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MicroRNA-181b Regulates ALX/FPR2 Receptor Expression and Proresolution Signaling in Human Macrophages*

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Background: The ALX/FPR2 receptor recognizes the proresolution mediators lipoxin A_4 (LXA_4) and resolvin (Rv) D1, thus modulating immune responses.
Results: miR-181b binds to the 3′-UTR of the ALX/FPR2 gene, regulating its expression. mir-181b blunted LXA_4- and RvD1-induced macrophage phagocytosis.
Conclusion: miR-181b controls ALX/FPR2 expression. This mechanism modulates proresolution signals in macrophages.
Significance: miR regulation of ALX/FPR2 expression may be exploited for innovative anti-inflammatory strategies.

Regulatory mechanisms of ALX/FPR2, the lipoxin A_4 receptor, expression have considerable relevance in inflammation resolution. Because microRNAs (miRs) are emerging as key players in inflammation resolution, here we examined microRNA-mediated regulation of ALX/FPR2 (lipoxin A_4 receptor/formyl peptide receptor 2) expression. By matching data from bioinformatic algorithms, we found 27 miRs predicted to bind the 3′-UTR of ALX/FPR2. Among these, we selected miR-181b because of its link with inflammation. Using a luciferase reporter system, we assessed miR-181b binding to ALX/FPR2 3′-UTR. Consistent with this, miR-181b overexpression in human macrophages significantly down-regulated ALX/FPR2 protein levels (−25%), whereas miR-181b knockdown gave a significant increase in ALX/FPR2 (+60%). miR-181b levels decreased during monocyte to macrophage differentiation (−50%), whereas ALX/FPR2 expression increased significantly (+60%). miR-181b overexpression blunted lipoxin A_4 (0.1–10 nM)- and resolvin D1 (0.01–10 nM)-stimulated phagocytic activity of macrophages. These results unravel novel regulatory mechanisms of ALX/FPR2 expression and ligand-evoked macrophages proresolution responses mediated by miR-181b, thus uncovering novel components of the endogenous inflammation resolution circuits.

Resolution of inflammation is an active process that prevents host damage and is essential for restoring tissue homeostasis. Several cellular and molecular processes orchestrate the return to homeostasis after an acute inflammatory challenge by limiting polymorphonuclear neutrophil (PMN)[2] infiltration and promoting their removal via non phlogistic phagocytosis by macrophages (MΦs) (*i.e.* efferocytosis) (1, 2). Endogenous chemical mediators derived from essential polyunsaturated fatty acids play key roles in acute inflammation and resolution via specific G protein-coupled receptors (GPCRs) (3). Contrary to the arachidonic acid-derived leukotrienes, which act on cognate GPCRs to enhance PMN recruitment, infiltration, and activation, lipoxins (LX) are autacoid, bestowing anti-inflammatory and pro-resolution bioactions (4). LXA_4 (5, 6, 15*S*-trihydroxy-7,9,11,13-*trans*-11-*cis*-eicosatetraenoic acid) is biosynthesized from arachidonic acid (5) during a lipid mediator class switch, characteristic of inflammation resolution (6), through a multienzymatic pathway involving 5-lipoxygenase and 12- or 15-lipoxygenase (7–9). In addition, aspirin initiates the biosynthesis of C15 epimers of LX, namely 15-epi-LX through the acetylation of cyclooxygenase-2 (10). Notably, 15-epi-LXA_4 (5, 6, 15*R*-trihydroxy-7,9,11,13-*trans*-11-*cis*-eicosatetraenoic acid) proved to be as efficacious as aspirin and dexamethasone in reducing acute inflammation in murine models (11) and to mediate anti-inflammatory actions of low-dose aspirin in humans (12).

The potent pro-resolution bioactions of LXA_4 and 15-epi-LXA_4 occur via the activation of a specific GPCR termed lipoxin A_4 receptor (ALX)/formyl peptide receptor 2 (FPR2) (13), which signals to inhibit NF-κB activation, stops leukocyte diapedesis, and enhances efferocytosis (9, 14). ALX/FPR2 also recognizes additional endogenous-immunoresolving lipid and peptide ligands, such as the omega-3-derived resolvin (Rv) D1 (15, 16) and the glucorticoid-induced protein annexin A1 and its derived peptides (11), signifying this receptor as a critical component of innate proresolution pathways. The important role of ALX/FPR2 in controlling immune responses stems from studies with genetically modified mice as well as from human studies. In particular, myeloid-driven overexpression of human

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[2] The abbreviations used are: PMN, polymorphonuclear neutrophils; MΦ, macrophage; LX, lipoxin; LXA_4, lipoxin A_4 (5*S*,6*R*,15*S*-trihydroxy-7*E*,9*E*,11*Z*,13*E*-eicosatetraenoic acid); ALX/FPR2, lipoxin A_4 receptor/formyl peptide receptor 2; GPCR, G protein-coupled receptor; miR, microRNA; RvD1, resolvin D1 (7*S*,8*R*,17*S*-trihydroxy-4*Z*,9*E*,11*E*,13*Z*,15*E*,19*Z*-docosahexaenoic acid); DPBS, Dulbecco's phosphate-buffered saline; Zym, zymosan.

ALX/FPR2 in mice resulted in reduced PMN infiltration during acute peritonitis and left-shifting in dose response to its ligands (14, 16), whereas ALX/FPR2 KO mice present an impaired resolution phenotype (16–18). Notably, in humans subjected to cantharidin-induced skin blisters and treated with low dose aspirin, levels of ALX/FPR2 and 15-epi-LXA_4 in exudate leukocytes and fluids predict the outcome of acute inflammation (19).

