miR-125b controls monocyte adaptation to inflammation through mitochondrial metabolism and dynamics

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miR-125b controls mitochondrial integrity

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Key points

- miR-125b reduces mitochondrial respiration and promotes elongation of mitochondrial network through BIK and MTP18 silencing, respectively
- miR-125b/BIK/MTP18 axis promotes adaptation of monocytes to inflammation.

Keywords

Monocytes, miR-125, mitochondria, BIK, MTP18, OCR, fusion, apoptosis
Abstract

Metabolic changes drive monocyte differentiation and fate. Although abnormal mitochondria metabolism and innate immune responses participate in the pathogenesis of many inflammatory disorders, molecular events regulating mitochondrial activity to control life and death in monocytes remain poorly understood. We show here that, in the human monocytes, miR-125b attenuates the mitochondrial respiration through the silencing of the BH3-only pro-apoptotic protein BIK and promotes the elongation of mitochondrial network through the targeting of the mitochondrial fission process 1 protein MTP18, leading to apoptosis. Proinflammatory activation of monocyte-derived macrophages is associated with a concomitant increase in miR-125b expression and decrease in BIK and MTP18 expression, which lead to reduced oxidative phosphorylation and enhanced mitochondrial fusion. In a chronic inflammatory systemic disorder, CD14⁺ blood monocytes display reduced miR-125b expression as compared with healthy controls, inversely correlated with BIK and MTP18 mRNA expression. Our findings not only identify BIK and MTP18 as novel targets for miR-125b that control mitochondrial metabolism and dynamics, respectively, but also reveal a novel function for miR-125b in regulating metabolic adaptation of monocytes to inflammation. Altogether, these data unravel new molecular mechanisms for miR-125b pro-apoptotic role in monocytes and identify potential targets for interfering with excessive inflammatory activation of monocytes in inflammatory disorders.
**Introduction**

Excessive and prolonged activation of monocytes/macrophages inflicts sustained tissue damage and targets organ inflammation. How the complex networks of survival and apoptotic regulators are integrated to tightly control the lifespan of infiltrating monocytes to avoid harmful reactions and allow healing and maintenance of tissue homeostasis remains elusive. Mitochondria play a pivotal role in innate immune cell homeostasis by supplying energy and integrating converging signals that lead to apoptosis or inflammation.\(^1\)\(^,\)\(^2\) In particular, the mitochondrial network is crucial for cell homeostasis, as the balance between fusion and fission is essential for mitochondria biogenesis and removal, optimized redistribution of mitochondria in response to local demand of ATP production, and progression of apoptosis.\(^3\) Furthermore, mitochondrial dysfunction is frequently associated with pathological conditions, including cancer and inflammatory disorders.\(^4\)\(^,\)\(^5\) The mechanisms that control mitochondrial activity and dynamics are however not fully understood.

Micro(mi)-RNAs are endogenous short non-coding RNA molecules that control gene expression mainly at the post-transcriptional level by pairing to the target transcripts.\(^6\) They participate in all the biological functions that have been investigated so far and dysfunction of miRNAs is implicated in major human disorders, including cancer and autoimmunity.\(^7\)\(^-\)\(^9\) The function of most of the mammalian miRNAs has yet to be determined. There is growing interest in exploring regulation of mitochondria by miRNAs. Few miRNAs have been demonstrated to control mitochondrial metabolism and dynamics, as well as mitochondria-mediated apoptosis. In addition, outer membrane of mitochondria is one of the destinations of miRNAs.\(^10\)

Among miRNAs that have been assigned apoptotic and immune functions, and that regulate nuclear encoded mitochondria-associated proteins, miR-125b is of particular interest.\(^11\) It is a highly conserved miRNA that has multiple targets, including proteins regulating apoptosis,
innate immunity, inflammation, and differentiation, and its dysregulation has been reported to occur in multiple cancer types.\textsuperscript{12-17} Depending on the cellular context, miR-125b shows different patterns of expression and effects, acting as tumour suppressor gene or as oncogene through the regulation of multiple genes involved in the mitochondrial pathways of apoptosis.\textsuperscript{18-21} In hematopoietic stem cells miR-125b is highly expressed, enhancing self-renewal and survival while providing resistance to apoptosis and blocking differentiation, and is up-regulated in lymphoblastic and myeloblastic leukemia.\textsuperscript{22} Importantly, miR-125b plays a critical role in myeloid biology,\textsuperscript{23} its expression being low in common myeloid progenitors,\textsuperscript{22} induced upon inflammatory stimuli in macrophages\textsuperscript{24,25} and deregulated in myeloid malignancies.\textsuperscript{15,26} Finally, miR-125b regulates adaptation of cell metabolism to cancer transformation\textsuperscript{27} and is one of the master miRNAs involved in the TLR4 signaling pathway during the development of endotoxin tolerance.\textsuperscript{25,28}

To provide novel insight into the integrated genetic regulatory network specifying cell fate, we have searched for novel target genes of miR-125b and further explored its role in the context of human monocytes, including the mechanisms on mitochondria metabolism.
Materials and methods

Cell culture and Reagents

THP-1 and HEK293 cell lines were grown in RPMI 1640 or DMEM supplemented with 10% fetal calf serum 1% penicillin/streptomycin and L-glutamine, respectively. Transfection was performed using the Lipofectamine 2000 (Invitrogen). For siRNA, pre-miRNA and antago-
miRNA, cells were transfected at 50 nM and harvested 48 hours after. For M1-polarized macrophages, THP-1 cells were treated 3 days with PMA (0.1 μg/ml), followed by 24 hours of stimulation with IFNγ (20 ng/ml) + LPS (0.1 μg/ml).

