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Lipoxin A_4 stimulates endothelial miR-126-5p expression and its transfer *via* microvesicles

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ABSTRACT: The proresolution lipid mediator lipoxin (LX)A_4 bestows protective bioactions on endothelial cells. We examined the impact of LXA_4 on transcellular endothelial signaling *via* microRNA (miR)-containing microvesicles. We report LXA_4 inhibition of MV release by TNF-α-treated HUVECs, associated with the down-regulation of 18 miR in endothelial microvesicles (EMVs) and the up-regulation of miR-126-5p, both in HUVECs and in EMVs. LXA_4 up-regulated miR-126-5p by ∼5-fold in HUVECs and promoted a release of microvesicles (LXA_4-EMVs) that enhanced miR-126-5p by ∼7-fold in recipient HUVECs. In these cells, LXA_4-EMVs abrogated the up-regulation of VCAM-1, induced in recipient HUVECs by EMVs released by untreated or TNF-α-treated HUVECs. LXA_4-EMVs also reduced by ∼40% the expression of SPRED1, which we validated as an miR-126-5p target, whereas they stimulated monolayer repair in an *in vitro* wound assay. This effect was lost when the EMVs were depleted of miR-126-5p. These results provide evidence that changes in miR expression and microvesicle packaging and transfer represent a mechanism of action of LXA_4, which may be relevant in vascular biology and inflammation.—Codagnone, M., Recchiuti, A., Lanuti, P., Pierdomenico, A. M., Cianci, E., Patruno, S., Mari, V. C., Simiele, F., Di Tomo, P., Pandolfi, A., Romano, M. Lipoxin A_4 stimulates endothelial miR-126-5p expression and its transfer *via* microvesicles. FASEB J. 31, 000–000 (2017). www.fasebj.org

KEY WORDS: proresolving mediators · vascular repair · microRNA · intercellular signaling · inflammation

Endothelial cells are key players in acute inflammation, an innate protective host response to pathogens. They regulate the initial steps of the inflammatory response that include vasodilation, leakage of plasma proteins, and leukocyte adhesion and transmigration into injured tissues (1). Endothelial cells also control inflammation-associated processes like neoangiogenesis, blood coagulation and platelet aggregation (2). Ideally, the acute inflammatory response is self-limited, ending with the cessation of leukocyte, mainly polymorphonuclear neutrophil (PMN), infiltration, the clearance of apoptotic PMNs by monocyte-derived macrophages, and tissue repair (3).

Endogenous chemical mediators produced from polyunsaturated fatty acids are potent regulators of vascular responses during inflammation. For instance, arachidonic acid (AA) (5*Z*, 8*Z*, 11*Z*, 14*Z* eicosatetraenoic acid) is converted into prostaglandin (PG)E_2, which promotes edema formation; PGI_2 (also termed prostacyclin), which induces vasodilation, and thromboxane (TX)A_2, which triggers vasoconstriction and platelet aggregation (4, 5). AA can also be converted by 5-lipoxygenase-driven reactions into leukotrienes, which carry potent vasoregulatory actions (6). On the other hand, lipoxygenases catalyze the formation of a series of tetraene-containing compounds termed lipoxins (LXs), which exhibit potent anti-inflammatory and proresolving bioactions (7–9). LX epimers, carrying the C15 alcohol in the *R* configuration and termed 15-epi-LX, can be produced by endothelial cells during interactions with PMNs in the presence of aspirin (10). Their biosynthesis occurs *in vivo* in humans and can be enhanced by pioglitazone or statins (11–14), providing clues for the vasoprotective effects of these drugs. LXA_4 inhibits PMN chemotaxis, endothelial adherence, and transmigration (15); enhances nonphlogistic recruitment of monocytes (16); and stimulates macrophage phagocytosis (17). It also

ABBREVIATIONS: Ann, annexin; AA, arachidonic acid; EGCF, endothelial cell growth factor; EM, endothelial medium; EMV, endothelial microvesicle; FBS, fetal bovine serum; FPR, formylpeptide receptor; LX, lipoxin; M-CAM, melanoma cell adhesion molecule; miR, microRNA; PECAM, platelet endothelial cell adhesion molecule; PG, prostaglandin; PMN, polymorphonuclear neutrophil; P/S, penicillin/streptomycin; Rv, resolvin; SPRED, sprout-related EVH1 domain-containing protein; VE-cadherin, vascular endothelial cadherin

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regulates endothelial cell migration and angiogenesis by modulating vascular endothelial growth factor (VEGF) and VEGF receptor expression (18, 19). LXA_4 bioactions depend on the activation of a GPCR termed ALX/formylpeptide receptor (FPR)-2 (20), which is expressed in myeloid cells and in endothelial cells (18).

