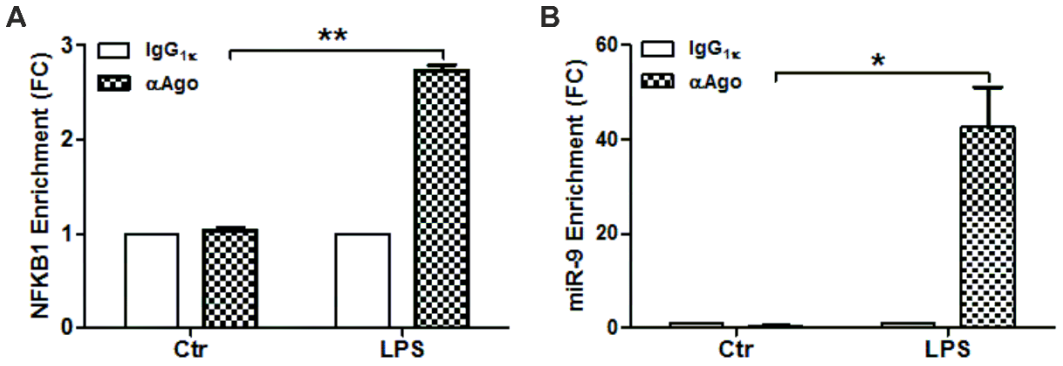


Figure 18. **NFKB1 mRNA and miR-9 simultaneously localize in Ago complexes.**
Freshly purified neutrophils were left untreated or were stimulated with 100ng/ml LPS for 8 h. Cells were lysed with N_2 cavitation for RIP assay and subjected to Ago or IgG (control) immunoprecipitation in parallel. The levels of NFKB1 (A) or miR-9 (B), were assessed by RT-qPCR, are expressed as normalized fold enrichment (FC) as described in Material and Methods, and are one representative of two independent experiments.

Enrichment of miR-23a and Fas mRNA into the RISC in LPS-stimulated neutrophils

Using the optimized protocol, RIP was performed in order to analyze whether miR-23a and/or miR-27a and Fas mRNA are simultaneously recruited to the RISC complex upon LPS stimulation. Neutrophils were incubated with or without LPS for 8h. Cells were lysed using N_2 cavitation and lysates were subjected to Ago immuno-precipitation. miR-23a and miR-27a and Fas mRNA level then were analyzed by RT-qPCR in the RNA fraction extracted from Ago and IgG immunoprecipitates. A significant recruitment of miR-23a-5p and miR-27a-5p, to the Ago complexes, was observed in LPS-stimulated neutrophils, (Figure 19A and B). Simultaneously, mRNA encoding for Fas was also significantly recruited to the Ago complexes upon LPS stimulation (Figure 19C), demonstrating that FAS gene is subjected to miRNA-mediated post-transcriptional regulation miR-23a, miR-27a and Fas mRNA were not detected in RIP from unstimulated cells, nor in IgG control immunoprecipitates, thus excluding the possibility that the observed increase in miR-23a, miR-27a and Fas mRNA is caused by a general, nonspecific enrichment of mRNAs in Ago immunoprecipitates.

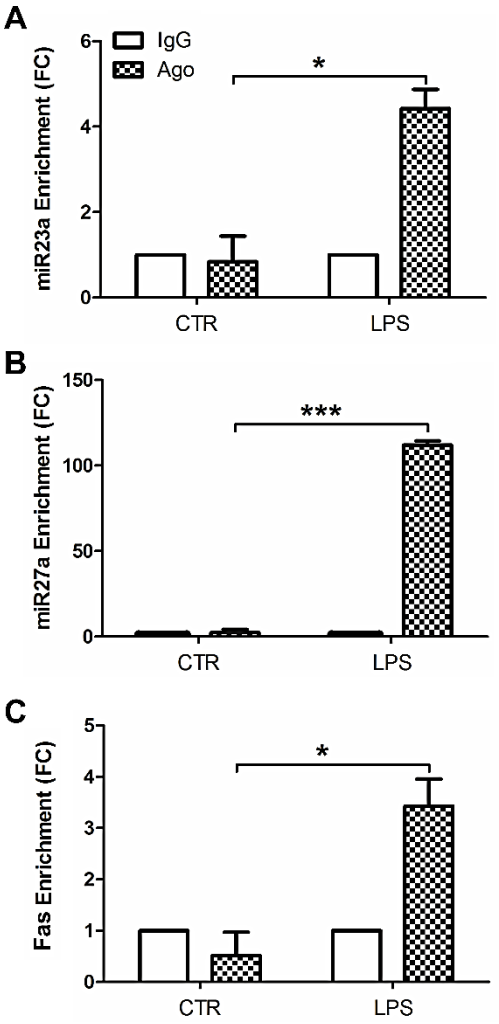


Figure 19. **Fas mRNA and miR23a/27a simultaneously localize in Ago complexes.**
Freshly purified neutrophils were left untreated or were stimulated with 100ng/ml LPS for 8 h. Cells were lysed for RIP assay and subjected to Ago or IgG (control) immunoprecipitation in parallel, as described in Section 3.10. The levels of the indicated miRNAs or mRNAs, were assessed by RT-qPCR, are expressed as normalized fold enrichment (FC) as described in Material and Methods, and are displayed as the mean ± SEM of three independent experiments for each panel. Asterisks indicate statistically significant (*P < 0.05, *** P<0.001) difference between LPS-stimulated versus resting neutrophils.

Overexpression of miR23a in neutrophils mirrors the effect of LPS on Fas expression

To provide direct evidence for Fas as a physiologic target of miR-23a, in a complementary approach to RIP assay, we tested whether miR-23a overexpression can directly influence the levels of the endogenous Fas protein expression. Pre-miR™ miRNA precursors were transiently transfected into neutrophils. Success of the microRNA transfection was evaluated in a dose response overexpression experiment. Freshly purified neutrophils were transfected with 50, 100 or 200 pmol of Pre-miR™ miRNA Precursor Molecules-Negative Control 2 (pre-miR-Ctr), Pre-miR™ miR-23a-5p precursor molecule (pre-miR-23a) or Pre-miR™ miR-27a-5p precursor molecule (pre-miR-27a). Cells were lysed for RNA extraction and the expression of miR-23a-5p and miR-27a-5p was detected Taqman miRNA assays. A dose dependent increase in miR-

23a and miR-27a was observed in neutrophils transfected with the pre-miRs as compared to the control pre-miR-Ctr (Figure 20A). The level of Fas mRNA (Figure 20B) and protein (Figure 20C and D) expression was then analyzed in neutrophils transfected with 200 pmol of miRNA precursors or relative negative control. Seven hours after transfection, miR-23a (Figure 20C), but not miR27a (Figure 20D), overexpression lead to a reduction in the expression of Fas protein while the expression of Fas mRNA remained comparable to that of pre-miR-Ctr-transfected neutrophils (Figure 20B). The reduction of the Fas membrane protein by pre-miR-23a overexpression was reproduced in three independent experiments (Figure 20D). Taken together these data provide clear evidence for Fas expression to be regulated in neutrophils by LPS via miR-23a.

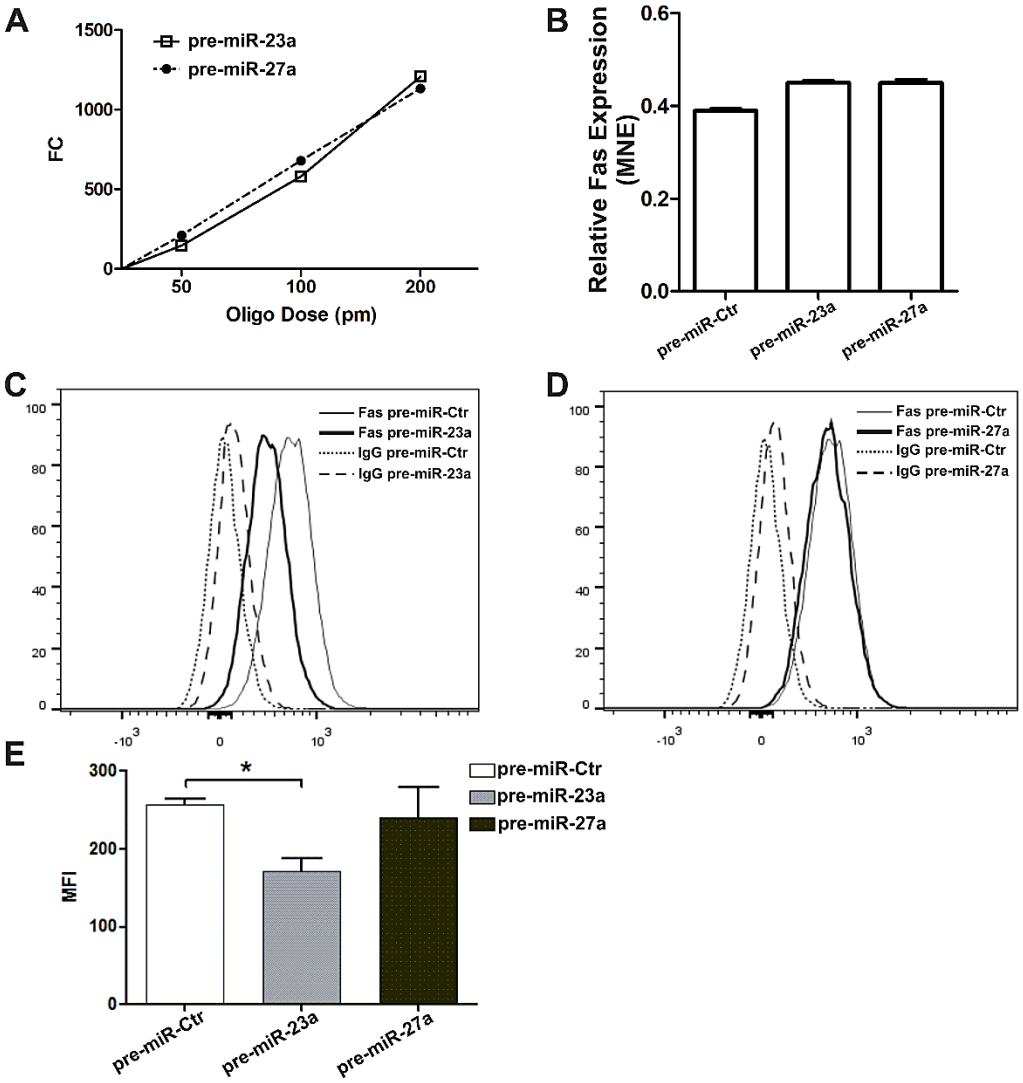


Figure 20. **Overexpression of miR-23a in neutrophils mirrors the effect of LPS on Fas expression.**

Freshly purified neutrophils were transfected with the indicated amount (A) or only 200pm (B, C, D) of pre-miR-Ctr, pre-miR-23a or pre-miR-27a. Cells were lysed for RNA extraction (A, B) or stained with anti-Fas/negative control mAbs for flow cytometry (C, D, E). The fold change expression of miRNAs normalized to RNU44 is represented versus their expression in cells transfected with pre-miR-Ctr (A). Fas mRNA expression relative to RPL32 is presented as MNE (B). Fas expression assessed by flow cytometry is presented for neutrophils transfected with pre-miR-Ctr or pre-miR-23a (C) and pre-miR-27a (D). Mean Fluorescence Intensity (MFI) of Fas in transfected cells is represented as ± SEM of three independent experiments (E) (* $p<0.05$).