Impairment of ALX/FPR2 and proresolution agonists in human diseases characterized by non resolving inflammation is well documented. For instance, subjects with severe asthma have an imbalance in the LX-ALX/FPR2 axis, with impaired biosynthesis of LX (20, 21) and increased levels of ALX/FPR2 on natural killer cells (22). Similarly, LX and RvD1 biosynthesis or glucorticoid-induced protein annexin A1 expression is significantly altered in patients suffering from cystic fibrosis (23–26), obesity (27), and atherosclerosis (28). Notably, therapeutic administration of ALX/FPR2 proresolution agonists in experimental models reverses disease progression and outcome, making the elucidation of the regulatory mechanisms of ALX/FPR2 expression and functions of strong interest. Along these lines, we recently identified a human ALX/FPR2 promoter sequence as well as genetic and epigenetic regulatory mechanisms of ALX/FPR2 transcription (29).

Accruing evidence demonstrates that microRNAs (miRs) (30–32), which act as fine tuners of gene output in cells, have important roles in resolution circuits and are part of the mechanisms of actions of LX and Rv. Several microRNAs have been recently identified in mouse inflammatory exudates. For instance, miR-21, miR-146b, miR-219, and miR-208a are regulated by RvD1 during self-limited inflammation (33) in a GPCR-dependent manner (16). In addition, Fredman *et al.* (34) compared microRNA expression in self-limited and delayed zymosan-induced inflammation, finding that the RvD1-regulated mir-208 was expressed to a lower extent in exudates from delayed resolution inflammation. Moreover, Li *et al.* (35) found that miR-466l is temporally regulated during inflammation resolution and enhances resolution by increasing RvD1 levels in mouse exudate-infiltrating leukocytes.

In humans, the LPS- and RvD1-regulated miR-21 controls the inflammatory response by down-regulating the translation of tumor suppressor programmed cell death 4 protein, an inhibitor of interleukin-10 production (36). Furthermore, the RvD1-regulated miR-219–5p reduces 5-lipoxygenase expression and leukotriene B_4 production in zymosan-induced peritonitis (33), whereas let-7c mediates the antifibrotic actions of LXA_4 in kidney fibrosis (37). Whether miRs control ALX/FPR2 expression as part of a more general host response during inflammation and resolution remains to be determined.

Herein, we provide the first evidence that miR-181b directly binds ALX/FPR2 3′-UTR and controls ALX/FPR2 protein expression. This mechanism has an impact on functional responses of human macrophages exposed to proresolution ALX/FPR2 agonists. Thus, miR-181b represents the first identified microRNA that can regulate the resolution process by targeting a pro-resolving GPCR.

EXPERIMENTAL PROCEDURES

Materials—Lipoxin A_4 (5*S*,6*R*,15*S*-trihydroxy-7*E*,9*E*,11*Z*,13*E*-eicosatetraenoic acid), purchased from Calbiochem, (Millipore, Billerica, MA), and resolvin D1 (7*S*,8*R*,17*S*-trihydroxy-4*Z*,9*E*,11*E*,13*Z*,15*E*,19*Z*-docosahexaenoic acid), from Cayman Chemicals (Ann Arbor, MI), were stored at −80 °C in ethanol, dissolved in the appropriate aqueous buffer immediately before each experiment, and kept in the dark until added to cells. Growth media, fetal bovine serum (FBS), and supplements were from Lonza (Walkersville, MD) unless otherwise indicated.

In Silico Analysis—In silico prediction analysis of human microRNA binding to ALX/FPR2 3′-UTR was carried out using two bioinformatic algorithms, TargetScanHuman and microRNA.org.

Monocyte Isolation and Macrophage Differentiation—Monocytes were isolated from peripheral blood of healthy subjects as follows. Twenty-five ml of blood, collected in sodium citrate (0.9%), were centrifuged (15 min, 150 × *g* without breaks and accelerator). Platelet-rich plasma was removed, and the remaining plasma was diluted with Ca^{2+}/Mg^{2+}-free Dulbecco's phosphate-buffered saline (DPBS) (10 ml) plus 6% dextran (8 ml) and allowed to sediment for 15 min. The upper layer was placed over 10 ml of Histopaque-1077 Ficoll (Sigma) and centrifuged (430 × *g* for 30 min without breaks and accelerator). Peripheral blood mononuclear cells containing buffy coat were gently aspirated, washed twice with Ca^{2+}/Mg^{2+}-free DPBS, and cells counted. Cells (12×10^6) were suspended with serum-free RPMI medium and allowed to adhere on polystyrene cell culture plates for 1–2 h. Non adherent cells were aspirated, whereas adherent monocytes were gently washed 3 times with DPBS. Monocyte purity was determined by flow cytometry using an anti-CD-14 antibody (TÜK4 clone, Miltenyi Biotech, Calderara di Reno, Bologna, Italy). MΦ differentiation was carried out as in Krishnamoorthy *et al.* (15) by maintaining monocytes in RPMI supplemented with 10% FBS, 1% L-glutamine (Gln), 1% penicillin/streptomycin, and GM-CSF (10 ng/μl, Prospec, East Brunswick, NJ) for 7 days.

Transfection of Macrophages with miR-181b Expressing Plasmid—Macrophages ($1–2 \times 10^6$ cells/well) were transfected with empty vector or miR-181b-expressing TW (miR-181b-TW), cloned as previously reported by Visone *et al.* (38), using Jet-Pei macrophages (Polyplus Transfection, Illkirch, France). Briefly, 1.5 μg of plasmids were diluted with 100 μl of NaCl (0.9%) and mixed with 3 μl of the JetPei reagent diluted with 100 μl of NaCl. After 20 min at room temperature, the DNA-JetPei mixture was added to macrophages kept in 1.8 ml of RPMI supplemented with 10% FBS, 1% L-Gln, and 1% penicillin/streptomycin.

Transfection of Macrophages with miR-181b Inhibitor—Human macrophages were transfected with 10 nM miR-181b inhibitor (miScript, Qiagen, Milan, Italy) or non targeting negative control single-strain RNA (also from Qiagen) using the INTERFERin transfection reagent (Polyplus Transfection). Briefly, miR-181b inhibitor and negative control were diluted with 200 μl of Opti-MEM (Invitrogen) and combined with 4 μl of INTERFERin. After 10 min at room temperature, the RNA-

reagent solution (200 μl) was added to macrophages kept in 1.8 ml of RPMI medium as above.