Microvesicles are small cell particles of heterogeneous size (100–1000 nm) that bud off parental cells in homeostatic conditions, upon cell activation. These microstructures contain a variety of proteins, nucleic acids, and lipids that can be delivered, in a paracrine, autocrine, or endocrine manner, to recipient cells, whereby they regulate functional responses (21). For instance, microvesicles from endothelial progenitor cells activate angiogenesis *in vitro* and *in vivo* and protect from kidney ischemia by transferring mRNA or microRNA (miR) (22, 23). Also, human PMN-derived microvesicles block leukocyte–endothelial cell interactions *in vivo* and *in vitro* by delivering annexin (Ann)A1 (24). Endothelial microvesicles (EMVs) are present in inflammatory lesions, such as atherosclerotic plaques or ischemic tissues (25). They may differ in concentration and phenotype between healthy subjects and patients, representing diagnostic markers of endothelial dysfunction and associated disorders (26). Microvesicles play a crucial role in inflammation and endothelial physiology, as they can deliver to recipient cells AA, which can be converted into PGI_2 and TXA_2 (27), or biogenic precursors of D-series resolvins (Rvs), which limit PMN infiltration and enhance keratinocyte healing (28).

miRs constitutes a class of small (~22 nucleotides long), noncoding RNAs that bind to mRNA to regulate posttranslational events (29). miRs are tissue- and cell-specific and control a variety of biologic processes, including cell proliferation, differentiation, and tissue development (30). They also play important roles in the inflammatory response (31) and vascular biology. For instance, the miR-17-92 cluster and miR-27a/b regulate angiogenesis and endothelial cell growth (32, 33). Moreover, miRs are part of the mechanisms triggered by LX and RvD1 to dampen acute inflammation and tissue fibrosis (34–36). miRs can be transferred across cells, packed into microvesicles for RNase protection. Also, significant differences can be observed in relative abundance of miRs between microvesicles and their parental cells (37). For instance, changes in miR content of microvesicles have been observed in metabolic and cardiovascular diseases (38), indicating a selective miR "packaging" into microvesicles.

In the present study, LXA_4 regulated EMV release and activity in inflammatory conditions and modulated miR content in endothelial cells and EMVs. Moreover, microvesicles from ECs exposed to LXA_4 were enriched in miR-126 and had enhanced capability to stimulate endothelial migration.

MATERIALS AND METHODS

Endothelial cell culture and EMV isolation

Umbilical cords were obtained from randomly selected healthy mothers who delivered at the Chieti University Hospital. All procedures were in agreement with the ethical standards of the Institutional Committee on Human Experimentation (Reference Number: 1879/09COET) and with the principles of the Declaration of Helsinki. After approval of the protocol by the Institutional Review Board, a signed informed consent form was obtained from each participating subject. HUVECs were obtained and cultured on a 1.5% gelatin matrix with endothelial medium (EM): DMEM low-glucose (50% vol/vol), M199 (50% vol/vol), penicillin/streptomycin (P/S, 1%), and L-glutamine (2 mM) supplemented with fetal bovine serum (FBS, 20%), heparin (18 U/ml), and endothelial cell growth factor (ECGF, 50 μg/ml). For EMV production, 80% confluent HUVECs between the 3rd and 6th passages were incubated with LXA_4 (0.1–100 nM) and TNF-α (1 ng/ml) for 24 h in modified EM with 10% FBS without ECGF and heparin, to minimize the influence of these agents on LXA_4 bioactions. In some experiments, cells were exposed to the ALX/FPR2 antagonist WRW_4 (WRWWWW; 10 μM, 30 min; Calbiochem, Billerica, MA, USA). For EMV isolation and purification, the cell medium was collected and centrifuged at 300 *g* for 10 min, then at 12,096 *g* for 15 min to remove cell debris. Supernatants were ultracentrifuged (152,000 *g*, 2 h) to collect EMVs. EMVs and cells were lysed with lysis solution from Norgen Biotek (Thorold, ON, Canada), and materials were stored at −20°C until miR isolation.