5. Discussion

The role of miRNAs in the post-transcriptional regulation of protein-coding gene expression is nowadays well established, and several works highlighted the involvement of these short non-coding RNA molecule in innate immunity [82-85]. Identification of miRNAs expressed in innate immune cells such as neutrophils and monocytes under different conditions has been addressed by several studies, which greatly increased our knowledge on the role of miRNAs in neutrophils and monocytes biology. However, these studies have been performed so far by TaqMan based arrays [58, 86, 87], miRCury LNA based arrays [88], miRNA microarrays [89-91] or nCounter platform [92]. Studies using small RNA-sequencing defining the differential microRNA profile in monocytes and macrophages have been performed [93, 94]. However, a thorough genome-wide analysis of the transcriptional profile of miRNAs in neutrophils has not been performed yet. A limiting factor in high-throughput analysis of miRNA expression in neutrophils is represented by the low level of pri-miRNAs expression, by the very low RNA content of these cells (average 1 $\mu g/10^7$ cells), and by the considerable quantity of proteases stored in neutrophils' granules. Thus, modulation of protein-coding gene expression at a post-transcriptional level in neutrophils is not the object of extensive investigation mainly for the difficulties of working with this type of cells, rather than for a scarce interest in neutrophils biology. Indeed, polymorphonuclear neutrophils, traditionally viewed as short-lived effector cells, are nowadays regarded as important components of effector and regulatory circuits in the innate and adaptive immune systems. Most of the physiological functions of neutrophils rely on their capacity to modulate the expression of a number of proinflammatory and immunoregulatory genes. This fact has reevaluated the importance, role, and physiological and pathological significance of neutrophils in the pathogenesis of inflammatory, infectious, autoimmune, and neoplastic diseases and has identified neutrophils as an important potential target for selective pharmacological intervention to both promote and restrain inflammation. In this context, understanding the mechanisms of modulation of gene expression represents a critical step toward a better understanding of how neutrophils may influence pathophysiological processes in

vivo. In this general scenario, this study represents the first high throughput analysis, performed using a deep sequencing-based strategy, that characterizes the pri-miRNA transcriptome of primary human neutrophils freshly purified or kept in culture under resting and/or LPS-stimulated conditions. To gain more insight on the functional relevance of the miRNAs identified, the same analysis has been conducted in parallel on autologous monocytes cultured under the same conditions as their neutrophil counterpart. These additional data should aid deciphering the role of certain miRNA selectively expressed in either cell type.

MiRNAs are transcribed first as a long primary transcript, meaning that any regulation in miRNA expression at the level of transcription is firstly affecting the expression of pri-miRNAs. Additionally, it has been reported that the majority of pri-miRNAs are transcribed by RNA polymerase II, indicating that pri-miRNAs are structurally analogous to mRNAs and bear a 5′ 7-methyl guanosine cap and a 3′ poly-A tail [93, 95]. Accordingly, in order to identify miRNA repertoire responsive to LPS challenge in neutrophils we performed RNAseq based on a Poly(A) paired end library. In this study we detected 381 pri-miRNAs expressed in freshly purified neutrophils, as compared to the 224 detected by TaqMan based array [87] and 282 detected by miRNA microarray [91] performed by other groups. RNAseq analysis of LPS-stimulated neutrophils showed modulation of 59 pri-miRNAs. A more detailed analysis of the LPS-modulated pri-miRNA transcriptome, identified that LPS-modulated pri-miRNAs can be clustered according to their kinetic of expression. 66% of the pri-miRNAs can be rapidly modulated after 90 minutes of stimulation, after which pri-miRNAs expression return to the basal levels (primary and transient), or remains consistent over the time (primary); 34% of the pri-miRNAs are modulated after 4 hours of stimulation (Secondary). The kinetic of expression may be useful for the future identification of miRNAs function.

Cell specific expression and function are of the well-known characteristics of microRNAs [96, 97]. Performing RNA-sequencing on autologous monocytes in parallel to neutrophils gave us also the opportunity to identify cell specific pri-miRNAs, which might be related to specific responses of each cell type to LPS. We identified the expression of 31 pri-miRNAs modulated specifically in

neutrophils after LPS treatment compared to freshly isolated cells. In addition, we found that LPS specifically upregulates 39 pri-miRNAs in monocytes, seven of which were further validated by RT-qPCR as LPS-induced. It is conceivable that the monocyte-specific pri-miRNAs are not upregulated in response to LPS in neutrophils because they are transcriptionally activated downstream TRIF-dependent pathway, which is not mobilized by TLR4 engagement in neutrophils. However, we found that only MIR4773-2 was found to be transcriptionally induced by LPS in a TRIF-dependent manner. Furthermore, we show that the chromatin at the promoters of pri-miR-155 and pri-miR-4470 is not accessible in neutrophils, thus explaining the failure of LPS to induce these MyD88-dependent pri-miRNA. A possible explanation for the failure of LPS to upregulate the remaining monocyte-specific MIR6775, MIR7976, MIR6738 and MIR146B primary transcripts in neutrophils may rely on the need for additional cell-specific chromatin modifications required for transcriptional activation.

In neutrophils, twelve pri-miRNAs were identified as LPS-induced, among which the increase in the mature forms of the two members of miR23a cluster, miR-23a-5p and miR-27a-5p, was validated. miR23a cluster consists of miR-23a, miR-27a, and miR-24-2 which are highly conserved in different species [98] and are described to play important roles in various biological and pathological processes such as cell development [99], proliferation [100], differentiation [101], invasion and metastasis [102], immune response [103] and apoptosis [104]. miR23a cluster is described in various vertebrate cells and their function has been studied in different cell types [98], but their role in neutrophil has not been described yet. To gain insight into the biological role of miRNA, the identification of miRNA-terget gene(s) is mandatory. However, this is not an easy task as demonstrated by the limited number of bona fide miRNA targets that have been experimentally validated so far (DIANA TarBase database; Sethupathy et al. 2006) [105]. 115 and 231 potential targets for miR-23a-5p and miR-27a-5p respectively were identified by PicTar and miRanda miRNA target prediction databeses. As a strategy to restrict the number of putative target to study, we focused on genes that fall in the cathegory of “immune-related” according to GO

term analysis. Among these, FAS was particularly interesting for neutrophil biology, and was choosen for the subsequent analysis.

Fas, also known as CD95/APO-1/DR2, is a cell surface transmembrane death receptor from the tumor necrosis factor superfamily. Fas receptor ligation by FasL triggers a series of secondray messagaes inside the target cell wich mediates apoptosis [106, 107]. FasL is constitutively expressed on immune cells and triggers apoptosis of activated inflammatory cells, so FasL/Fas system plays an important role in the down-regulation of immune reactions [108]. During infection, the neutrophil life-span is extended, as exposure to bacterial LPS at the site of infection protects neutrophils from apoptosis [79, 109, 110]. Fas:FasL system has been shown to play a major role in neutrophil lifespan and clearance from the site of inflammation [108]. Most importantly, deregulation of Fas-mediated neutrophil apoptosis correlates with incidence of autoimmune disorders [111-113]. Cosistent with the current knowledge [79, 109, 110] , we show that neutrophils cultured in the presence of LPS have a prolonged half life and display a decreased level of membrane Fas. This occurs in the absence of downregulation of Fas mRNA expression, thus suggesting that a post-transcriptional mechanism regulating Fas protein expression is likely active. Several evidence demonstrate that Fas is a true endogenous miR-23a target gene: i) upon LPS stimulation the increase in miR-23a expression is paralleled by a decrease in Fas membrane protein expression; ii) the recruitment of both Fas mRNA and miR-23a to the RISC complex is induced by LPS, iii) overexpression of miR-23a in primary neutrophils results into a decreased Fas antigen expression, thus mimicking the effect of LPS. A role of miR-23a as antiapoptotic miRNA has been previously described in osteoblast cell lines, in which miR-23a protects cells from apoptosis by decreasing the expression of membrane Fas [104].

Herein, it is important to highlight that these results we succesfully achieved thanks to the optimization of protocols that have never been applied to primary human neutrophils: the detection of RISC-associated miRNAs/mRNAs and overexpression of miRNA mimics. These technical achievements are of great value and open the way for future molecular studies in primary human neutrophils.

In conclusion, based on the results obtained in this study, we propose a novel molecular mechanism for the pro-survival effect of LPS in neutrophils: LPS upregulates the expression of miR-23a, that in turn promotes the recruitment of Fas mRNA to the RISC complex where a translational silencing is achieved, leading to a reduced expression of the pro-apoptotic Fas antigen (Figure 21).