ALX/FPR2 3′-UTR Cloning—ALX/FPR2 3′-UTR was cloned in the psiCHECK-2 vector (Promega, Milan, Italy) (ALX-psiCHECK-2) using the primers 5′-GGATGGGGTCAGGGATATTTT-3′ (forward) and 5′-CACTGGTGAATTTTTCTGAATATT-3′ (reverse) with the addition of consensus sequences for NotI and XhoI (both from New England Biolabs, Ipswich, MA). The PCR thermal profile consisted of 3 min at 94 °C, 35 cycles (30 s at 94 °C; 30 s at 58 °C; 50 s at 72 °C), and 5 min at 72 °C. After NotI/XhoI digestion at 37 °C overnight and ligation (T4 DNA ligase, Promega), plasmids were sequenced to confirm the correctness of cloning.

3′-UTR Luciferase Reporter Assay—Breast cancer MDA-MB-231 cells, grown in DMEM supplemented with 10% FBS, 1% L-Gln, and penicillin/streptomycin, were co-transfected with ALX-psiCHECK-2 and miR-181b-TW or empty TW plasmid as a control (1.5 μg each) using Lipofectamine 2000 (12 μl, Invitrogen) according to the manufacturer's protocol. Twenty-four hours post transfection, cells were lysed, and the luciferase activity was determined as in Simiele *et al.* (29).

miRNA Extraction and Real Time PCR Analysis—miRNA-enriched fractions were extracted using a silica-based spin column system (Norgen, Thorold, ON, Canada), UV-quantified using a NanoDrop spectrophotometer (Thermo Scientific, Waltham, MA), and reverse-transcribed with the miScript II RT kit (Qiagen). Real time PCR analyses were carried out with 1.5 ng of cDNA using specific primers (miScript Primer Assays) and a SYBR Green master mix (also from Qiagen) with a 7900HT Fast thermal cycler (Invitrogen). Relative abundance of miR-181b was determined by the $2^{-\Delta Ct}$ method (39), and the small nucleolar RNA 1A was used to normalize input cDNA.

Flow Cytometry—For flow cytometric analysis of ALX/FPR2 protein, cells (1×10^6/sample) were incubated with 0.5 μg of anti-ALX/FPR2 primary antibody (Genovac/Aldevron, Freiburg, Germany) and Alexa Fluor 647-labeled secondary antibody (Invitrogen). Analyses were carried out using a FACS Calibur flow cytometer equipped with the CellQuest software (BD Bioscience).

Zymosan Phagocytosis by Macrophages—Human peripheral blood mononuclear cell-derived macrophages were transfected with 1.5 μg of miR-181b-TW or empty TW vector using Jet-Pei Macrophages (Polyplus Transfection™) as reported above and seeded in 24-well plates ($2.5–5 \times 10^5$ cells/well) 48 h post transfection. The following day cells were washed twice with DBPS and treated with LXA_4, RvD1, or vehicle (0.01% EtOH) in DPBS. After 15 min at 37 °C, fluorescein isothiocyanate (FITC)-labeled serum opsonized zymosan (Zym) A (from *Saccharomyces cerevisiae*) particles (15 ng/well) were added to cells for 30 min at 37 °C. Macrophages were then washed twice with DPBS, 100 μl of trypan blue (0.03% in DPBS) was added to quench fluorescence from extracellular FITC-Zym, and phagocytosis was assessed by measuring fluorescence on a SpectraMAX Gemini XS plate reader (Molecular Devices, Sunnyvale, CA) as in Recchiuti *et al.* (40).

PMN Isolation and Apoptosis—PMN were isolated from 25 ml of blood by dextran sedimentation as reported previously (33). Briefly, after removal of platelet-rich plasma, blood was diluted with Ca^{2+}/Mg^{2+}-free DPBS (10 ml) plus 6% dextran (8 ml) and allowed to sediment for 15 min. The upper layer was placed over 10 ml of Histopaque-1077 Ficoll (Sigma) and centrifuged ($430 \times g$ for 30 min without breaks and accelerator). PMN-rich pellet was lysed, and PMN was isolated as reported (33). Apoptosis was induced by incubating PMN with RPMI supplemented with 10% FBS, 1% Gln, 1% penicillin/streptomycin overnight. Apoptosis was assessed by trypan blue exclusion and flow cytometry with annexin V and propidium iodide double staining.

TNF-α Release—Human Peripheral blood mononuclear cell-derived macrophages were transfected with 1.5 μg of miR-181b-TW or empty TW vector and seeded as reported above. Cells were exposed to LXA_4, RvD1, or vehicle (0.01% EtOH) in DPBS at 37 °C 15 min before the addition of Zym (see above). TNF-α levels in the supernatants were evaluated using a TNF-α standard ELISA development kit (Peprotech, London, UK).

Statistical Analysis—Results are reported as the arithmetic mean ± S.E. unless otherwise indicated. Statistical significance was evaluated by the Student's *t* test with $p < 0.05$ taken as significant.

RESULTS

Selection of miRs That Putatively Bind to ALX/FPR2 3′-UTR—ALX/FPR2 has pivotal roles in the resolution of inflammation as well as in the outcome of acute inflammatory reactions (12). Therefore, the elucidation of the mechanisms that regulate its expression is relevant. Because miRs are important modulators of gene/protein expression (41), we sought to identify miRs that may regulate ALX/FPR2 expression. To this end, we searched for predicted binding sites for human miRs within the 3′-UTR of ALX/FPR2 by matching data from two bioinformatic algorithms, namely TargetScanHuman and microRNA.org, which rely on different criteria for prediction and ranking, *e.g.* stringent or loose Watson-Crick pairing of the seed region of miRNAs, site context, and accessibility and sequence conservation (42). Among the 27 miRs (Table 1) predicted by both algorithms to bind the 3′-UTR of ALX/FPR2, we selected miR-181b as the primary candidate, as it is strongly expressed in lymphocytes (36), and it appears to be related to inflammation (43–45).