Blood CD14–CD16+ monocytes were selected from PBMCs by magnetic separation (Dynabeads Untouched Human Monocytes, Invitrogen), and grown in complemented RPMI 1640 media (purity > 97%). For rheumatoid arthritis (RA) patients and healthy controls, informed consents were provided in accordance with procedures approved by the local human ethics committee (Comité de Protection des Personnes Sud Méditerranée IV: NCT02909998). Fresh peripheral blood was obtained from 6 healthy donors with no history of autoimmune diseases (75% of woman) and from 8 age- and sex-matched patients with RA (Table I).

Gene expression analysis

Total RNAs were extracted using a miRNeasy Mini Kit with a Qiacube (QIAGEN). Expression levels of miRNAs and mRNAs were quantified using the TaqMan microRNA and gene expression assays (Life Technologies). The expression of RNU6B and GAPDH was used as endogenous control for miRNA and mRNA data normalization, respectively. Specific primers were used for quantification (Table S1). TaqMan® Array Human Inflammation Panel (Life Technologies) was used according to the manufacturer's protocols and relative gene expression was calculated using the comparative threshold cycle (C_T) method.
Luciferase assay

HEK293 cells transfected with 50 ng of either psiCHECK-2 vector encoding BIK 3’UTR or luciferase reporter plasmid GoClone Reporter (SwitchGear Genomics) encoding MTP18 3’UTR (see supplemental information), together with 1 to 100 nM of pre- or antago miR-125b. Luciferase activity was measured 48 hours using Dual-luciferase Reporter Assay System (Promega). Normalization was performed with the number of living cells.

Protein extraction and western blotting

Cells were lysed with CelLytic™ MT (Sigma-Aldrich). Proteins were separated on NuPAGE® 12% Bis-TRIS gels (10 µg/lane) and transferred to nitrocellulose membranes, which was blocked with primary antibody. Anti-BIK antibody was purchased from Santa-cruz and anti-MTP18 antibody was kindly provided by Dr. Daniel Tondera (Silence Therapeutics, Berlin, Germany). Table S2 describes the antibodies and dilutions used for the immunoblot analysis. Proteins were visualized and quantitated using the Odyssey Infrared Imaging System (LI-COR Biosciences). Autophagy and autophagic flux were assessed according to previously reported methods.29

Real-time monitoring of the mitochondrial energy metabolism

Cells were suspended 48 hours after transfection in XF assay buffer supplemented with 1 mM pyruvate, and 12 mM D-glucose at pH 7.4 and plated (250000 cells/well) in 100 µl on the cellTak-coated assay plates (BD Biosciences). Oxygen-consumption rate was measured with a Seahorse Bioscience XF24 extracellular flux analyser (Seahorse Bioscience).30 Test compounds (Sigma-Aldrich) were injected during the assay at the following final concentrations: 0.9 µM oligomycin, 0.5 µM FCCP, 0.9 µM rotenone and 0.9 µM antimycin A
(see supplemental information). Normalization was performed with the number of living cells.

**Fluorescence microscopy**

THP-1 and CD14+ cells were seeded at 50% confluence, treated 12 hours with PMA (0.1 μg/ml) and fixed with 4%, permeabilized in 0.2% Triton X-100 and incubated in blocking solution (DAKO) before staining with TOM20 and secondary antibody Alexa® 488. Cells were visualized using a Leica TCS SP5 confocal laser-scanning microscope model DM 6000S and the LAS AF acquisition software (Wetzlar, Germany). Images were captured on the Cellomics ArrayScan VTi platform (Thermo Scientific) using the cell health-profiling algorithm to quantify the percentage of cells containing Tom20 mitochondrial staining.

**Cell viability and apoptosis assay**

Cytotoxic effects and Caspase 3/7 activation were measured 48 hours post-transfection using CellTiter-Glo® Reagent (Promega) and the Caspase-Glo 3/7 Assay (Promega), respectively.

**Microarray hybridization and data analysis**

Quality control and quantification of total RNA was ensured with Bioanalyser (Agilent Technologies) and NanoDrop ND-1000 (Thermo Scientific), respectively. The generation of cRNA, sample hybridisation (using Affymetrix HG U133 plus 2.0 arrays) and scanning with a GeneChip Scanner 3000 (Affymetrix) were performed as described previously. Data from three pairs of conditions were analyzed using BioRetis database (supplemental information). The chip data discussed in this publication have been deposited in NCBI’s Gene Expression Omnibus and are accessible through GEO Series accession number GSE62693.
Statistical analysis.

GraphPad software was used to perform unpaired non-parametric Mann-Whitney or Student’s t-test according to the experimental design for two group comparisons. One-way ANOVA following Tukey’s multiple comparisons post-test analysis was performed for statistical tests of more than two groups. Correlations were evaluated with Spearman correlation analyses. P values less than 0.05 were considered statistically significant.