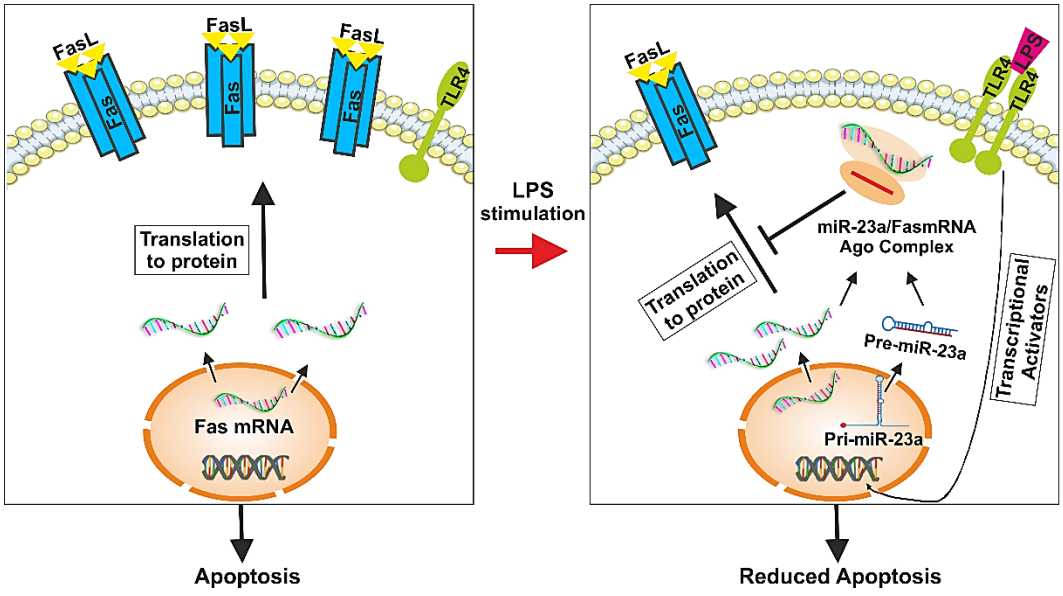


Figure 21. **The proposed model for pro-survival effect of LPS on neutrophils via mir-23a/Fas.**

Appendices

Supplementary Table S1. **Primers used in RT-qPCR analysis for pri-miRNAs.**

Target	Sense Oligonucleotide	Antisense oligonucleotide
Pri-miR103a-2	TGTAGCATTCAGGTCAAGCAG	ACACAGGGACAAGCCATAAG
Pri-miR106b	CTCCTTACCGTGCTCTCATTG	CAACCACCCTCTCAGTGAAG
Pri-miR1199	GCTGCGGGCTTTGAGAGTC	TCGGGGCACGGCGGAAAAC
Pri-miR1250	TCCCTGCCCCTGGTATGTC	GCTCCTCACTGGTTGATGG
Pri-miR1273h	GACTAAGGCAGGATTGCTTG	CCCACCCTCCAGAACCATG
Pri-miR133a-1	CCAAATCGCCTCTTCAATGG	GTTGTCCCGTAGTAATCAATGC
Pri-miR142	GTTGGGGGGATCTTAGGAAG	CAGTGCTGTTAGTAGTGCTTTC
Pri-miR143	AGTTGGGAGTCTGAGATGAAG	CAAGTGGCTGATAGTATGGAG
Pri-miR145	TCCTCACGGTCCAGTTTTCC	GACAGCCTTCTTCTTGAACC
Pri-miR145	TCCTCACGGTCCAGTTTTCC	GACAGCCTTCTTCTTGAACC
Pri-miR146a	CTGAAAAGCCGATGTGTATCC	CAAGCCCACGATGACAGAG
Pri-miR146b	CGGGAGACGATTCACAGAAG	CCTTGGCATTGATGTTGTAGC
Pri-miR155	CAAACCAGGAAGGGGAAATC	GCTAGTAACAGGCATCATACAC
Pri-miR15a	GCAGCACATAATGGTTTGTGG	CCTTACTTCAGCAGCACAGTT
Pri-miR17	CTGAAGATTGTGACCAGTCAG	CCATAATGCTACAAGTGCCTTC
Pri-miR181c	GTTTGGGGGAACATTCAACC	GCCTCAGGGTCCACTCAAC
Pri-miR181D	CACAGCCGAGGTCACAATC	AGCCACAGTGACATTCATCC
Pri-miR193a	GCTGAGGGCTGGGTCTTTG	CGAGAACTGGGACTTTGTAGG
Pri-miR194-2	GAAGCCTCGGTGAAAAGACC	CCTCGCCCCAGATAACAGC
Pri-miR2117	AGGAACTGGAGAGAATGCTC	CCTCAAGGCAATCAGATCAG
Pri-miR22	GAGCCGCAGTAGTTCTTCAG	CCAGATGATGGGGAAAGCAG
Pri-miR221	CCCAGCATTTCTGACTGTTG	AAACCCAGCAGACAATGTAGC
Pri-miR222	CTGCTGGAAGGTGTAGGTAC	GCTAGAAGATGCCATCAGAG
Pri-miR223	ATCTCACTTCCCCACAGAAG	GACAAACTGACACTCTACCAC
Pri-miR23a-27a	ACATTGCCAGGGATTTCCAAC	GAACTTAGCCACTGTGAACAC
Pri-miR24-2	CGCCTGTCTGCCTGCCATC	CCCTGTTCCTGCTGAACTG

Pri-miR25	GTTGAGAGGCGGAGACTTG	CTCACAAAACAGGAGTGGAATC
Pri-miR29A	CTAGCACCATCTGAAATCGG	CCCCAACGGTCACCAATAC
Pri-miR302b	GCTCCCTTCAACTTTAACATGG	GACACCTCCACTGAAACATGG
Pri-miR3065	GCCCTCTTCAACAAAATCACTG	GTCCTCTCCAACAATATCCTG
Pri-miR3138	CTGTGGACAGTGAGGTAGAG	GAAGATGCTGGGAAACTTCTG
Pri-miR3153	CCTGAAGCACCGTACTAGAAC	AAGTGTCCCGCATACAGTAAG
Pri-miR3164	GCACACATCCACAGTAAGGC	TGAGAGGTCCTGGGAAACTG
Pri-miR3174	CGTTACCTGGTAGTGAGTTAG	GTCCATCAGTGCTTTACAGAC
Pri-miR3176	TGTGGGGCGTGTGTTAGAC	CAGGGTGAAAGAGAAAGAGC
Pri-miR3179-a	TCTGTGATCCTGGGGTTGTTG	GTAAGAAGGGCTGCTGTTTC
Pri-miR3591	GCTCTGCTAAGGAAACTCTG	GCTTGTACCCGTGATGCTTC
Pri-miR3671	GTAAACACCAAGCCAGCATC	CACTCCCGTGTGGGCAACA
Pri-miR3682	CATAGCCCCCGTCCGTATC	TACCTCCACCTGTATCATCATG
Pri-miR373	CTTCCCCCATAGCAAGCAAG	AAGCACTTCCCAGTACAGAC
Pri-miR378D2	GGTTACAAGGAGAGAACACTG	GGAACAGAAATGGGACTTAGAG
Pri-miR3909	GACAGGGAAAAGCACACAAG	CCTCCAGGACAGCACTTAG
Pri-miR3945	CCCAGTACCCTAAAAGGAAC	CTGTGGACCAGTCAGGAATC
Pri-miR4257	GGGGACTGAGCCTTAGTTGG	GGAGCAGGGAGGATCACTG
Pri-miR4260	TGACACCCACCTTCAACAAG	CACCAGAAACAGCCAGACATC
Pri-miR4263	GCTCTTCAGGGTTTTACTTGG	TGAGATCCACACTGCTTGTG
Pri-miR4313	GTGGGGACACTGGGATCAG	CTCCTGCTCGTCACATTCAC
Pri-miR4313	GTGGGGACACTGGGATCAG	CTCCTGCTCGTCACATTCAC
Pri-miR4435	TACATCTCCTCAGAGTAAGGC	CTTCCCTGATAGCATCTGTTG
Pri-miR4441	TTGGAGCAGCAGTTTGTCAG	TGCGGTTCTGTCTTGTTCTG
Pri-miR4453	CTTGGTCTGTAGCGGTTTCC	ATCAGGCTGTAGGGTAACTC
Pri-miR4458	GTGTCTGTGTTACCAGTATGTG	CCTCACTCATTTCTTCTCCAC
Pri-miR4482	CTTCTCGTGGTGGTGATGTG	CCCCTTCAAATCCTTCCTATC
Pri-miR4635	GCAAAGTCCTGGTTTCTTCC	CAGCGTGACAGTCTCTATCC
Pri-miR4637	GTTAGGACCCTTACTTGGATC	AGAACCCTCACTTGAATCTGC
Pri-miR4645	AAGTTGCGACGAGACAGTAG	ACTCCAGTAGTGACAAACCG
Pri-miR4658	CCTTCACTCAGAGCATCTAC	AGCGGTCATCACAAAACACG