Analysis of miR-181b Binding to ALX/FPR2 3′-UTR—To validate results from bioinformatic predictions, we examined miR-181b direct binding to ALX/FPR2 3′-UTR. To this end we generated a reporter construct containing the renilla luciferase gene 684 bp upstream of the 3′-UTRs of the ALX/FPR2 mRNA, encompassing the putative miR-181b target sites (ALX-psiCHECK-2). This was co-transfected into MDA-MB-231 cells with a miR-181b-expressing plasmid (miR-181b-TW).

The miR-181b sequence cloned in this plasmid is conserved within the two known miR-181b-encoding genes (Fig. 1). Twenty-four hours post transfection, luciferase activity was measured. We observed a significant (~70%; $p = 0.006$) reduction in luciferase activity in MDA-MB-231 cells co-expressing miR-181b-TW and ALX-psiCHECK-2 compared with cells transiently transfected with the empty TW vector together with ALX-psiCHECK-2 (Fig. 2). These results establish that miR-181b binds to the 3′-UTR of ALX/FPR2.

TABLE 1
miRs predicted by both microrna.org and TargetScanHuman to bind the 3′-UTR of ALX/FPR2

miRs predicted to bind the 3′-UTR of ALX/FPR2
hsa-miR-181b
hsa-miR-20b
hsa-miR-106a
hsa-miR-17
hsa-miR-374a
hsa-miR-374b
hsa-miR-519d
hsa-miR-93
hsa-miR-181c
hsa-miR-181a
hsa-miR-375
hsa-miR-101
hsa-miR-139–5p
hsa-miR-335
hsa-miR-411
hsa-miR-410
hsa-miR-144
hsa-miR-20a
hsa-miR-106b
hsa-miR-212
hsa-miR-200a
hsa-miR-132
hsa-miR-141
hsa-miR-203
hsa-miR-433
hsa-miR-543
hsa-miR-181d

miR-181b Controls ALX/FPR2 Surface Expression in Monocytes/Macrophages—Given the direct binding of miR-181b to the ALX/FPR2 3′-UTR, we tested the effect of miR-181b on ALX/FPR2 surface expression. To this end, we selected monocyte/macrophages as the cellular system because they express both miR-181b and ALX/FPR2 and play key roles in inflammation resolution. As shown in Fig. 3*A*, transfection of human monocyte-derived MΦs with the miR-181b-TW plasmid resulted in an ~4-fold increase in miR-181b levels 24 h post transfection. This was associated with an ~25% ($p = 0.0001$) reduction in ALX/FPR2 protein (Fig. 3*B*), which is in line with the reported impact of microRNAs on protein output (32) as well as of miR-181b on other validated target genes such as importin-3, cyclooxygenase-2, and insulin growth factor 1 receptor (46). Notably, miR-181b overexpression reduced ALX/FPR2 levels equally well in permeabilized ($p = 0.016$) and non permeabilized cells ($p = 0.0001$), indicating that miR-181b downregulates ALX/FPR2 expression by acting on translation, not on trafficking.

To obtain further evidence of a "cause and effect" relationship between miR-181b and ALX/FPR2, we transfected human MΦs with a miR-181b inhibitor. Real time PCR analysis showed that miR-181b levels decreased by ~2.5-fold ($p = 0.004$) in MΦs transfected with the miR-181b inhibitor (Fig. 4*A*). In these cells, ALX/FPR2 protein expression increased by ~60% ($p = 0.0002$) (Fig. 4*B*).

On the other hand, ALX/FPR2 regulation by mir-181b appears to be selective, as the expression of GPR32, another G-coupled protein receptor recognized by RvD1 (15), did not change in MΦs transfected with the miR-181b inhibitor (Fig. 4*D*). Together, these results establish that ALX/FPR2 is a direct target of miR-181b.

miR-181b and ALX/FPR2 Levels Change during Differentiation of Monocytes to Macrophages—To evaluate the inverse relationship between miR-181b and ALX/FPR2 expression