Results

miR-125b controls the expression of genes involved in inflammatory and apoptotic functions

To identify novel genes and pathways regulated by miR-125b in human monocytes, we profiled the gene expression changes of the human monocytic THP-1 cell line following miR-125b gain- and loss-of-function experiments (Figure 1). Analysis revealed that 419 genes were significantly up or downregulated in cells transfected with miR-125b mimics as compared with control conditions. We selected mRNAs with fold change (FC) higher than 1.5, p-value less than 0.05 and false discovery rate less than 5% (Figure 1A). DAVID32 analysis revealed high enrichment of specific biological processes including apoptosis, inflammation, and immune responses (Figure 1B). Selecting the specific down-regulated mRNAs in cells overexpressing miR-125b, we identified four putative miR-125b gene targets predicted by two major target prediction algorithms (microRNA.org and TargetScan). All putative target genes were involved in the apoptosis pathway: TMEM77 (alias DRAM2), HTATIP2 (alias TIP30), MTP18 (alias MTFP1), and BIK.

BIK and MTP18 are two novel targets for miR-125b
The 3’UTR of the four predicted targets were cloned downstream from luciferase gene, HEK-293 cells were co-transfected with the synthetic miR-125b precursor or antagonist, and relative luciferase activities were determined (Figure 1C-H and Supplementary Figure S1). Over-expression of miR-125b only significantly decreased the luciferase activity of the *BIK* and *MTP18* reporter systems. For both cases, increasing doses of miR-125b antisense dose-dependently reversed the inhibitory effect of basal miR-125b expression on the reporter system. To prove that miR-125b regulates endogenous BIK and MTP18 mRNA expression in monocytes, we transfected THP-1 cells with synthetic miR-125b precursor or antagonist. Data revealed that BIK and MTP18 mRNA and protein levels were significantly reduced by enforced expression of miR-125b to levels comparable with BIK and MTP18 siRNA-mediated knockdown (Figure 1E-H).

**miR-125b promotes apoptosis of monocytes**

BIK is a BH3-only pro-apoptotic protein localized in the endoplasmic reticulum that sensitizes mitochondrial apoptosis. MTP18 is localized in the mitochondria and induces mitochondrial fission and apoptosis. Since miR-125b targets two pro-apoptotic genes, we studied the effect of miR-125b on THP-1 apoptosis. Unexpectedly, enforced expression of miR-125b, as well as BIK or MTP18 siRNAs, induced apoptosis as determined by decreased cell viability and increased caspase 3/7 activation (Figure 2A-B). To further understand the functional role of miR-125b in apoptosis, we quantified several other anti- and pro-apoptotic genes that have been previously identified as directly targeted by miR-125b. The quantification of mRNA and protein levels revealed that BAK1, BMF, and BCL-2 expression were not controlled by miR-125b in THP-1 (Supplementary Figure S2).

As BIK can induce autophagy by displacing Bcl-2 from Beclin 1 and NAF-1 we investigated the effect of miR-125b on autophagy. We first analyzed the accumulation of
LC3-II, which reflects the number of autophagosomes. Neither the over-expression of miR-125b nor the silencing of BIK modified the levels of LC3-II as compared with controls (Figure 2C). Second, to assess modulation of the autophagic flux, we analyzed the expression of the autophagic cargo SQSTM1/p62. The expression levels of SQSTM1/p62 mRNA and protein were unaltered in all conditions tested (Figure 2C-D). Overall these results strongly suggest that miR-125b does not modulate autophagy in THP-1 cells.

**miR-125b dampens mitochondrial respiration rate**

To further explore the underlying mechanisms by which miR-125b-mediated silencing of BIK promotes apoptosis, we monitored the mitochondrial metabolism through quantification of the oxygen consumption rate (OCR) using the Seahorse technology, in a basal state and after the addition of oligomycin (to block ATP synthesis), FCCP (to uncouple ATP synthesis from the electron transport chain, ETC), and rotenone plus antimycin A (to block complex I and III of the ETC, respectively). Both ectopic expression of miR-125b and BIK silencing significantly reduced basal OCRs, decreased maximal mitochondria respiration and spare respiratory capacity when compared with control conditions (Figure 2E-F). MTP18 loss-of-function neither altered basal respiration, nor ATP production or respiratory capacities (Figure S3A-B). Rescue of BIK expression restored mitochondrial respiration parameters (Figure S4D-F). Overall, our data suggest that miR-125b induces apoptosis in THP-1 by regulating mitochondrial respiration, at least in part through BIK silencing, but not through MTP18 silencing.

**Increase of mitochondrial fusion by miR-125b**

As BIK activates mitochondrial fragmentation with little release of cytochrome c to the cytosol and MTP18 loss-of-function promotes fusion of mitochondria, we assessed the
effects of miR-125b ectopic expression on mitochondrial dynamics as compared with BIK and MTP18 silencing. DRP1 and MFN1 knockdown were used as positive controls for mitochondria fusion and fission, respectively. Using staining with an antibody against the outer membrane TOM20, we evidenced changes in the pattern of mitochondrial networks following enforced expression of miR-125b (Figure 2G-H). The hyperfused mitochondrial structure observed was comparable to mitochondrial networks observed with siDRP1 or siMTP18. Neither BIK silencing (Figure 2G) nor miR-125b inhibition (Figure 2H) affected mitochondrial dynamics. Importantly, neither mitochondrial content nor membrane potential was affected by miR-125b as evidenced by unmodified MitoTracker Red accumulation in mitochondria and staining of the mitochondrial protein TOM20, respectively (Figure S5A-B). Finally, rescue experiments co-transfecting cells with miR-125b mimics and either BIK or MTP18 expression plasmids have been performed. While co-transfection of miR-125b mimics together with mock- or BIK-expressing plasmids significantly increased the mitochondrial fusion rate, rescue of MTP18 expression restored fusion to levels comparable to the control condition (Figure S4G). In addition, rescue of MTP18 expression significantly increased the mitochondrial fission rate as compared with the 3 other conditions (Figure S4H). These results suggest that miR-125b regulates mitochondrial fusion at least in part through MTP18 silencing, but not through BIK silencing.