Pri-miR4726	CAGCCTCCCCGCACTCTTC	CACCCCTCCTCACTGTTGAC
Pri-miR4742	ATCTGTATTCTCCTTTGCCTGC	CCACTAGCGATGAAGTCTTC
Pri-miR4772	CTCTATGGTCAGGTTGAGTC	GGAAGACACAAGGGTATTGC
Pri-miR4773-2	AAACGCTGTCTCAGAATCAGTG	AGAAAGGCTGGGGAGCATC
Pri-miR503	TCGTGGGGAAGGTAGAAGG	GCCTAGCCAGGAATACTGC
Pri-miR5187	CTGGATGGGGAAAAGACTAAG	CACCTGAGGAAAAGAGGATTC
Pri-miR5188	AGGTATGGAAAGGTCCTACTTG	GGGTCCGATTATTGAATGGATC
Pri-miR5684	CAGTTGTGGTGTGCTTGTAG	GTTGCTCAGGCTAGAGTTCAG
Pri-miR5685	GCAGCACAGTAGGCACTATG	CCTTGTAATCACCTCTTGTTCC
Pri-miR5696	AGTCTGATGCCTACTACTGATG	CGCAGGAAGATGAGTCTATC
Pri-miR570	GGTTTTGGGTGGAGAAAGAG	CTGTGGATGTTATGCTGACTTC
Pri-miR573	GCGGTTTCTCCCTGAAGTG	ACTCTCCTCACCCTGACAAG
Pri-miR579	TGGACAACCTCTAACCTTTCTG	AATCGTCATCTGGCACAAACC
Pri-miR589	TGCTCATAATGCCATCTCTGTG	CCACCTGCTCCTTCCACTG
Pri-miR600	GGAAGGGAAACTGACAAACTG	CACTGCCTGACAGACAAGAG
Pri-miR6075	TTGCTCCAGAGACACCACATG	TTGGCACCAGTGTCGTCAC
Pri-miR616	GTAATTCCTCCACTCAAAACCC	GCAACTCTATTCACCATACAGC
Pri-miR645	GCAGTCCAGTTCCTAACAGG	CAGTCTCCAGGCTTCTCTG
Pri-miR657	TGGGGAGCCGAGAGTGATAG	GCCTAGAGAGGGTGAGAAC
Pri-miR6738	CTAAATACCCTCCCAATCCAAG	AACCCCTGTGCTCTTCTACC
Pri-miR6749	GCTCTGTCCTGTCTCACTC	GGTCACGCTGGTTGATGAG
Pri-miR6750	GCTGTGTTAGCGAAGGGAAC	GGAGCAGAGGGTGAGTTCT
Pri-miR6766	TTCTCCTGCTCCTGATTGTC	GCTCTGTGGTCCCCTTCAG
Pri-miR6769A	TGGTATGGAAGCCTGGTAAG	CTCCTGGTTGTTCTTGTTCTC
Pri-miR6772	CCACAGCCACATCAGTCATC	CTTCTCCTTCAGGTGTTCTAC
Pri-miR6775	GGCGGCTCAGGGTTTCCAG	GGGGCTCAGGCGGCTTATG
Pri-miR6807	GTGAGCCAGTGGAATGGAG	CCAGCACAGCAGAGAACTTG
Pri-miR6821	CCTTAGGCACCCGAGTGTAC	GATGTCCCCACACACCATATC
Pri-miR6895	CCAGGCACAGAGTAAGCATC	TCCAGCAGCGTGTAGCAAG
Pri-miR744	TAGGGCTAACAGCAGTCTTAC	TGGCACGACACAATAAGGAC
Pri-miR7976	CTCAGGGCAGATTTTCATTGG	TGGTAAGAGCACCGTTTTCC

Pri-miR93	TGTGCTACAGACCTTCCTTTC	AGACCAGACCCTTTTGAACG
Pri-miR940	TTCAAGCCGAAGCATCCAAC	GCCTTCCTTCCTCAACACAC
Pri-miRLet7b	GTAGGTTGTGTGGTTTCAGG	CAGGGAAGGCAGTAGGTTG
Pri-miRLet7i	TGGCTGAGGTAGTAGTTTGTG	AGCACTAGCAAGGCAGTAGG

Supplementary Table S2. **List of miRNA genes detected in freshly isolated neutrophils and/or monocytes.**

	PMN	Mono		PMN	Mono		PMN	Mono
MIR3180-3	0.0069	0.0000	**MIR4322**	0.0707	0.0000	**MIR548AT**	0.2580	0.0000
MIR4523	0.0129	0.0000	**MIR553**	0.0721	0.0000	**MIR7155**	0.2719	0.0000
MIR1284	0.0408	0.0000	**MIR181D**	0.0754	0.0000	**MIR140**	0.2792	0.0000
MIR766	0.0438	0.0000	**MIR6806**	0.0766	0.0000	**MIR5700**	0.2798	0.0000
MIR582	0.0438	0.0000	**MIR4692**	0.0778	0.0000	**MIR491**	0.2816	0.0000
MIR92B	0.0450	0.0000	**MIR4459**	0.0782	0.0000	**MIR1227**	0.2904	0.0000
MIR148B	0.0450	0.0000	**MIR6846**	0.0817	0.0000	**MIR4285**	0.2945	0.0000
MIR30E	0.0457	0.0000	**MIR4726**	0.0845	0.0000	**MIR548H1**	0.3011	0.0000
MIR365B	0.0465	0.0000	**MIR6833**	0.0846	0.0000	**MIR4646**	0.3236	0.0000
MIR181C	0.0469	0.0000	**MIR6504**	0.0846	0.0000	**MIR6815**	0.3257	0.0000
MIR504	0.0474	0.0000	**MIR548AK**	0.0860	0.0000	**MIR5696**	0.3552	0.0000
MIR624	0.0505	0.0000	**MIR340**	0.0883	0.0000	**MIR33A**	0.3628	0.0000
MIR617	0.0505	0.0000	**MIR6131**	0.0899	0.0000	**MIR4773-2**	0.4599	0.0000
MIR607	0.0511	0.0000	**MIR450A2**	0.0930	0.0000	**MIR3939**	0.4821	0.0000
MIR132	0.0511	0.0000	**MIR1976**	0.0993	0.0000	**MIR6867**	0.5199	0.0000
MIR141	0.0516	0.0000	**MIR6826**	0.1027	0.0000	**MIR101-1**	0.5987	0.0000
MIR548Q	0.0516	0.0000	**MIR576**	0.1027	0.0000	**MIR6812**	0.8588	0.0000
MIR602	0.0527	0.0000	**MIR6075**	0.1032	0.0000	**MIR548E**	0.9825	0.0000
MIR3688-1	0.0527	0.0000	**MIR629**	0.1038	0.0000	**MIR3065**	2.2566	0.0000
MIR595	0.0538	0.0000	**MIR320C1**	0.1144	0.0000	**MIR5685**	4.8998	0.0000
MIR326	0.0543	0.0000	**MIR3135A**	0.1307	0.0000	**MIR137HG**	0.0021	0.0031
MIR558	0.0549	0.0000	**MIR190B**	0.1313	0.0000	**MIR7515HG**	0.0029	0.0016
MIR5192	0.0561	0.0000	**MIR4667**	0.1336	0.0000	**MIR3180-1**	0.0067	0.0288
MIR4296	0.0587	0.0000	**MIR6876**	0.1379	0.0000	**MIR497HG**	0.0079	0.0071
MIR938	0.0591	0.0000	**MIR302B**	0.1379	0.0000	**MIR490**	0.0088	0.0118
MIR4511	0.0593	0.0000	**MIR4480**	0.1381	0.0000	**MIR181A1HG**	0.0124	0.2841
MIR548L	0.0600	0.0000	**MIR941-3**	0.1398	0.0000	**MIR3654**	0.0124	0.0441
MIR4802	0.0613	0.0000	**MIR4533**	0.1418	0.0000	**MIR3180-4**	0.0143	0.1672
MIR548P	0.0615	0.0000	**MIR6737**	0.1438	0.0000	**MIR133A1HG**	0.0169	0.0045
MIR4753	0.0622	0.0000	**MIR6852**	0.1525	0.0000	**MIR205HG**	0.0186	0.0061
MIR15A	0.0622	0.0000	**MIR6089**	0.1532	0.0000	**MIR3911**	0.0201	0.1436
MIR8087	0.0628	0.0000	**MIR5091**	0.1581	0.0000	**MIR503HG**	0.0209	0.1801
MIR4783	0.0630	0.0000	**MIR604**	0.1648	0.0000	**MIR600HG**	0.0243	0.0381
MIR4284	0.0637	0.0000	**MIR548AY**	0.1857	0.0000	**MIR4454**	0.0251	0.0096
MIR4294	0.0645	0.0000	**MIR3609**	0.1871	0.0000	**MIR3179-1**	0.0294	0.0769
MIR1200	0.0645	0.0000	**MIR378E**	0.1894	0.0000	**MIR7851**	0.0323	0.1290
MIR7974	0.0653	0.0000	**MIR4433A**	0.1948	0.0000	**MIR4500HG**	0.0325	0.0131
MIR5693	0.0672	0.0000	**MIR4718**	0.2024	0.0000	**MIR99AHG**	0.0337	0.0202
MIR4752	0.0681	0.0000	**MIR548C**	0.2075	0.0000	**MIR3615**	0.0365	0.0669
MIR3165	0.0688	0.0000	**MIR99B**	0.2212	0.0000	**MIR335**	0.0398	0.0235
MIR6853	0.0698	0.0000	**MIRLET7G**	0.2255	0.0000	**MIR17HG**	0.0418	0.1715
MIR4699	0.0698	0.0000	**MIR548AC**	0.2287	0.0000	**MIR191**	0.0434	0.0506
MIR3159	0.0698	0.0000	**MIR6803**	0.2303	0.0000	**MIR1255A**	0.0434	0.0506
MIR4689	0.0700	0.0000	**MIR4673**	0.2537	0.0000	**MIR1301**	0.0438	0.0444