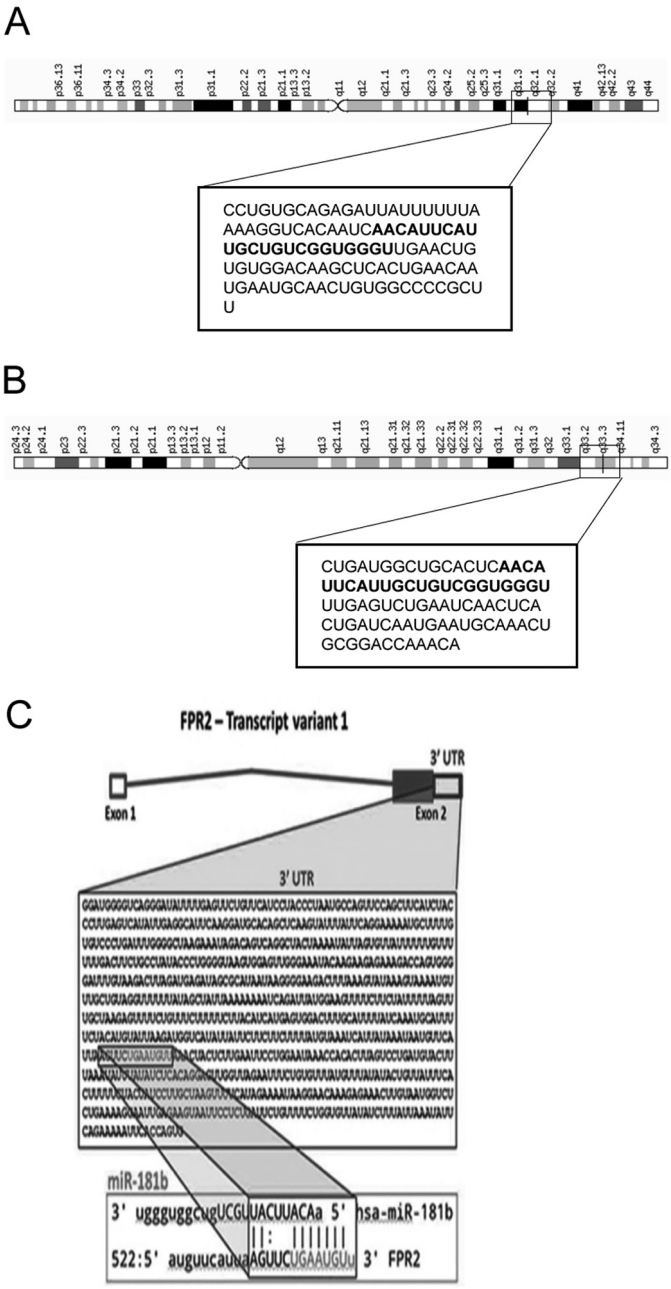

FIGURE 1. ***In silico* analyses.** mir-181b is encoded by genes located on chromosome 1 (*A*) and on chromosome 9 (*B*). Conserved sequences complementary to ALX/FPR2 3′-UTR are in *bold*. *C*, predicted binding of miR-181b sequences to a specific seed region within the ALX/FPR2 3′-UTR.

within a pathophysiological context, we determined their levels during monocyte to macrophage differentiation, a key cellular event in inflammation resolution. Relative expression of miR-181b in peripheral blood monocytes from healthy subjects, calculated using the $2^{-\Delta Ct}$ method with nucleolar RNA 1A as housekeeping miRNA, was 0.043 ± 0.012 ($2^{-\Delta Ct}$). This significantly decreased to 0.025 ± 0.008 after macrophage differentiation with GM-CSF for 7 days ($p = 0.048$) (Fig. 5*A*). On the contrary, ALX/FPR2 protein expression significantly increased in MΦs (44.5 ± 4.2 mean fluorescence intensity (MFI)) compared with monocytes (29.2 ± 1.5 MFI) ($p = 0.01$) (Fig. 5*B*). Thus, miR-181b and ALX/FPR2 displayed an inverse expression pattern during macrophage differentiation.

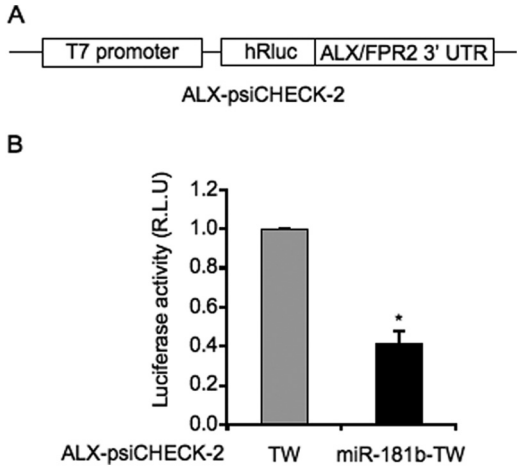

FIGURE 2. **Luciferase reporter assay.** *A*, schematic representation of the reporter construct containing the ALX/FPR2 3′-UTR (*ALX-psiCHECK-2*). *B*, MDA-MB-231 cells were co-transfected with ALX-psiCHECK-2 plus TW empty vector or miR-181b-expressing TW vector (*miR-181b-TW*). The luciferase activity was normalized for total proteins in cell lysates (mean ± S.E. from $n = 3$ independent experiments carried out in duplicate. *, $p = 0.006$). *R.L.U.*, relative luciferase units.

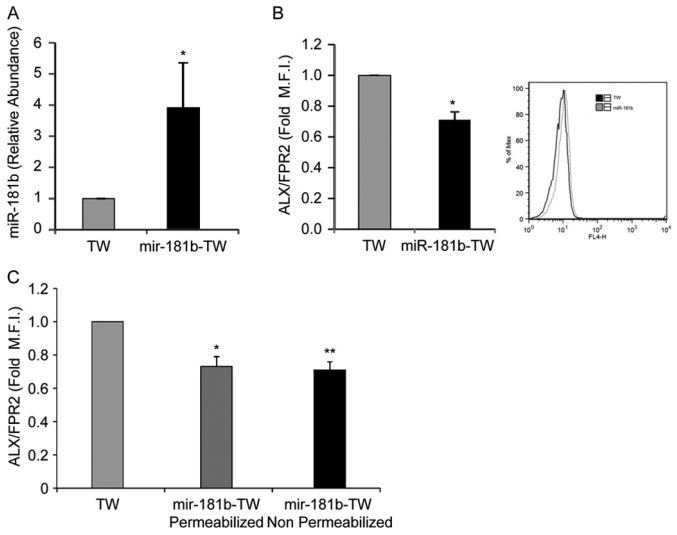

FIGURE 3. **Overexpression of mir-181b in human macrophages downregulates ALX/FPR2 protein expression.** *A*, relative abundance of miR-181b in MΦs transfected with a miR-181b expression plasmid (*miR-181b-TW*) or with an empty plasmid (*TW*). Expression levels of miR-181b were determined 24 h post transfection. Data are the mean ± S.E. from $n = 5$ independent experiments carried out in duplicate. *, $p = 0.03$. *B*, ALX/FPR2 protein expression in MΦs transfected with miR-181b-TW or mock-transfected (*TW*). ALX/FPR2 expression was evaluated by flow cytometry 72 h after transfection. *M.F.I.*, mean fluorescence intensity; *FL4H*, fluorescence intensity of the APC fluorochrome (height). *Bars* represent the mean ± S.E. of mean fluorescence intensity values from $n = 5$ independent experiments carried out in duplicate. *, $p = 0.0001$. *C*, ALX/FPR2 protein expression in MΦs transfected with miR-181b-TW or mock-transfected (*TW*) in permeabilized (*, $p = 0.016$ *versus* TW) and non permeabilized cells (**, $p = 0.0001$ *versus* TW).

miR-181b Regulation by Efferocytosis and Exposure to Zymosan—To assess the impact of key events in inflammation and resolution on miR-181 expression, we incubated macrophages with apoptotic PMN or zymosan and evaluated miR-181b relative expression after 24, 48, and 72 h by real time PCR. Coincubation with apoptotic PMN (Fig. 6*A*) significantly decreased miR-181b expression ($p = 0.011$) after 48 h. On the contrary exposure to zymosan gave a significant increment ($p = 0.011$) (Fig. 6*B*).