**TLR4 engagement induces the miR-125b-mediated silencing of BIK**

LPS is a potent activator of monocytes that controls survival and apoptosis through TLR4 engagement. TLR4 is not only a component of innate immunity but also a modulator in metabolic systems. To further address the functional role for miR-125b in regulating the mitochondrial metabolism in monocytes, we investigated the link between TLR4 stimulation, BIK downregulation, and metabolic switch. First, consistent with previous studies, miR-
125b was significantly up-regulated by TLR4 engagement in THP-1 cells (Figure 3A). Both paralogs coding for the same mature miR-125b sequence and located on two different polycistronic miRNA clusters (hsa-miR-125b-1 and hsa-miR-125b-2 on chromosomes 11 and 21, respectively)\(^4\) were over-expressed upon LPS challenge. Although the induction of miR-125b-2 expression was higher than miR-125b-1 following LPS stimulation, there was 4 times more miR-125b-1 expressed in the cells than miR-125b-2 (Figure 3B). These data suggest that the majority of the mature forms of miR-125b detected were probably processed from the hsa-miR-125b-1. Second, consistent with an induction of miR-125b by LPS and a control of BIK expression by miR-125b, we found that LPS-induced activation of THP-1 cells promoted a decrease of BIK mRNA and protein (Figure 3C), as well as changes in mitochondrial respiration (Figure 3D). Similar to what we observed following miR-125b over-expression or BIK knockdown (Figure 2E-F), LPS significantly reduced oxygen consumption from mitochondria (Figure 3D-E). Importantly, when the LPS-induced miR-125b increase was prevented using introduction of synthetic miRNA antagonists, BIK expression (Figure 3F) and mitochondrial maximal respiration (Figure 3G-H) were restored to near normal levels. Thus, blocking LPS-induced miR-125b increase preserved the reserve respiratory capacity of the cells. Altogether, these data suggest that miR-125b regulates metabolic adaptation of monocytes to inflammation.

**miR-125b promotes classical proinflammatory activation of THP-1 cells**

To further explore the mechanisms of miR-125b-induced metabolic adaptation to inflammation, we quantified a set of genes implicated in the inflammatory response (Figure 4A). Following miR-125b enforced expression, a variety of genes were elevated as compared with miR-125b inhibition, particularly chemokines and proinflammatory mediators: *CXCL10*, *CXCL11*, *TNF*, and *NOS2A* genes. All these genes provide a likely mechanism for the THP-1
cells that occurs following increased miR-125b expression, as they are indicative of the M1-type polarization known to induce distinct genetic and metabolic profiles, switching metabolism from oxidative phosphorylation to glycolysis and triggering specific proinflammatory cytokines.44

To induce classic (M1) polarization, PMA differentiated-THP-1 macrophages (Mφ) were incubated in the presence of IFN-γ plus LPS.45 Polarization was first confirmed using a panel of established markers (Figure 4B) and second by real-time monitoring of the mitochondrial energy metabolism (Figure 4C). M1-polarized THP-1 macrophages showed increased expression of TNF, CXCL10, and CXCL11, as well as decreased OCR, compared with M0 or Mφ. As previously reported for mouse monocytes,14 we observed enhanced miR-125b expression in THP-1 macrophages (Mφ), that is maintained when polarized into M1-like macrophages (Figure 4D). These data suggest that miR-125b may be involved in the induction and maintenance of the activated nature of macrophages.

Since the expression of BIK and MTP18 in M1-like macrophages was inversely correlated to that of miR-125b (Figure 4D), we determined the effects of M1 polarization on mitochondria respiration and dynamics. We showed that M1 polarization reproduces the effect of miR-125b overexpression or BIK silencing on the mitochondrial respiration of macrophages (Figure 2E and 3G), and the effect of miR-125b overexpression or MTP18 silencing on the mitochondrial dynamics (Figure 2C), i.e. reduced OCR (Figure 4C) and increased fusion of mitochondrial structures (Figure 4E), respectively. Ectopic expression of miR-125b promoted M1-like polarization as determined by increased expression of M1 markers compared with control miRNA or knockdown of miR-125b (Figure 4F-G). Importantly, rescue of BIK and MTP18 expression inhibited miR-125b-induced TNF and CXCL11 transcription (Figure S4I-J). These data showed that miR-125b contributes to the M1-like macrophage polarization, mimicking
IFNγ/LPS stimulation effect, and suggest that BIK and MTP-18 may mediate the regulatory role of miR-125b on M1-like macrophage activation.

The miR-125b/BIK/MTP18 axis plays a role in the adaptation of human CD14+ monocytes to inflammation

Finally, we tested the mechanisms of miR-125b-mediated metabolic adaptation to inflammation in human primary monocytes. We showed that purified CD14+ monocytes challenged with LPS displayed reduced OCR (Figure 5A-B) and increased mitochondrial fusion (Figure 5C). Furthermore, LPS induced the rapid increase in the miR-125b expression level, which is associated with reduced MTP18 and BIK expression levels, as well as parallel increased expression of the proinflammatory TNF cytokine and CXCL11 chemokine (Figure 5D). These data support the hypothesis that metabolic adaptation of CD14+ monocytes to inflammatory stimulus involves the control of mitochondrial respiration and dynamics through miR-125b/BIK/MTP18 axis.