	PMN	Mono		PMN	Mono		PMN	Mono
MIR3657	0.0441	0.1829	MIR342	0.1017	0.1080	MIR378J	0.2770	0.8057
MIR7-3HG	0.0445	0.0190	MIR3179-3	0.1027	0.0550	MIR6719	0.2847	0.3775
MIRLET7I	0.0454	0.2580	MIR7845	0.1043	0.5829	MIR941-1	0.2868	0.0691
MIR146A	0.0456	0.0690	MIR4754	0.1102	0.1202	MIR6855	0.2965	0.1708
MIR331	0.0465	0.0516	MIR2117	0.1102	0.2016	MIR6858	0.3082	0.4566
MIR212	0.0469	0.0520	MIR4644	0.1167	0.0592	MIR3153	0.3084	0.0607
MIR210HG	0.0477	0.0805	MIR6077	0.1196	0.0698	MIR6842	0.3097	0.2296
MIR4782	0.0478	0.0921	MIR1238	0.1213	0.0689	MIR6781	0.3104	0.0894
MIR579	0.0500	0.0508	MIR1285-1	0.1229	0.2458	MIR8075	0.3129	0.0622
MIR4440	0.0500	0.1168	MIR499A	0.1248	0.0469	MIR3127	0.3185	0.4370
MIR550A1	0.0505	0.0513	MIR3192	0.1360	0.1466	MIR7107	0.3194	0.8551
MIR151B	0.0511	0.1710	MIR219A1	0.1361	0.3301	MIR3936	0.3238	0.1493
MIR143HG	0.0522	0.0269	MIR3181	0.1379	0.0681	MIR548AR	0.3440	2.4350
MIR145	0.0536	0.0274	MIR6752	0.1418	0.3013	MIR371B	0.3506	0.2365
MIR4999	0.0539	0.0629	MIR6810	0.1438	0.6326	MIR4668	0.3539	0.1528
MIR6728	0.0551	0.1202	MIR6851	0.1463	0.1485	MIR194-2	0.3543	0.0458
MIR4258	0.0567	0.1175	MIR6885	0.1525	0.0867	MIR1254-1	0.3618	1.6310
MIR1273D	0.0570	0.0665	MIR6754	0.1525	0.1507	MIR302A	0.3665	0.2163
MIR6124	0.0577	0.0673	MIR3128	0.1525	0.3015	MIR8086	0.3746	0.1150
MIR378H	0.0591	0.0689	MIR1827	0.1588	0.0880	MIR3198-1	0.3774	0.0715
MIR4737	0.0605	0.9336	MIR769	0.1684	0.0970	MIR6729	0.3891	0.2296
MIR1255B1	0.0610	0.0540	MIR1537	0.1693	0.0815	MIR6738	0.3911	0.6802
MIR3146	0.0621	0.4252	MIR6808	0.1750	0.0843	MIR6793	0.3973	0.1817
MIR1290	0.0628	0.0638	MIR425	0.1750	0.0658	MIR940	0.4003	0.6026
MIR4766	0.0645	0.0753	MIR4727	0.1783	0.2985	MIR568	0.4183	0.3012
MIR4469	0.0653	0.0630	MIR328	0.1790	0.0964	MIR647	0.4248	2.3243
MIR4767	0.0662	0.6665	MIR222	0.1797	0.0444	MIR302C	0.4440	0.2926
MIR3591	0.0670	0.0237	MIR374B	0.1797	0.2865	MIR302D	0.4478	0.0732
MIR6797	0.0681	0.2177	MIR3178	0.1813	0.1363	MIR6838	0.4516	0.1910
MIR4734	0.0700	0.0711	MIR4786	0.1838	0.0622	MIR548AA1	0.4602	0.6027
MIR941-4	0.0717	0.1486	MIR4451	0.1888	0.2050	MIR4730	0.4618	0.1964
MIR941-2	0.0717	0.2280	MIR1199	0.1934	0.0986	MIR5194	0.4624	0.0829
MIR7111	0.0717	2.5152	MIR3161	0.1977	0.0646	MIR3926-1	0.4808	0.0784
MIR155HG	0.0721	0.0554	MIR641	0.2007	0.2085	MIR4263	0.4850	1.0310
MIR4442	0.0732	0.4159	MIR4259	0.2019	0.0492	MIR4313	0.5013	0.0302
MIR1229	0.0748	0.2992	MIR4534	0.2186	0.1428	MIR601	0.5063	0.2613
MIR367	0.0759	0.0842	MIR1249	0.2235	1.4770	MIR4685	0.5124	0.2380
MIR3677	0.0817	0.4395	MIR6804	0.2239	0.6914	MIR5187	0.5229	1.3814
MIR8072	0.0828	0.2221	MIR1273A	0.2481	0.2004	MIR548O	0.5297	0.2247
MIR4669	0.0833	0.1605	MIR6736	0.2537	0.2656	MIR4530	0.5345	2.7182
MIR6515	0.0860	0.2008	MIR5581	0.2538	0.3441	MIR4688	0.5472	0.0599
MIR202HG	0.0867	0.0572	MIR6895	0.2547	0.0734	MIR3189	0.5550	0.1465
MIR6740	0.0868	0.2774	MIR877	0.2649	0.0938	MIR3174	0.5874	0.4432
MIR27B	0.0908	0.1060	MIR421	0.2673	0.1910	MIR4674HG	0.6110	0.6932
MIR181B1	0.0939	0.3121	MIR519A2	0.2736	0.2167	MIR1302-9	0.6196	1.4324
MIR1539	0.0944	0.1667	MIR4324	0.2759	0.2177	MIR623	0.6215	0.3198

	PMN	Mono
MIR4477B	0.6245	3.1020
MIR6779	0.6250	0.4003
MIRLET7BHG	0.6544	0.2506
MIR6818	0.6551	0.2524
MIR6892	0.6666	0.6339
MIR3173	0.6679	0.2195
MIR3138	0.6703	0.2609
MIR2355	0.6941	0.0572
MIR6832	0.6953	0.5943
MIR646HG	0.6960	0.2313
MIR6509	0.7165	0.8136
MIR26A2	0.7251	0.1184
MIR4292	0.7394	4.9676
MIR6822	0.7531	0.1876
MIR4645	0.7639	1.0725
MIR6802	0.7702	0.4057
MIR4453	0.7988	0.2653
MIRLET7A1	0.8162	0.1163
MIR33B	0.8333	0.2747
MIR5684	0.8496	2.1813
MIR644A	0.9581	0.1058
MIR135A1	0.9606	0.6329
MIR6746	0.9750	0.5212
MIR199A1	0.9959	0.4414
MIR1273H	1.0034	0.2637
MIR3164	1.0291	1.3667
MIR4326	1.0323	1.2228
MIR3613	1.0501	0.3031
MIR29A	1.0563	0.1996
MIR628	1.0713	0.6485
MIR6514	1.0802	1.5992
MIR6864	1.0839	1.2439
MIR26B	1.1050	4.5684
MIR4513	1.1072	0.8446
MIR7847	1.1376	0.3816
MIR4632	1.1635	0.5261
MIR5010	1.1698	1.5631
MIR6775	1.1707	1.3991
MIR4690	1.1915	1.1651
MIR3136	1.2308	0.3380
MIR4426	1.2493	14.1109
MIR373	1.3127	1.5069
MIR6766	1.3297	1.6550
MIR6763	1.3301	0.5932
MIRLET7F1	1.3326	0.7779
MIR6835	1.3509	1.7649

	PMN	Mono
MIR645	1.4085	0.2196
MIR6723	1.4415	60.9185
MIR4482	1.4452	1.6029
MIR765	1.4578	1.5449
MIR4441	1.5149	0.3706
MIR4435-1	1.5481	0.7625
MIR573	1.5770	1.0502
MIR4312	1.5823	0.6087
MIR744	1.5985	0.0584
MIR4260	1.6064	0.3305
MIR6080	1.6122	1.1675
MIR6807	1.6409	0.9761
MIR4648	1.6665	7.6460
MIR570	1.7665	3.0773
MIR6894	1.7794	0.8773
MIR589	1.8535	0.8871
MIR221	1.8768	2.9717
MIR3682	1.9139	0.3317
MIR186	1.9804	2.4789
MIR3945	2.0013	0.6041
MIR320E	2.0253	0.2421
MIR6769A	2.0716	0.5180
MIR7152	2.1506	2.4692
MIR6848	2.3116	1.2332
MIR29B1	2.3601	0.3070
MIR3690	2.3640	0.5705
MIR4298	2.5030	0.1363
MIR6777	2.8309	0.4862
MIR6865	3.0533	0.2526
MIR6813	3.1381	1.1194
MIR4742	3.2607	1.1823
MIR4639	3.4334	1.3340
MIRLET7D	3.5622	4.3407
MIR4658	3.6366	1.5461
MIR590	3.7218	4.4932
MIR3909	4.0512	1.8899
MIR6069	4.4627	1.6499
MIR657	4.5984	0.3351
MIR3671	4.9558	0.5427
MIR1250	5.8695	0.2707
MIR106B	6.0109	0.7129
MIR6819	6.1515	2.1490
MIR324	6.3224	12.0582
MIR7848	7.0883	1.8867
MIR93	7.1063	0.5442
MIR6821	7.3513	5.5096