Flow cytometric analysis of EMVs

For EMV characterization, HUVEC supernatants (5 μl/sample) were added to 200 μl of binding buffer (BD Bioscience, Franklin Lakes, NJ, USA) 2X, to control Ca^{2+} concentration, which is relevant for AnnV binding and processed with a "no lyse and no wash" method (39). In brief, samples were stained with AnnV (allophycocyanin-conjugated) and Mitotracker green (Thermo Fisher Scientific, Waltham, MA, USA). After staining (30 min, 4°C in the dark), 500 μl of binding buffer 2X (BD Bioscience) were added to each tube, and 1×10^6 events/samples were acquired by flow cytometry with a FACS Canto cytometer (BD Bioscience) with a 3-laser, 8-color configuration, set at a low threshold (200) on the FITC channel. Signal amplifications for forward scatter and side scatter, as well as for any fluorescence channel, were set in the logarithmic mode. EMV morphology was confirmed by running Megamix beads (Biocytex, Marseille, France). Instrument performances and data reproducibility were checked by the Cytometer Setup & Tracking Module and further validated by the acquisition of Rainbow Beads (BD Bioscience). Fluorescence Minus One controls were used to detect nonspecific fluorescence. Compensation was assessed by using CompBeads (BD Bioscience) and Mitotracker green single-stained samples. Data were analyzed with the FACSDiva ver. 6.1.3 (BD Bioscience), and FlowJo, ver. 8.8.6 (TreeStar, Ashland, OR, USA) software. EMVs were enumerated by a single-platform counting method with Trucount tubes (BD Bioscience). EMV concentration was calculated with the following formula: (number of events for AnnV in the Trucount bead region) × (number of Trucount beads per test/test volume).

miR measurements

miR were evaluated by real-time PCR. To this end, miR, contained in EMVs, were isolated with a purification kit (Norgen Biotek) and quantified using a Nanodrop Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). A hundred nanograms miR was reverse transcribed with miScript II RT Kit (Qiagen, Milan, Italy), and the relative abundance of 86 miR was determined using miScript miR PCR Array (Qiagen). Real-time

PCR was outperformed on a ABI 7900HT thermocycler (Thermo Fisher Scientific), using the manufacturer's recommended program. miR expression was determined by the $2^{-\Delta\Delta Ct}$ method (40) and cel-mir-39 was used to normalize input cDNA.

miR-126-5p inhibition

HUVECs (1–1.5 $\times$ 10^6 cells), seeded into a 10 cm culture dish, were transfected with the human serum albumin-miR-126-5p inhibitor (MIN0000444; Qiagen) or a scramble negative control miRNA (200 pmol/dish in 10 ml of complete growth medium) using the Interferin reagent (40 μl/dish; Polyplus transfection; Illkirch, France) according to the manufacturer's protocol. Optimal efficiency was achieved when cells were 50% confluent on the day of transfection. EMVs were collected and analyzed as reported above.

EMV uptake by HUVECs and miR-126 expression

Conditioned media from untreated or LXA_4-treated HUVECs were incubated with PKH26 (1:250; Sigma-Aldrich, Milan, Italy) and ultracentrifuged for 2 h, as described above. PKH26-labeled EMVs were counted using FACS Verse (BD Biosciences), and 1×10^6 EMVs were added directly to confluent HUVECs cultured in 6-well plates (2 ml). After 4 and 24 h at 37°C, the HUVECs were washed twice with PBS. To assess uptake, the cells were detached and visualized with the Image Stream Amnis (EMD Millipore, Billerica, MA, USA). miR-126 levels in recipient HUVECs were measured as above.

Analysis of VCAM-1 expression

HUVECs (70–80% confluent) were treated with LXA_4 (10 nM) and/or TNF-α (1 ng/ml) for 24 h in modified EM with 10% FBS without ECGF and heparin. After 24 h, EMVs were isolated by ultracentrifugation for 2 h and enumerated on the FACS Verse (BD Biosciences) flow cytometer. EMVs (2×10^6) derived from untreated HUVECs (CTRL-EMV) or from HUVECs exposed to TNF-α (TNFα-EMV), TNF-α plus LXA_4 (TNFα+LXA_4-EMV), or LXA_4 (LXA_4-EMV), were added to confluent HUVECS cultured in 6-well plates (2 ml). After a 24 h incubation at 37°C, HUVECs were washed twice with PBS and labeled with an anti-VCAM-1 antibody (PE-A-anti-CD106; BioLegend, San Diego, CA, USA). The cells were analyzed with a FACS Verse flow cytometer (BD Biosciences).