We then analyzed the expression profile relationships between miR-125b and its target genes on monocytes in the context of a systemic inflammatory disorder. As CD14+ monocytes are important contributors to inflammation in patients with rheumatoid arthritis (RA) through the production of proinflammatory cytokines, we determined whether miR-125b-mediated regulation of BIK and MTP18 expression was affected on activated monocytes. In CD14+ blood monocytes from RA subjects, miR-125b exhibited decreased expression as compared with healthy controls (Figure 5E), and was inversely correlated with BIK (r=0.46, p=0.032) and MTP18 mRNA (r=0.19) expression, while no correlation was found in CD14+ monocytes isolated from healthy donors (Figure 5F). Co-factors could not account for the observed modifications (data not shown). The observation of a reduced expression of the pro-apoptotic miR-125b in CD14+ monocytes from RA patients is consistent with previous findings.
showing that CD14\(^+\) monocytes are resistant to oxidative stress and to apoptosis, and thus abundantly present in RA tissues.\(^{46,47}\)

**Discussion**

Our present work identifies the endoplasmic reticulum BH3-only protein BIK and the mitochondrial fission process 1 protein (MTFP1, also known as MTP18) as two novel cellular targets of miR-125b. In addition, we show that increased miR-125b through TLR4 affects mitochondrial respiration and dynamics through BIK and MTP18 silencing, respectively, promoting pro-inflammatory activation and apoptosis of monocytes (Figure 6). Our data suggest a novel role for miR-125b in regulating metabolic adaptation of monocytes to inflammation, thus extending its involvement in cell biology and disease pathogenesis as well as providing potential candidates for therapeutic intervention.

Monocytes differentiation, polarization and plasticity allow them tailoring an immune response upon stimulation of a range of receptors, including TLRs.\(^{48}\) Changes in cell activation, in cell fate and functions are coupled to changes in cellular metabolism and, vice versa. As regulator of oxygen consumption and ATP production, mitochondria are instrumental to metabolic adaptation of monocytes to environmental signals\(^2,4\) that drives effector functions such as migration, cytokine production, phagocytosis, antigen presentation and polarization.\(^{49}\) Ligation of TLR4 induces metabolic changes characterized by a decrease in oxidative phosphorylation (OxPhos) and an increase in glycolysis.\(^{50,51}\) This switch is observed when monocytes undergo differentiation into macrophages, and inhibition of this transition limits cell activation and lifespan. Profound metabolic reprogramming also occurs under macrophage polarizing conditions, glycolysis being an hallmark of the inflamed state that occurs in M1 macrophages while OxPhos occurs in immuno-regulatory M2 macrophages.\(^{44}\) M1 polarization uses anaerobic glycolysis to generate large quantities of ATP.
and to maintain a high mitochondrial membrane potential despite the complete inhibition of respiration, allowing cells to quickly adapt functions and cope with hypoxic tissue environment while providing a defence mechanism against cell death induced by endogenous ROS. In contrast, M2 macrophages preferentially use OxPhos metabolism to provide sustained ATP supply for tissue remodelling and repair and immunomodulation.\textsuperscript{52,53} The molecular events controlling the decrease in OxPhos and survival response to LPS and to M1 polarization remain unclear. Here we showed that one element controlling metabolic adaptation of monocytes/macrophages to these signals is miR-125b through coordinated targeting of BIK and MTP18. Consistent with previous studies, we found that TLR4 ligation and M1 polarization reduced OxPhos.\textsuperscript{44,50,54} We showed that response to TLR4 agonist is accompanied by a decrease in mitochondrial respiration, and that this effect is reversed when miR-125b is blocked. We also showed that miR-125b alone mimics metabolic adaptation of monocytes to TLR4 ligation as evidenced by decreased OCR. More specifically, miR-125b regulates the spare respiratory capacity, which represents the amount of extra ATP that can be produced by OxPhos in case of a sudden increase in energy demand, permitting rapid adaptation to metabolic changes. If the respiratory reserve is not sufficient to provide the cellular ATP levels required, cells are induced to apoptosis. This is consistent with our observation that miR-125b ectopic expression reduces cell proliferation and promotes apoptosis, while miR-125b blockade prevents cells from apoptosis. Although observed in a different cell context, miR-125b was recently reported to induce loss of mitochondrial membrane potential and sensitization to apoptosis in human breast cancer cell line.\textsuperscript{20} In addition to identifying a miR-125b/BIK/MTP18 pathway controlling energy production and cellular survival in human monocytes, our work also substantially clarifies the nature of the complex crosstalk between ER and mitochondria in human monocytes. The pro-apoptotic proteins BAK1, BMF, PUMA and p53, and the anti-apoptotic protein MCL-1, BCL-W and
BCL2, have been experimentally confirmed as cellular targets of miR-125b.\textsuperscript{18} Therefore, miR-125b can simultaneously modulate multiple genes playing opposite roles in apoptosis, acting as molecular hub that integrates various signals and takes different routes to modulate mitochondrial apoptosis pathway. Our expression profiling evidenced that miR-125b redirects genetic programs of human monocytes towards metabolic cascades that promote apoptosis and inflammation, by modulating several genes implicated in mitochondrial functions. Here, we evidenced that, in human monocytes, miR-125b does not directly control \textit{BCL2}, \textit{BAK1}, and \textit{BMF} expression, but promotes proinflammatory macrophage activation and apoptosis through direct regulation of \textit{BIK} and \textit{MTP18} expression. Several miRNAs have been reported to regulate mitochondrial functions,\textsuperscript{55} few of them are associated or localized in mitochondria. These so-called mitomiRs are different according to species and cell types, and their modulation is involved in inflammation and age-related diseases.\textsuperscript{56} Although miR-125b is considered as a mitomiR in human skeletal muscular cells,\textsuperscript{57} we could not detect it in mitochondria isolated from human monocytes (data not shown).