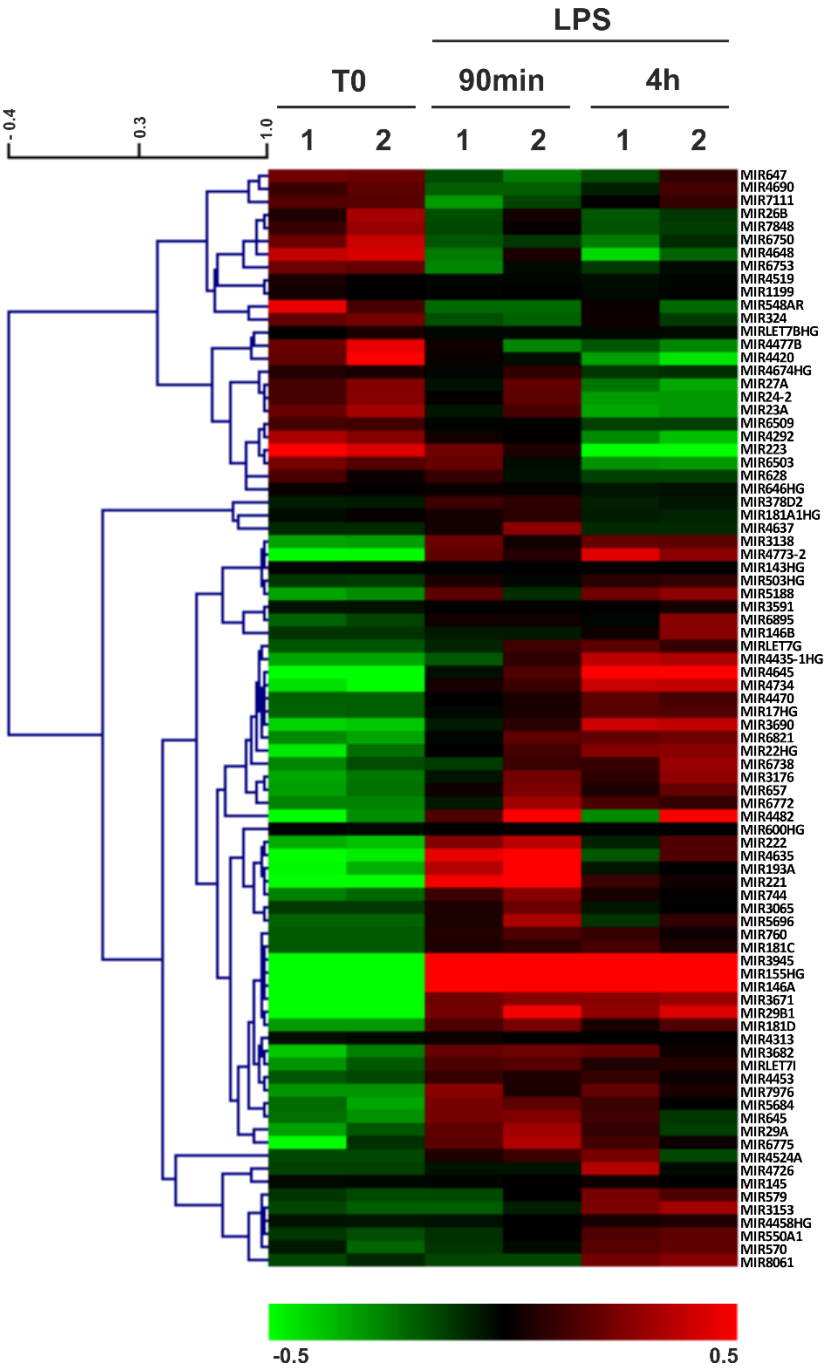
	PMN	Mono
MIR25	7.8617	1.9783
MIR635	8.2184	4.4779
MIR616	8.5348	10.1431
MIR103A2	9.4058	2.7078
MIR6891	10.7981	12.5049
MIR6753	11.2449	5.1511
MIR4489	12.5203	2.0100
MIR3916	13.2495	20.0386
MIR142	16.4481	14.0816
MIR4772	17.6743	0.0734
MIR4257	27.0833	6.5200
MIR6749	27.5500	1.1790
MIR4420	36.6021	35.6141
MIR23A	40.6472	53.1642
MIR24-2	40.6585	67.9095
MIR6750	49.0498	2.6071
MIR27A	53.8689	73.1656
MIR22HG	57.3325	34.7720
MIR223	76.5551	23.5203
MIR4697HG	0.0000	0.0019
MIR663AHG	0.0000	0.0022
MIR451A	0.0000	0.0064
MIR31HG	0.0000	0.0077
MIR4458HG	0.0000	0.0378
MIR4461	0.0000	0.0385
MIR5572	0.0000	0.0418
MIR3149	0.0000	0.0444
MIR378D2	0.0000	0.0451
MIR1256	0.0000	0.0481
MIR4305	0.0000	0.0488
MIR5006	0.0000	0.0520
MIR1262	0.0000	0.0535
MIR7849	0.0000	0.0540
MIR3680-2	0.0000	0.0572
MIR619	0.0000	0.0578
MIR345	0.0000	0.0584
MIR5089	0.0000	0.0592
MIR4779	0.0000	0.0599
MIR3162	0.0000	0.0607
MIR3202-1	0.0000	0.0614
MIR7850	0.0000	0.0630
MIR5088	0.0000	0.0630
MIR544B	0.0000	0.0638
MIR4516	0.0000	0.0665
MIR28	0.0000	0.0665
MIR4771-2	0.0000	0.0672

	PMN	Mono
MIR4787	0.0000	0.0681
MIR3922	0.0000	0.0681
MIR4671	0.0000	0.0681
MIR4653	0.0000	0.0689
MIR429	0.0000	0.0689
MIR3158-1	0.0000	0.0706
MIR6799	0.0000	0.0721
MIR6757	0.0000	0.0721
MIR4323	0.0000	0.0721
MIR6801	0.0000	0.0724
MIR7975	0.0000	0.0732
MIR5692C2	0.0000	0.0743
MIR4757	0.0000	0.0743
MIR4512	0.0000	0.0743
MIR8061	0.0000	0.0763
MIR3118-2	0.0000	0.0784
MIR6731	0.0000	0.0795
MIR4681	0.0000	0.0795
MIR4316	0.0000	0.0806
MIR32	0.0000	0.0818
MIR98	0.0000	0.0836
MIR6856	0.0000	0.0854
MIR4641	0.0000	0.0867
MIR4449	0.0000	0.0867
MIR7856	0.0000	0.0888
MIR215	0.0000	0.0904
MIR6880	0.0000	0.0923
MIR200B	0.0000	0.0964
MIR486-1	0.0000	0.1022
MIR361	0.0000	0.1022
MIR659	0.0000	0.1103
MIR8064	0.0000	0.1105
MIR574	0.0000	0.1192
MIR6762	0.0000	0.1244
MIR5094	0.0000	0.1258
MIR153-1	0.0000	0.1272
MIR5092	0.0000	0.1301
MIR3188	0.0000	0.1346
MIR4537	0.0000	0.1421
MIR5195	0.0000	0.1428
MIR185	0.0000	0.1438
MIR5188	0.0000	0.1453
MIR3940	0.0000	0.1463
MIR3944	0.0000	0.1520
MIR6829	0.0000	0.1597
MIR4519	0.0000	0.1605
MIR6893	0.0000	0.1659
MIR661	0.0000	0.1677
MIR6776	0.0000	0.1686
MIR6784	0.0000	0.1708
MIR4642	0.0000	0.1820
MIR339	0.0000	0.1860
MIR6747	0.0000	0.1876
MIR937	0.0000	0.1909
MIR3186	0.0000	0.1932
MIR193A	0.0000	0.1951
MIR4660	0.0000	0.2017
MIR6512	0.0000	0.2035
MIR6761	0.0000	0.2073
MIR4635	0.0000	0.2078
MIR4444-1	0.0000	0.2118
MIR378I	0.0000	0.2160
MIR378A	0.0000	0.2287
MIR6816	0.0000	0.2374
MIR4677	0.0000	0.2674
MIR3942	0.0000	0.2807
MIR4505	0.0000	0.2828
MIR3155B	0.0000	0.3216
MIR6825	0.0000	0.3241
MIR3176	0.0000	0.3649
MIR4425	0.0000	0.3731
MIR621	0.0000	0.5131
MIR4664	0.0000	0.5220
MIR3685	0.0000	0.5978
MIR6739	0.0000	0.6568
MIR593	0.0000	0.7985
MIR5587	0.0000	0.8214
MIR4740	0.0000	0.9635
MIR4722	0.0000	1.8782
MIR6503	0.0000	2.8260
MIR6774	0.0000	4.0228

Data obtained from RNA-sequencing are shown as FPKM related to each miRNA gene.

Supplementary Table S3. **List of immune related genes targeted by the members of miR23a-5p and miR27a-5p.**

miRNA	Potential immune related target genes
miR23a-5p	IRF1, COLQ, COL11A2, PTK2B, BNI3BL, FAS
miR27a-5p	IRF4, ZFHX4, DLL4 ,GRB2, NGFR, NRP2, CSF1, NPTX2, RUNX1, CADM1, ARHGEF7, CNTNAP2, NLK, MAP3K12, STRBP, TMEM25, BNIP3L, MAPK14



Supplementary Figure S1. **LPS stimulation modulates pri-miRNA expression in monocytes.**

Hierarchical Clustering Analysis of pri-miRNA expression in monocytes freshly purified or cultured for 90 min and 4h in the presence of LPS. The expression levels of LPS-modulated pri-miRNAs ($|Log_2FC|>1$, $Pval<0.05$) in monocytes are shown as $Log_{10}(FPKM+1)$, centered to the mean across the genes in row.

References

[1] Wells CA, Ravasi T and Hume DA. Inflammation suppressor genes: please switch out all the lights. *J Leukoc Biol* 2005; 78: 9-13.

[2] Henson PM. Dampening inflammation. *Nat Immunol* 2005; 6: 1179-81.

[3] Cassatella MA. Neutrophil-derived proteins: selling cytokines by the pound. *Adv Immunol* 1999; 73: 369-509.

[4] Kantari C, Pederzoli-Ribeil M and Witko-Sarsat V. The role of neutrophils and monocytes in innate immunity. *Contrib Microbiol* 2008; 15: 118-46.

[5] Bardoel BW, Kenny EF, Sollberger G, et al. The balancing act of neutrophils. *Cell Host Microbe* 2014; 15: 526-36.

[6] Jaillon S, Galdiero MR, Del Prete D, et al. Neutrophils in innate and adaptive immunity. *Semin Immunopathol* 2013; 35: 377-94.

[7] Davidson D, Zaytseva A, Miskolci V, et al. Gene expression profile of endotoxin-stimulated leukocytes of the term new born: control of cytokine gene expression by interleukin-10. *PLoS One* 2013; 8: e53641.

[8] Lara-Astiaso D, Weiner A, Lorenzo-Vivas E, et al. Immunogenetics. Chromatin state dynamics during blood formation. *Science* 2014; 345: 943-9.

[9] Tamassia N, Le Moigne V, Calzetti F, et al. The MyD88-independent pathway is not mobilized in human neutrophils stimulated via TLR4. *J Immunol* 2007; 178: 7344-56.

[10] Davey MS, Tamassia N, Rossato M, et al. Failure to detect production of IL-10 by activated human neutrophils. *Nat Immunol* 2011; 12: 1017-8; author reply 1018-20.

[11] Davidson D, Patel H, Degoy AC, et al. Differential effect of exogenous interleukin-10 versus glucocorticoids on gene expression and pro-inflammatory cytokine release by polymorphonuclear leukocytes and monocytes of the newly born. *Am J Transl Res* 2013; 5: 103-15.

[12] Ostuni R, Natoli G, Cassatella MA, et al. Epigenetic regulation of neutrophil development and function. *Semin Immunol* 2016; 28: 83-93.

[13] Tecchio C, Micheletti A and Cassatella MA. Neutrophil-derived cytokines: facts beyond expression. *Front Immunol* 2014; 5: 508.

[14] Brubaker SW, Bonham KS, Zanoni I, et al. Innate immune pattern recognition: a cell biological perspective. *Annu Rev Immunol* 2015; 33: 257-90.