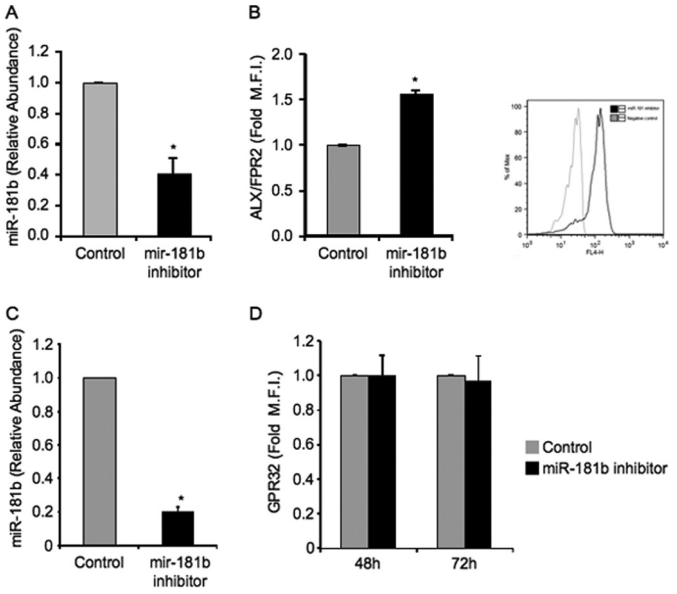

FIGURE 4. **Inhibition of mir-181b in human macrophages enhances ALX/FPR2 protein expression.** *A*, relative abundance of mir-181b 24 h post transfection of MΦs with mir-181b inhibitor or control plasmid. Data are the mean ± S.E. from $n = 3$ independent experiments carried out in duplicate. *, $p = 0.004$. *B*, ALX/FPR2 expression 48 h post transfection of MΦs with mir-181b inhibitor (mean ± S.E. from $n = 3$ independent experiments with duplicates. *, $p = 0.0002$). A representative histogram is shown in the *right panel*. *M.F.I.*, mean fluorescence intensity; *FL4H*, fluorescence intensity of the APC fluorochrome (height). *C*, see the legend to *panel A*. Data are the mean ± S.E. from $n = 3$ independent experiments carried out in duplicate. *, $p = 0.0001$. *D*, GPR32 expression at 48 h and 72 h post transfection of macrophages with mir-181b inhibitor (mean ± S.E. from $n = 3$ independent experiments with duplicates).

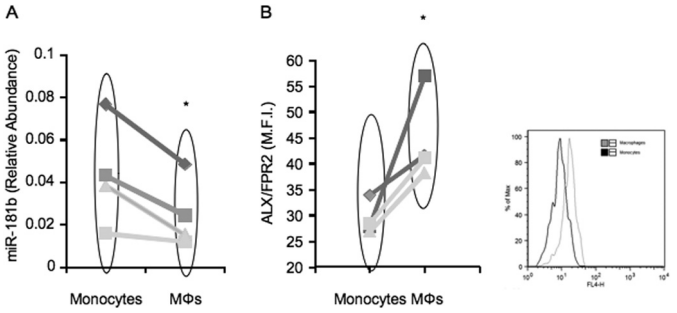

FIGURE 5. **Changes in miR-181b and ALX/FPR2 levels during human monocyte to macrophage differentiation.** Peripheral blood monocytes were differentiated into macrophages (*MΦs*) by exposure to human serum and GMCSF for 7 days. *A*, miR-181b relative abundance in MΦs was determined by real time PCR (*, $p = 0.048$). *B*, ALX/FPR2 protein expression was assessed by flow cytometry (*, $p = 0.01$). A representative histogram is shown in the *right panel*. Data are from experiments with cells from four healthy subjects carried out in duplicate. *M.F.I.*, mean fluorescence intensity; *FL4H*, fluorescence intensity of the APC fluorochrome (height).

Overexpression of miR-181b Affects Macrophage Anti-inflammatory and Proresolution Responses to ALX/FPR2 Agonists—Next, we sought to determine whether miR-181b-mediated regulation of ALX/FPR2 had an impact on agonist-evoked biological responses. Because macrophage phagocytosis is the hallmark of resolution and it is strongly enhanced by pro-resolving lipid mediators, such as LX and Rv (9, 15, 16), we assessed phagocytosis of fluorescent-labeled zymosan particles, which mimics bacterial clearance from inflamed tissue, by miR-181b overexpressing MΦs exposed to the ALX/FPR2 agonists LXA_4 and RvD1. miR-181b overexpression *per se* did not alter baseline

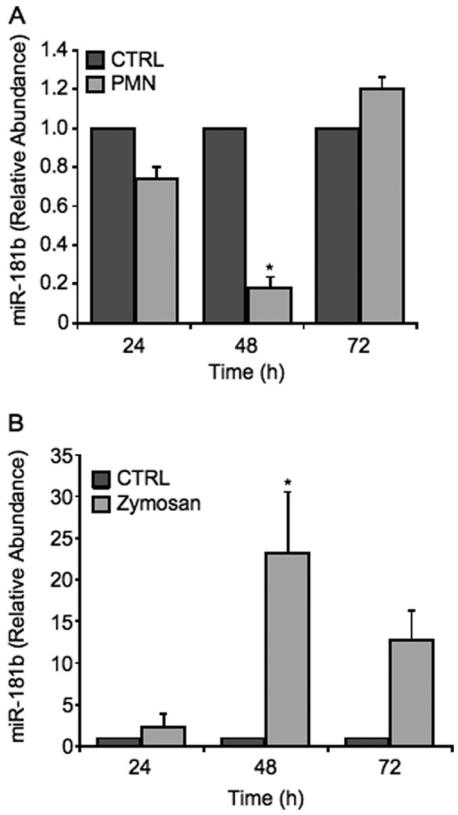

FIGURE 6. **Apoptotic PMN and zymosan regulate miR-181b expression in human macrophages.** Relative abundance of mir-181b evaluated in MΦs incubated for 24, 48, and 72 h with apoptotic PMN (*A*) (*, $p = 0.011$) or Zym (*B*) (*, $p = 0.035$).