\textit{BIK} is an apoptosis-sensitizing protein anchored to the ER membrane\textsuperscript{33} that mediates release of ER Ca\textsuperscript{2+} and contributes to apoptosis through the mitochondria.\textsuperscript{40,41} Upon induction, \textit{BIK} neutralizes pro-survival Bcl2 family members, relieving pro-apoptotic BAX and BAK proteins\textsuperscript{58} that are translocated to the mitochondrial membrane and induce mitochondrial fission, causing apoptotic cell death. \textit{BIK} can also contribute to the regulation of autophagy by displacing BCL-2 from Beclin 1 and NAF-1.\textsuperscript{35,36} We showed that \textit{BIK} silencing mimics miR-125b over-expression and TLR4 stimulation, decreasing OCR and promoting cell death, with no impact on either mitochondrial network or autophagy. Our present work on human monocytes definitively reveals a totally new role for \textit{BIK} in controlling mitochondrial respiration that fits with the reported role of BH3-only proteins in restraining inflammatory diseases.\textsuperscript{59} In addition, we observed an inverse correlation between low miR-125b expression
levels and high mRNA levels of BIK and MTP18 in CD14+ blood monocytes from patients with rheumatoid arthritis, a systemic inflammatory condition in which CD14+ monocytes are abundant and abnormally activated in tissues and harbour a reduced propensity to undergo apoptosis.47

MTP18 is localized in the mitochondria and involved in shaping the mitochondrial network through the induction of fission.34 MTP18 loss-of-function promotes fusion of mitochondria leading to increased susceptibility to apoptosis,42 as we observed in THP-1 cells using either siRNA against MTP18 or miR-125b mimics. Mitochondria fission facilitates the redistribution of mitochondria in response to local changes in the demand for ATP and for mitophagy, while mitochondria fusion promotes exchange of mitochondrial components to strengthen the mitochondrial network. When cells become committed to apoptosis, massive fragmentation of the mitochondrial network through fission induces simultaneous release of cytochrome c from all mitochondria, promoting cell death. An alternative pathway termed stress-induced mitochondrial hyperfusion (SIMH) however promotes mitochondrial fusion to confer some resistance to low levels of stress but can also lead to apoptosis if further insults occur.60 Dynamic changes and balanced fusion/fission is crucial for cell survival but how different forms of cellular stress converge on the mitochondria and are regulated remains unknown. Our data reveal a novel model regulating the mitochondrial network in monocytes, which is composed of miR-125b and MTP18.

MicroRNAs are fine-tuners of TLR signaling and some of them have been involved in macrophage plasticity and polarization.61-66 In THP-1, the expression of miR-125b is upregulated in macrophages, either following PMA-induced differentiation or M1 polarizing conditions, as well as in monocytes following LPS stimulation. Several reports showed that miR-125b is enriched in macrophages and is an LPS-regulated miRNA. Its expression is decreased shortly after LPS stimulation of macrophages27,67,68 but increased following
sustained LPS exposure. Contrasting results from different studies show that miR-125b acts either as a negative regulator of the NF-κB pathway by stabilizing NKIRAS2 and repressing TNFα, or as a positive regulator that strengthen and prolong NF-κB activity by targeting TNFAIP3. In mouse macrophages miR-125b controls an activated phenotype through IRF4 silencing and regulates the differentiation/proliferation balance through Stat3 repression, two genes that promote M2 polarization. Both in THP-1 cells and primary CD14+ we showed that the M1 phenotype was associated with decreased mitochondrial metabolism and increased mitochondrial fusion, as well as repressed expression of BIK and MTP18, as observed when increasing miR-125b expression. The lack of autophagy induction upon miR-125b over-expression in human monocytes is consistent with early works showing that elongated mitochondria escape autophagy and that inhibition of autophagy switches macrophages from M2 to M1 polarization.

Understanding how immune cells commit to particular microenvironment and metabolic fates, and the immunologic consequences of reaching a metabolic endpoint is key. Here, we provide molecular clues for miR-125b involvement on the complex metabolic adaptation of human monocytes when activated by LPS and polarized towards M1 phenotype. Our data suggest that miR-125b contributes to inflammation by controlling mitochondria integrity through BIK and MTP18 silencing to adapt oxygen consumption and mitochondrial dynamics of monocytes to environmental cues. These results identify important mechanistic connections between microRNAs and mitochondrial functions, with broad implications for cellular metabolism and adaptation to cellular stress, and deepen our understanding of the molecular events of human monocyte biology.

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**Author Contributions**

DRI and AF designed the experiments, analysed data and wrote the manuscript. DRI contributed to experiments in all figures. RC, AM, PJ, GJR, LCH, HT, CP, BV, EJ, JC, PYM and GA contributed to experiments, data analyses, and helped writing the manuscript. All authors read and approved the final manuscript.

**Disclosure of Conflicts of Interest**

The author(s) declare(s) that there is no conflict of interests regarding the publication of this article.