[15] Tamassia N, Calzetti F, Ear T, et al. Molecular mechanisms underlying the synergistic induction of CXCL10 by LPS and IFN-gamma in human neutrophils. *Eur J Immunol* 2007; 37: 2627-34.

[16] McDonald PP, Bovolenta C and Cassatella MA. Activation of distinct transcription factors in neutrophils by bacterial LPS, interferon-gamma, and GM-CSF and the necessity to overcome the action of endogenous proteases. *Biochemistry* 1998; 37: 13165-73.

[17] Glass CK and Natoli G. Molecular control of activation and priming in macrophages. *Nat Immunol* 2016; 17: 26-33.

[18] Tamassia N, Zimmermann M, Castellucci M, et al. Cutting edge: An inactive chromatin configuration at the IL-10 locus in human neutrophils. *J Immunol* 2013; 190: 1921-5.

[19] Mehta A and Baltimore D. MicroRNAs as regulatory elements in immune system logic. *Nat Rev Immunol* 2016; 16: 279-94.

[20] Wang J CJaSU. MicroRNA as Biomarkers and Diagnostics. *Journal of cellular physiology* 2015; 231: 6.

[21] Lee RC, Feinbaum RL and Ambros V. The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. *Cell* 1993; 75: 843-54.

[22] Piletic K and Kunej T. MicroRNA epigenetic signatures in human disease. *Arch Toxicol* 2016; 90: 2405-19.

[23] Sayed D and Abdellatif M. MicroRNAs in development and disease. *Physiol Rev* 2011; 91: 827-87.

[24] Barca-Mayo O and Lu QR. Fine-Tuning Oligodendrocyte Development by microRNAs. *Front Neurosci* 2012; 6: 13.

[25] Cloonan N, Wani S, Xu Q, et al. MicroRNAs and their isomiRs function cooperatively to target common biological pathways. *Genome Biol* 2011; 12: R126.

[26] Li SC, Tsai KW, Pan HW, et al. MicroRNA 3' end nucleotide modification patterns and arm selection preference in liver tissues. *BMC Syst Biol* 2012; 6 Suppl 2: S14.

[27] Wery M, Kwapisz M and Morillon A. Noncoding RNAs in gene regulation. *Wiley Interdiscip Rev Syst Biol Med* 2011; 3: 728-38.

[28] Ameres SL and Zamore PD. Diversifying microRNA sequence and function. *Nat Rev Mol Cell Biol* 2013; 14: 475-88.

[29] Kiriakidou M, Tan GS, Lamprinaki S, et al. An mRNA m7G cap binding-like motif within human Ago2 represses translation. *Cell* 2007; 129: 1141-51.

[30] Mathonnet G, Fabian MR, Svitkin YV, et al. MicroRNA inhibition of translation initiation in vitro by targeting the cap-binding complex eIF4F. *Science* 2007; 317: 1764-7.

[31] Petersen CP, Bordeleau ME, Pelletier J, et al. Short RNAs repress translation after initiation in mammalian cells. *Mol Cell* 2006; 21: 533-42.

[32] Bazzini AA, Lee MT and Giraldez AJ. Ribosome profiling shows that miR-430 reduces translation before causing mRNA decay in zebrafish. *Science* 2012; 336: 233-7.

[33] Eulalio A, Behm-Ansmant I and Izaurralde E. P bodies: at the crossroads of post-transcriptional pathways. *Nat Rev Mol Cell Biol* 2007; 8: 9-22.

[34] Bhattacharyya SN, Habermacher R, Martine U, et al. Stress-induced reversal of microRNA repression and mRNA P-body localization in human cells. *Cold Spring Harb Symp Quant Biol* 2006; 71: 513-21.

[35] Parker R and Sheth U. P bodies and the control of mRNA translation and degradation. *Mol Cell* 2007; 25: 635-46.

[36] Lee S and Vasudevan S. Post-transcriptional stimulation of gene expression by microRNAs. *Adv Exp Med Biol* 2013; 768: 97-126.

[37] Vasudevan S. Posttranscriptional upregulation by microRNAs. *Wiley Interdiscip Rev RNA* 2012; 3: 311-30.

[38] Vasudevan S, Tong Y and Steitz JA. Switching from repression to activation: microRNAs can up-regulate translation. *Science* 2007; 318: 1931-4.

[39] Ma F, Liu X, Li D, et al. MicroRNA-466l upregulates IL-10 expression in TLR-triggered macrophages by antagonizing RNA-binding protein tristetraprolin-mediated IL-10 mRNA degradation. *J Immunol* 2010; 184: 6053-9.

[40] Didiano D and Hobert O. Perfect seed pairing is not a generally reliable predictor for miRNA-target interactions. *Nat Struct Mol Biol* 2006; 13: 849-51.

[41] Martinez-Sanchez A and Murphy CL. MicroRNA Target Identification-Experimental Approaches. *Biology (Basel)* 2013; 2: 189-205.

[42] Saito T and Saetrom P. MicroRNAs--targeting and target prediction. *N Biotechnol* 2010; 27: 243-9.

[43] Peterson SM, Thompson JA, Ufkin ML, et al. Common features of microRNA target prediction tools. *Front Genet* 2014; 5: 23.

[44] Doench JG and Sharp PA. Specificity of microRNA target selection in translational repression. *Genes Dev* 2004; 18: 504-11.

[45] Orom UA and Lund AH. Experimental identification of microRNA targets. *Gene* 2010; 451: 1-5.

[46] Nahid MA, Satoh M and Chan EK. MicroRNA in TLR signaling and endotoxin tolerance. *Cell Mol Immunol* 2011; 8: 388-403.

[47] Taganov KD, Boldin MP, Chang KJ, et al. NF-kappaB-dependent induction of microRNA miR-146, an inhibitor targeted to signaling proteins of innate immune responses. *Proc Natl Acad Sci U S A* 2006; 103: 12481-6.

[48] Taganov KD, Boldin MP and Baltimore D. MicroRNAs and immunity: tiny players in a big field. *Immunity* 2007; 26: 133-7.

[49] He X, Jing Z and Cheng G. MicroRNAs: new regulators of Toll-like receptor signalling pathways. *Biomed Res Int* 2014; 2014: 945169.

[50] Johnnidis JB, Harris MH, Wheeler RT, et al. Regulation of progenitor cell proliferation and granulocyte function by microRNA-223. *Nature* 2008; 451: 1125-9.

[51] Ward JR, Heath PR, Catto JW, et al. Regulation of neutrophil senescence by microRNAs. *PLoS One* 2011; 6: e15810.

[52] Gurol T, Zhou W and Deng Q. MicroRNAs in neutrophils: potential next generation therapeutics for inflammatory ailments. *Immunol Rev* 2016; 273: 29-47.

[53] Yang J, Han H, Zhao Y, et al. Specific miRNA and its target in neutrophils after traumatic injury. *Acta Biochim Biophys Sin (Shanghai)* 2015; 47: 749-54.

[54] Chen CZ, Li L, Lodish HF, et al. MicroRNAs modulate hematopoietic lineage differentiation. *Science* 2004; 303: 83-6.

[55] Landgraf P, Rusu M, Sheridan R, et al. A mammalian microRNA expression atlas based on small RNA library sequencing. *Cell* 2007; 129: 1401-14.

[56] Li Y, Dalli J, Chiang N, et al. Plasticity of leukocytic exudates in resolving acute inflammation is regulated by MicroRNA and proresolving mediators. *Immunity* 2013; 39: 885-98.

[57] Murata K, Yoshitomi H, Furu M, et al. MicroRNA-451 down-regulates neutrophil chemotaxis via p38 MAPK. *Arthritis Rheumatol* 2014; 66: 549-59.

[58] Bazzoni F, Rossato M, Fabbri M, et al. Induction and regulatory function of miR-9 in human monocytes and neutrophils exposed to proinflammatory signals. *Proc Natl Acad Sci U S A* 2009; 106: 5282-7.

[59] Nagalakshmi U, Wang Z, Waern K, et al. The transcriptional landscape of the yeast genome defined by RNA sequencing. *Science* 2008; 320: 1344-9.

[60] Mortazavi A, Williams BA, McCue K, et al. Mapping and quantifying mammalian transcriptomes by RNA-Seq. *Nat Methods* 2008; 5: 621-8.

[61] Tamassia N, Cassatella MA and Bazzoni F. Fast and accurate quantitative analysis of cytokine gene expression in human neutrophils. *Methods Mol Biol* 2014; 1124: 451-67.

[62] Livak KJ and Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 2001; 25: 402-8.

[63] Keene JD, Komisarow JM and Friedersdorf MB. RIP-Chip: the isolation and identification of mRNAs, microRNAs and protein components of ribonucleoprotein complexes from cell extracts. *Nat Protoc* 2006; 1: 302-7.

[64] McDonald PP, Bald A and Cassatella MA. Activation of the NF-kappaB pathway by inflammatory stimuli in human neutrophils. *Blood* 1997; 89: 3421-33.

[65] Rossato M, Curtale G, Tamassia N, et al. IL-10-induced microRNA-187 negatively regulates TNF-alpha, IL-6, and IL-12p40 production in TLR4-stimulated monocytes. *Proc Natl Acad Sci U S A* 2012; 109: E3101-10.

[66] Vermi W, Micheletti A, Lonardi S, et al. slanDCs selectively accumulate in carcinoma-draining lymph nodes and marginate metastatic cells. *Nat Commun* 2014; 5: 3029.

[67] Trapnell C, Roberts A, Goff L, et al. Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. *Nat Protoc* 2012; 7: 562-78.

[68] Anders S, Pyl PT and Huber W. HTSeq--a Python framework to work with high-throughput sequencing data. *Bioinformatics* 2015; 31: 166-9.

[69] Love MI, Huber W and Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol* 2014; 15: 550.

[70] Akira S, Uematsu S and Takeuchi O. Pathogen recognition and innate immunity. *Cell* 2006; 124: 783-801.

[71] Tamassia N, Le Moigne V, Rossato M, et al. Activation of an immunoregulatory and antiviral gene expression program in poly(I:C)-transfected human neutrophils. *J Immunol* 2008; 181: 6563-73.