Zym phagocytosis (Fig. 7*A*). Both LXA_4 (0.1–10 nM) and RvD1 (0.1–10 nM) concentration-dependently stimulated FITC-Zym phagocytosis in mock-transfected MΦs. This response was blunted in miR-181b-overexpressing cells (Fig. 7, *B* and *C*). We also examined the impact of miR-181b overexpression on RvD1 regulation of TNF-α expression as readout of proinflammatory signals. To this end, MΦs, transfected with TW or miR-181b-TW, were exposed to zymosan plus or minus increasing concentrations of RvD1. TNF-α levels were then assessed by ELISA. Transfection with miR-181b-TW gave a slight, not statistically significant, reduction in TNF-α release (Fig. 7*D*). RvD1 (0.01–10 nM) significantly reduced TNF-α release by TW-transfected MΦs exposed to zymosan. This effect was lost in miR-181b overexpressing cells (Fig. 7*E*). Together, these results indicate that the down-regulation of ALX/FPR2 expression by miR-181b is associated with suppression of anti-inflammatory, proresolution cellular responses triggered by endogenous ligands of this receptor.

DISCUSSION

In this study we identified miR-181b as regulator of human ALX/FPR2 expression as well as of ALX/FPR2 agonist-induced proresolution functions in human macrophages.

The ALX/FPR2 receptor conveys proresolution signals triggered by a number of endogenous anti-inflammatory mediators (8, 9). *In vivo* and *in vitro* data indicate that ALX/FPR2 expression levels represent *per se* a determinant of inflammation resolution even in the absence of exogenously added agonists (9, 14). Strikingly, a recent clinical trial demonstrated safety and effectiveness of a LXA_4 analog in infantile eczema, opening the road to resolution pharmacology in humans (47). Thus, the elucidation of mechanisms that control ALX/FPR2 expression may provide useful information for designing innovative strategies to combat human disease with an inflammatory background. In this respect we have recently uncovered a single nucleotide mutation (−220 Adenine/Guanine) in the human ALX/FPR2 core promoter of one patient with a history of acute cardiovascular events and of his two daughters, both affected by arterial hypertension and metabolic syndrome (29). This mutation reduces promoter activity *in vitro*, and it is associated with strong inhibition of ALX/FPR2 expression in circulating PMN (29). Thus, it is likely that a number of mechanisms concur to alter ALX/FPR2 expression in disease, both at the genetic and epigenetic level.

In the present report we focused on microRNAs, which are emerging as key tuners of genes related to inflammation (41). To identify miR that may regulate ALX/FPR2 expression, we employed a "bottom up" method based on *in silico* prediction of putative binding of mapped human miRs to the 3′-UTR sequence of ALX/FPR2. This system avoids time-consuming miR manipulation in cells by forced expression or inhibition and subsequent target validation. Using two bioinformatic tools, TargetScanHuman and microRNA.org, we found 27 miRNAs predicted to bind human ALX/FPR2 3′-UTR, which represent ~1.4% of annotated human miRNA mature sequences (mirbase.org) (42) (Fig. 1*C*). Among these, we selected miR-181b for further analysis, mainly because of its documented link with inflammation (45).

The human genome encompasses two miR-181b genes (NCBI Entrez gene MIR181B1 and MIR181B2) located on chromosome 1 and 9. Both genes encode for the same hairpin precursor and mature sequence of 23 nucleotides (42). mir-181b is highly expressed in leukocytes, mainly of the lymphoid lineage, and is considered a biomarker of chronic lymphocytic leukemia (38, 48, 49). Besides its involvement in cancer, miR-181b has been associated with inflammatory disorders. Increased miR-181b levels have been detected in serum or plasma from patients with liver cirrhosis (50, 51). Moreover, miR-181b controls chondrocyte differentiation and maintains cartilage integrity, being up-regulated in chondrocytes isolated from the cartilage of osteoarthritic patients (52). Increased miR-181b levels were also denoted in periodontitis gingiva (53). Along these lines, NF-κB regulation by miR-181b has been reported (45). Notably, a decrease in miR-181b after cerebral ischemic injury in mouse is regarded as a neuroprotective mechanism (54). Thus, it is likely that miR-181b is involved in the regulation of the inflammatory response in different cells and clinical settings.

Our present data support this hypothesis, as we show for the first time that miR-181b binds the 3′-UTR of the ALX/FPR2 gene, thus regulating ALX/FPR2 protein expression in human MΦs (Figs. 3 and 4). Notably, monocyte to macrophage differentiation was associated with significant down-regulation of miR-181b expression, paralleled by ALX/FPR2 up-regulation (Fig. 5). This represents an unappreciated aspect of the macrophage differentiation program, which carries functional conse-