In vitro wound healing

HUVECs were seeded in 6-well plates (2×10^5 cells/well) with basal medium until monolayers reached 80–90% confluence. Wounds were created by manually scraping the monolayers with a 200 μl pipet tip and the plates were then vigorously rinsed with PBS to remove cellular debris. After the wound was created, 2×10^6 EMVs from untreated or LXA_4-treated HUVECs were added to each well and incubated at 37°C, in a humidified atmosphere containing 5% CO_2 for 6 or 20 h. HUVECs in 1% or 10% FBS medium were respectively used as negative and positive controls. Wound closure was evaluated at 6 and 20 h with an optical microscope at ×10 magnification. Two zones of each wound were photographed at different time intervals along the wound boundary, and for each of the 2 images, wound widths were quantified in 5 locations with ImageJ software (National Institutes of Health, Bethesda, MD, USA). Duplicate wells of each condition were examined for each experiment.

Western blot analysis

HUVECs from the wound-healing assay were homogenized at 4°C with RIPA buffer [150 mM NaCl; 1.0% Nonidet P-40; 0.5% deoxycholate; 0.1% SDS, and 50 mM Tris (pH 8.0)] containing 1 mM Na_3VO_4, 5 mM NaF and a cocktail of protease inhibitors (Sigma-Aldrich). Protein concentration was determined with the Lowry protein assay (Bio-Rad, Hercules, CA, USA). Equal amounts of proteins (30 μg) were resolved by 12% SDS gel electrophoresis, transferred onto PVDF membranes, and blocked with 10% nonfat dry milk for 1 h. Blots were incubated with a primary anti-sprouty-related EVH1 domain–containing 1 (SPRED1) antibody (1:500 dilution; Thermo Fisher Scientific) or with an anti-GAPDH antibody (Santa-Cruz Biotechnologies, Inc., Dallas TX, USA), followed by the correspondent HRP-conjugated secondary antibodies. Proteins were revealed by chemiluminescence with the ECL kit (Euroclone, Milan, Italy). GAPDH was used as loading control. Chemiluminescence signals were quantitated with an imaging scanner (Alliance 4.7; Uvitec, Cambridge, United Kingdom).

3′UTR luciferase reporter assay

Breast cancer MDA-MB-231 cells, grown in DMEM supplemented with 10% FBS, 1% L-Glu and P/S, were cotransfected with SPRED1 3′UTR (pEZX-MT05; Genecopoeia, Rockville, MD, USA), plus the pCMV-miR-126-5p plasmid (Origene, Rockville, MD, USA) or an empty pCMV-MIR plasmid (Origene) as a control (1.5 μg each) using Lipofectamine 2000 (12 μl; Thermo Fisher Scientific), according to the manufacturer's protocol. Twenty-four hours after transfection, cells were lysed, and the luciferase activity was determined as previously reported (41).

Statistical analysis

Results are reported as arithmetic means ± SEM, unless otherwise indicated. Statistical significance was assessed with the Student's t test. A value of $P < 0.05$ indicatied significance. Statistical analysis was performed with Prism 5 (GraphPad, La Jolla, CA, USA).

RESULTS

Impact of LXA_4 on TNF-α-induced EMV release and activity

LXA_4 exerts potent regulatory bioactions on endothelial cells. Therefore, we asked whether LXA_4 regulates EMV release. To this end, we initially set the appropriate flow cytometric conditions to identify EMVs in conditioned medium, by exposing confluent HUVEC monolayers to TNF-α (1 ng/ml) for 24 h and incubating the conditioned medium with Mitotracker Green fluorescent medium. EMVs were detected and distinguished from cell debris based on positive MitoTracker staining (39). They were gated on the basis of their mitochondrial activity and morphologic parameters (**Fig. 1*A***). This method, by avoiding morphologic recognition, maximized the probability of including all EMV events in the analysis and helped to exclude debris, immune complexes, and calcium phosphate precipitates.

TNF-α increased EMV release by ~2.2 fold ($P = 0.002$). This effect was abrogated by LXA_4 (0.1–100 nM, 24 h) in