[72] Barski A, Cuddapah S, Cui K, et al. High-resolution profiling of histone methylations in the human genome. *Cell* 2007; 129: 823-37.

[73] Benayoun BA, Pollina EA, Ucar D, et al. H3K4me3 breadth is linked to cell identity and transcriptional consistency. *Cell* 2014; 158: 673-88.

[74] Hon GC, Hawkins RD and Ren B. Predictive chromatin signatures in the mammalian genome. *Hum Mol Genet* 2009; 18: R195-201.

[75] Griffiths-Jones S, Hui JH, Marco A, et al. MicroRNA evolution by arm switching. *EMBO Rep* 2011; 12: 172-7.

[76] Guo L, Yu J, Yu H, et al. Evolutionary and expression analysis of miR-#-5p and miR-#-3p at the miRNAs/isomiRs levels. *Biomed Res Int* 2015; 2015: 168358.

[77] Krek A, Grun D, Poy MN, et al. Combinatorial microRNA target predictions. *Nat Genet* 2005; 37: 495-500.

[78] John B, Enright AJ, Aravin A, et al. Human MicroRNA targets. *PLoS Biol* 2004; 2: e363.

[79] O'Donnell JA, Kennedy CL, Pellegrini M, et al. Fas regulates neutrophil lifespan during viral and bacterial infection. *J Leukoc Biol* 2015; 97: 321-6.

[80] Becker C, Hammerle-Fickinger A, Riedmaier I, et al. mRNA and microRNA quality control for RT-qPCR analysis. *Methods* 2010; 50: 237-43.

[81] Russell JN, Clements JE and Gama L. Quantitation of gene expression in formaldehyde-fixed and fluorescence-activated sorted cells. *PLoS One* 2013; 8: e73849.

[82] Chen CZ, Schaffert S, Fragoso R, et al. Regulation of immune responses and tolerance: the microRNA perspective. *Immunol Rev* 2013; 253: 112-28.

[83] Jeker LT and Bluestone JA. MicroRNA regulation of T-cell differentiation and function. *Immunol Rev* 2013; 253: 65-81.

[84] Montagner S, Deho L and Monticelli S. MicroRNAs in hematopoietic development. *BMC Immunol* 2014; 15: 14.

[85] Tsitsiou E and Lindsay MA. microRNAs and the immune response. *Curr Opin Pharmacol* 2009; 9: 514-20.

[86] Eigsti RL, Sudan B, Wilson ME, et al. Regulation of activation-associated microRNA accumulation rates during monocyte-to-macrophage differentiation. *J Biol Chem* 2014; 289: 28433-47.

[87] Surmiak M, Hubalewska-Mazgaj M, Wawrzycka-Adamczyk K, et al. Neutrophil MiRNA-128-3p is Decreased During Active Phase of Granulo-matosis with Polyangiitis. *Curr Genomics* 2015; 16: 359-65.

[88] Ma J, Li N, Lin Y, et al. Circulating Neutrophil MicroRNAs as Biomarkers for the Detection of Lung Cancer. *Biomark Cancer* 2016; 8: 1-7.

[89] Dang TM, Wong WC, Ong SM, et al. MicroRNA expression profiling of human blood monocyte subsets highlights functional differences. *Immunology* 2015; 145: 404-16.

[90] Luers AJ, Loudig OD and Berman JW. MicroRNAs are expressed and processed by human primary macrophages. *Cell Immunol* 2010; 263: 1-8.

[91] Radom-Aizik S, Zaldivar F, Jr., Oliver S, et al. Evidence for microRNA involvement in exercise-associated neutrophil gene expression changes. *J Appl Physiol (1985)* 2010; 109: 252-61.

[92] Naqvi AR, Zhong S, Dang H, et al. Expression Profiling of LPS Responsive miRNA in Primary Human Macrophages. *J Microb Biochem Technol* 2016; 8: 136-143.

[93] Cobos Jimenez V, Bradley EJ, Willemsen AM, et al. Next-generation sequencing of microRNAs uncovers expression signatures in polarized macrophages. *Physiol Genomics* 2014; 46: 91-103.

[94] Ma Y, Vilanova D, Atalar K, et al. Genome-wide sequencing of cellular microRNAs identifies a combinatorial expression signature diagnostic of sepsis. *PLoS One* 2013; 8: e75918.

[95] Cullen BR. Transcription and processing of human microRNA precursors. *Mol Cell* 2004; 16: 861-5.

[96] Preusse M, Theis FJ and Mueller NS. miTALOS v2: Analyzing Tissue Specific microRNA Function. *PLoS One* 2016; 11: e0151771.

[97] Sood P, Krek A, Zavolan M, et al. Cell-type-specific signatures of microRNAs on target mRNA expression. *Proc Natl Acad Sci U S A* 2006; 103: 2746-51.

[98] Elton TS, Selemon H, Elton SM, et al. Regulation of the MIR155 host gene in physiological and pathological processes. *Gene* 2013; 532: 1-12.

[99] Kong KY, Owens KS, Rogers JH, et al. MIR-23A microRNA cluster inhibits B-cell development. *Exp Hematol* 2010; 38: 629-640 e1.

[100] Peng H, Wang X, Zhang P, et al. miR-27a promotes cell proliferation and metastasis in renal cell carcinoma. *Int J Clin Exp Pathol* 2015; 8: 2259-66.

[101] Hassan MQ, Gordon JA, Beloti MM, et al. A network connecting Runx2, SATB2, and the miR-23a~27a~24-2 cluster regulates the osteoblast differentiation program. *Proc Natl Acad Sci U S A* 2010; 107: 19879-84.

[102] Li X, Liu X, Xu W, et al. c-MYC-regulated miR-23a/24-2/27a cluster promotes mammary carcinoma cell invasion and hepatic metastasis by targeting Sprouty2. *J Biol Chem* 2013; 288: 18121-33.

[103] Lin R, Sampson JH, Li QJ, et al. miR-23a blockade enhances adoptive T cell transfer therapy by preserving immune-competence in the tumor microenvironment. *Oncoimmunology* 2015; 4: e990803.

[104] Dong J, Cui X, Jiang Z, et al. MicroRNA-23a modulates tumor necrosis factor-alpha-induced osteoblasts apoptosis by directly targeting Fas. *J Cell Biochem* 2013; 114: 2738-45.

[105] Vlachos IS, Paraskevopoulou MD, Karagkouni D, et al. DIANA-TarBase v7.0: indexing more than half a million experimentally supported miRNA:mRNA interactions. *Nucleic Acids Res* 2015; 43: D153-9.

[106] Kiraz Y, Adan A, Kartal Yandim M, et al. Major apoptotic mechanisms and genes involved in apoptosis. *Tumour Biol* 2016; 37: 8471-86.

[107] Planells-Ferrer L, Urresti J, Coccia E, et al. Fas apoptosis inhibitory molecules: more than death-receptor antagonists in the nervous system. *J Neurochem* 2016; 139: 11-21.

[108] Brint E, O'Callaghan G and Houston A. Life in the Fas lane: differential outcomes of Fas signaling. *Cell Mol Life Sci* 2013; 70: 4085-99.

[109] Kotani J, Avallone NJ, Lin E, et al. Fas-mediated neutrophil apoptosis and associated A1 protein expression during systemic inflammation are regulated independently of both tumor necrosis factor receptors. *Shock* 2003; 19: 201-7.

[110] Takeshita S, Tsujimoto H and Nakatani K. Intravenous immunoglobulin preparations promote apoptosis in lipopolysaccharide-stimulated neutrophils via an oxygen-dependent pathway in vitro. *APMIS* 2005; 113: 269-77.

[111] Renshaw SA, Timmons SJ, Eaton V, et al. Inflammatory neutrophils retain susceptibility to apoptosis mediated via the Fas death receptor. *J Leukoc Biol* 2000; 67: 662-8.

[112] Slight-Webb SR, Bagavant H, Crowe SR, et al. Influenza A (H1N1) virus infection triggers severe pulmonary inflammation in lupus-prone mice following viral clearance. J Autoimmun 2015; 57: 66-76.

[113] Wright HL, Moots RJ, Bucknall RC, et al. Neutrophil function in inflammation and inflammatory diseases. *Rheumatology (Oxford)* 2010; 49: 1618-31.

List of Publications

Conference abstracts

1. MicroRNA profiling in human neutrophils during activation. **S. Ghasemi**, B. Mariotti, N. Tamassia, F. Bianchetto, MA. Cassatella and F. Bazzoni. 10th National Congress of the Italian Society of Immunology, Clinical Immunology and Allergology (25-28 May 2016) Pavova, Italy. Abstract Book; P1.13, page.43.

2. The role of microRNAs in modulation of neutrophils and monocytes gene expression. **S. Ghasemi**, N. Tamassia, F. Bianchetto, B. Mariotti, MA. Cassatella and F. Bazzoni. EMBO/EMBL symposium: The Non-Coding Genome (18-21 Oct 2015) Heidelberg, Germany. Abstract Book; 116, page.161.

3. Optimizing the purification and analysis of miRNAs and mRNAs from RIP in primary human neutrophils**. S. Ghasemi**, M. Castellucci, S. Gasperini, B. Mariotti, N. Tamassia and F. Bazzoni. 4th European Congress of Immunology (ECI) (6-9 Sep2015) Vienna, Austria. Abstract Book; P.C.24.07, page.545.

Acknowledgment

My first and foremost gratitude goes to my supervisor Professor Flavia Bazzoni for giving me this wonderful opportunity to join such a dedicated and enthusiastic research group and for being both challenging and supportive in the right extent throughout these years. Moreover, it was an amazing feeling of confidence to know whenever I am in trouble my supervisor would be there to help me.

I would like also to thank Prof. Marco Cassatella for all his support and appreciated critical evaluations and suggestions. I am truly thankful of all my friends in general pathology specifically Monica, Barbara, Sara, Nicola, Francisco, Alessandra and Olivia for their kind and thoughtful help. I highly appreciate all the experimental/technical and theoretical knowledge, you have taught me.

As other steps in my life, this PhD also would not be possible without the help and fortitude of my beloved parents and family. Even though my decisions usually have not been in line with your desires but you have always been supportive and I am grateful for that.