**References**


**Figure legends**

**Figure 1. miR-125b represses the expression of BIK and MTP18.** (A) Transcriptomic analysis of miR-125b-overexpressing THP-1 cells (miR-125b) as compared with negative control miRNA-precursor-transfected cells (CTRL) and miR-125b antagomir (Anti-125b) transfected cells. Hierarchical clustering of the 419 genes with p < 0.05 and fold change of at least 1.5 was generated from two independent experiments (S=2). (B) Analysis of differentially expressed genes in miR-125b-overexpressing THP-1 cells versus negative controls using the functional annotation of DAVID, National Institute of Allergy and Infectious Diseases (NIAID). (C-D) Luciferase activity levels upon cotransfection of HEK293 cells with a luciferase construct (50 ng) containing 3’UTR of BIK (C) or MTP18 (D) together with control miRNA, miR-125b mimics or antagomir (n=3). The results are shown 48 hours after transfection and expressed as mean ± SD. Data are representative of two independent experiments. **p< 0.01 compared with control condition. (E-H) mRNA (E-F) and protein (G-H) levels of BIK (E and G) and MTP18 (F and H) in THP-1 cells transfected with control miRNA (CTRL), miR-125b mimics or antagomir, or siRNA targeting human BIK or MTP18 (50 nM). Results are expressed as mean ± SD of 4 independent experiments. *p<0.05, **p<0.01 and ***p<0.001. Abbreviations: miR-125b: miR-125b mimics; Anti-125b: miR-125b antagomir; A.U.: Arbitrary Units.

**Figure 2. miR-125b modulates mitochondrial metabolism and dynamics through BIK and MTP18 silencing, respectively.** THP-1 cells were transfected with si-DRP1, si-MPT18, si-MFN1, si-BIK, control miRNA, miR-125b mimics or antagomir and analyzed 48 hours after. (A-B) Cell apoptosis was assessed by measuring cell viability and caspase-3/7 activity. The data are presented as percentage (A) or a fold change (B) of the cell transfected with control miRNA. Data represent mean ± SD of 4 independent experiments. (C-D) Autophagy
was monitored using LC3/p62 immunoblotting to track the conversion of LC3-I into LC3-II and the expression of p62 for autophagic activity. Representative western blot of LC3-II/LC3-I, p62 and β-actin is shown and quantitative analysis of LC3-II and p62 are plotted as mean ± SD of 3 exposures of 2 independent experiments (C). Quantification of SQTIM1 mRNA (D) was performed using RT-qPCR. Data represent 2 technical independent experiments. (E) Oxygen consumption rate (OCR) was measured in real time under basal conditions; oligomycin, ATP-synthetase-inhibited rate; FCCP, uncoupled rate and rotenone + antimycin A, inhibited rate. OCR was normalized by number of cells in each condition (n=6/group). (F) Representative graph and quantification of various parameters of mitochondrial respiratory profiles presented as mean ± SD of 4 independent experiments. (G-H) Monitoring of mitochondrial fusion and fission in cells stained with anti-TOM20 antibodies (n=8). Representative images of mitochondria stained with TOM20 (bars=10 μm) and quantification of fluorescence using Cellomics ArrayScan VTi platform (n=20/experimental replicate) are shown. Results are expressed as mean ± SD *p<0.05, **p<0.01 and ***p<0.001. Abbreviations: CTRL: control miRNA; miR-125b: miR-125b mimics; Anti-125b: miR-125b antagonir.

Figure 3. TLR4 engagement mimics BIK silencing. (A-B) Expression levels of mature miR-125b (A) or pre-miR125b-1 and pre-miR-125b-2 (B) in THP-1 monocytes following or not 4 hours of LPS stimulation (1 μg/ml). RNU6B and GAPDH expression were used as endogenous control for miRNA and pre-mRNA data normalization. Mean ± SD of 2-8 duplicates are shown. (C) Kinetics of BIK mRNA expression in THP-1 cells following LPS stimulation (1 μg/ml). RNA quantification presents fold change to T0. Error bars represent the SD of 3-7 independent experiments. BIK protein levels were analyzed by immunoblot at the indicated time-points. (D) Kinetics of the oxygen consumption rate (OCR) measured in THP-
1 following LPS stimulation (1 μg/ml). All data are mean ± SD (n=6), representative of 3 independent experiments. (E) OCR measurement on THP-1 after 12 hours of LPS stimulation. Error bars represent the mean ± SD of 2-3 replicates of 3 independent experiments. (F) BIK mRNA expression levels 48 h after transfection with miR-125b antagomir and 4 hours of LPS stimulation (1 μg/ml). Data are presented as fold change (FC) with THP-1 transfected with control pre-miRNA. Error bars represent the SD of 4 independent experiments. (G) Oxygen consumption rate (OCR) measured on THP-1 after 48 hours of transfection with control miRNA or miR-125b antagomir (50 nM), including 12 hours of LPS treatment (1 μg/ml), normalized by number of cells. OCR were measured in real time under basal conditions; oligomycin, ATP-synthetase-inhibited rate; FCCP, uncoupled rate and rotenone + antimycin A, inhibited rate. Data are representative of four independent experiments. (H) Parameters of respiratory profiles are presented as mean ± SD for 4 independent experiments. *p<0.05, **p<0.01 and ***p<0.001. Abbreviations: CTRL: control miRNA; miR-125b: miR-125b mimics; Anti-125b: miR-125b antagomir.