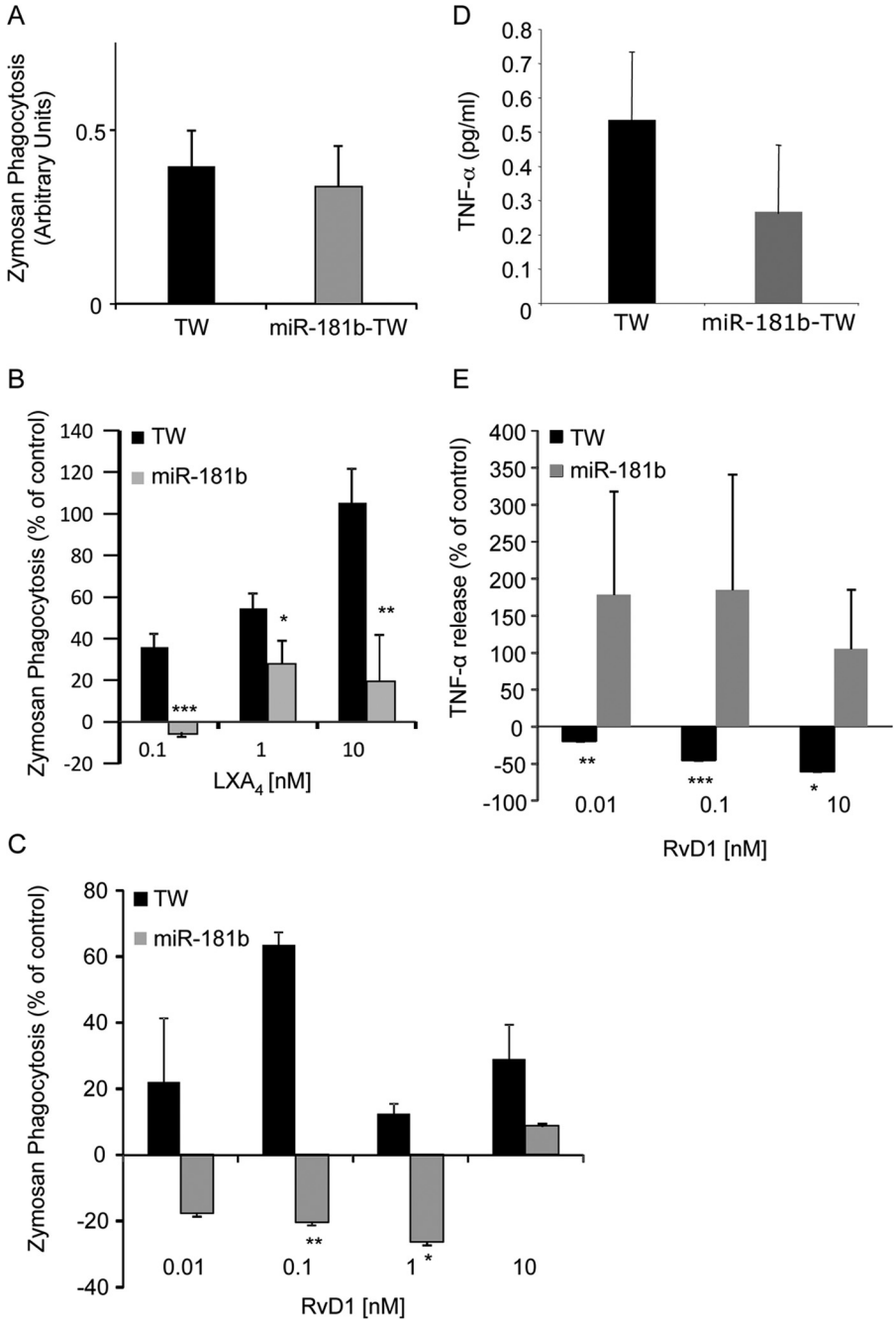

FIGURE 7. **miR-181b overexpression dampens anti-inflammatory, proresolution responses of human macrophages exposed to ALX/FPR2 endogenous agonists.** MΦs, transfected with either empty vector (*TW*) or miR-181b expressing vector (*miR-181b-TW*) were exposed for 30 min to vehicle (*A*) or to increasing concentrations of LXA_4 (*B*) (*, $p = 0.041$, **, $p = 0.036$, ***, $p = 0.033$) or RvD1 (*C*) (*, $p = 0.006$, **, $p = 0.0013$). Phagocytosis of FITC-zymosan was assessed by measuring fluorescence as under "Experimental Procedures." *Bars* represent the mean ± S.E. of four independent experiments. *D*, human MΦs were transfected with miR-181b-TW or empty TW vector and exposed to Zym for 30 min at 37 °C. TNF-α levels in the supernatants were determined by ELISA. Results are the mean ± S.E. from $n = 3$. *E*, cells were processed as in *D* and exposed to increasing concentrations of RvD1 30 min before zymosan. TNF-α was determined by ELISA. Results are expressed as the % of vehicle control and are the mean ± S.E. from $n = 3$. *, $p = 0.02$; **, $p = 0.004$; ***, $p = 0.0001$.

quences. Indeed, when the physiological decrement in miR-181b expression during macrophage differentiation was reversed by miR-181b overexpression, the stimulation by ALX/FPR2 agonists of macrophage phagocytosis (key proresolution mechanism) as well as the down-regulation of TNF-α release (proinflammatory mediator) were suppressed (Fig. 7). Along these lines, efferocytosis was accompanied by the drastic reduction of miR-181 expression, whereas zymosan potently up-regulated it (Fig. 6), suggesting that bacterial particles trigger distinct phagocytic signaling in macrophages. This interesting aspect requires further investigation. Collectively, these results indicate that miR-181b can control proresolution signals, triggered by ALX/FPR2 agonists, by regulating receptor expression, although additional mechanisms cannot be excluded. In this respect, it has to be pointed out that this mechanism appears to be rather selective, as miR-181b did not modify the expression of the GPR32 receptor, which can be activated in human leukocytes by LXA_4 and RvD1 (15, 16, 33) and it is not

expressed in mouse cells (15). Therefore, increased miR-181b levels in inflammatory disorders may sustain disease development by impairing resolution mechanisms. On the other hand, the possibility that other miRs among those listed in Table 1 may regulate ALX/FPR2 expression cannot be excluded. This should be investigated in future studies.

In conclusion, we provide here the first evidence of a miR that regulates the expression of the ALX/FPR2 receptor and, as a consequence, of agonist-induced proresolution responses in human macrophages. Together, these results uncover novel regulatory mechanisms of ALX/FPR2 protein expression, which could be exploited for innovative approaches to inflammation-based diseases.

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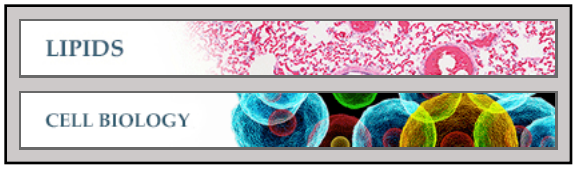
Lipids:

MicroRNA-181b Regulates ALX/FPR2 Receptor Expression and Proresolution Signaling in Human Macrophages



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