**Figure 4. miR-125b promotes M1 macrophage polarisation.** (A) Total RNA was isolated from THP-1 transfected with control miRNA, miR-125b mimics or miR-125b antagomir. After reverse transcription, analysis of the expression level of 96 inflammatory genes was performed using a TaqMan-based Low Density Array Human Inflammation Panel. The volcano plot displays statistical significance versus fold-change pre-miR-125b/antagomiR-125b on the y- and x-axes respectively. Red dots represent M1 specific genes: TNF, NOS2A, CXCL10 and CXCL11. Graphs represent RNA expression levels of these genes as mean ± SD of 3 technical experiments. (B) THP-1 monocytes (MO) were differentiated into macrophages (Mφ) following 3 days of culture with PMA (0.1 μg/ml), then polarized into M1 macrophages using IFNγ and LPS stimulation for 24 hours. M1 polarization phenotype was monitored by
quantification of CXCL11 mRNA expression using RT-qPCR, and of human TNF secretion using ELISA. (C) Real time analysis of OCR for THP-1 monocytes differentiated into macrophages, polarized or not (white circles) towards M1 phenotype (black circles). (D) Expression levels of miR-125b, BIK and MTP18 mRNA in monocytes (MO), macrophages (Mφ) or M1-polarized macrophages (M1) using real-time PCR. Error bars represent the mean ± SD of 3 independent experiments. (E) Unpolarized (Mφ) or M1-polarized macrophages (M1) were fixed and stained with anti-TOM20 antibodies. Representative pictures (left panel, bars=10 μm) and quantification of captured images (right panel) are shown. (F-G) Expression levels of CXCL11 and TNF mRNAs (F) and human TNF secretion (G) in THP-1 cells transfected with control miRNA, miR-125b mimics or miR-125b antagomir. RNA quantification is presented as fold change to the controls, and error bars represent the SD of 4 independent experiments. *p<0.05, **p<0.01 and ***p<0.001. Abbreviations: CTRL: control miRNA; miR-125b: miR-125b mimics; Anti-125b: miR-125b antagomir.

Figure 5. miR-125b and mitochondrial metabolism in human CD14+ monocytes under inflammatory conditions. CD14+ monocytes were negatively selected from PBMCs by magnetic separation isolated from either healthy donors (A-D) or patients with rheumatoid arthritis (E-G). (A) Kinetics of the oxygen consumption rate (OCR) measured in CD14+ following LPS stimulation (0.1 μg/ml), in real time under basal conditions; oligomycin, ATP-synthetase-inhibited rate; FCCP, uncoupled rate and rotenone + antimycin A, inhibited rate. Data are representative of 3 independent experiments. (B) Quantification of respiratory profiles parameters presents 6 technical replicates. (C) Monitoring of mitochondrial fusion in CD14+ stained with anti-TOM20 antibodies (n=3). Representative images of mitochondria stained with TOM20 (bars=5 μm) and quantification of fluorescence using Cellomics ArrayScan VTi platform are shown (n=20/experimental replicate). (D) Kinetics of expression
levels of miR-125b, BIK, MTP18, TNF, and CXCL11 mRNA in CD14+ monocytes following LPS stimulation using real-time PCR. RNA quantification presents fold change to T0, and error bars represent the SD of 3 independent experiments. (E) miR-125b expression was quantified in CD14+ peripheral blood monocytes isolated from healthy controls (HC, n=5) or patients with rheumatoid arthritis (RA, n=8). (F) Correlation study of miR-125b and BIK or MTP18 expression levels in CD14+ monocytes from healthy controls (HC, n=5) or patients with rheumatoid arthritis (RA, n=8) was performed using a Spearman correlation analysis. All results are expressed as mean ± SD. *p<0.05, **p<0.01 and ***p< 0.001. Abbreviation: SRC: Spare respiratory capacity.

**Figure 6- miR-125b/BIK/MTP18 axis controls monocyte mitochondrial metabolism**

The endoplasmic reticulum BH3-only protein BIK and the mitochondrial fission process 1 protein (MTP18) are two novel cellular targets of miR-125b. An increase of miR-125b expression through TLR4 engagement affects mitochondrial respiration and dynamics through BIK and MTP18 silencing, respectively. Mitochondrial fusion induction and decrease of mitochondrial respiration promote apoptosis of monocytes. The figure was produced using Servier Medical Art.
Table 1

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<td>Number of samples</td>
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<td>Positive RF (%)</td>
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ACPA: Anti-citrullinated protein antibodies; RF: Rheumatoid factor; DAS28: Disease activity score referring to the 28 joints examined.
Figure 1

A

miR-125b
miR-125b
CTRL
Anti-125b
CTRL

B

miR-125b over-expression signatures

Enrichment

-Log10(p-values)

Hits per Biological Process category

C

Luciferase activity (AU)

D

Luciferase activity (AU)

E

Relative EK expression

G

CTRL
miR-125b
Anti-125b
siEK

H

CTRL
miR-125b
Anti-125b
siMTP18

MTP18
GAPDH

GAPDH
Figure 2
Figure 3

A

B

C

D

E

F

G

H
Figure 4
Figure 5

A

Oligomycin
FCCP
Rotenone + Antimycin

OCR (pMoles/min)

Time (min)

CD14
CD14+LPS

B

Basal Respiration
Maximal Respiration

OCR (pMoles/min)

CD14
CD14+LPS

C

LPS
+ LPS

D

mR-123b
MTP18
BIK
TNF
CXCL11

Fold change

T0 T0.5 T4 T24

E

CD14

mR-1255 expression

RA HC

F

Gene expression

mR-1255

0.10 0.11 0.12 0.13 0.14 0.15

MTP18 BIK

Gene expression

mR-1255

0.04 0.06 0.08 0.10

MTP18 BIK
Figure 6
miR-125b controls monocyte adaptation to inflammation through mitochondrial metabolism and dynamics