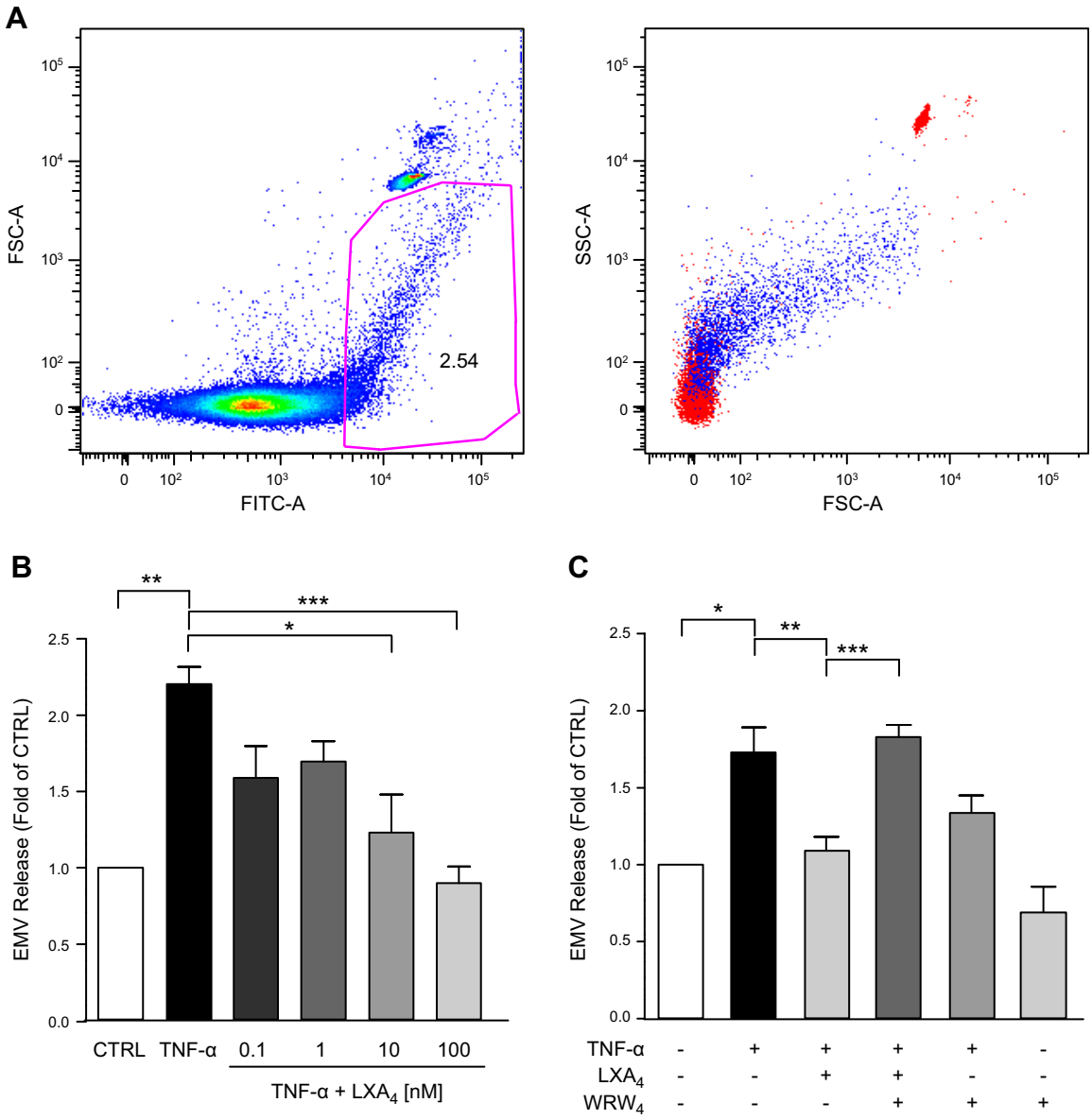

Figure 1. LXA_4 receptor-dependent regulation of TNF-α-induced EMV release. (*A*) Left: flow cytometric analysis of MitoTracker+EMV. Right: EMV morphologic parameters. (*B*) HUVECs were either left untreated (CTRL) or exposed to TNF-α (1 ng/ml), in the presence or not of the indicated concentrations of LXA_4. EMVs were collected after 24 h and enumerated by flow cytometry. Results are means ± SEM of 4 different experiments, each performed in duplicate. **P* = 0.02, ***P* = 0.002, ****P* = 0.0005. (*C*) HUVECs were exposed for 24 h to TNF-α (1 ng/ml), with or without LXA_4 (100 nM). When present, WRW_4 (10 μM) was added to cells alone or 30 min before LXA_4. Results are means ± SEM of 3 different experiments, each performed in duplicate. **P* = 0.04, ***P* = 0.03, ****P* = 0.004.

a concentration-dependent manner, with maximum inhibition at 10 nM (~80% reduction; $P = 0.02$) and at 100 nM LXA_4 (~100% reduction; $P = 0.0005$; Fig. 1*B*). LXA_4 itself did not modify the number of released EMVs (results not shown). To establish receptor dependence of this effect, we exposed HUVECs to WRW_4, a selective ALX/FPR2 antagonist (42). As illustrated in Fig. 1*C*, WRW_4 suppressed the effect of LXA_4 on EMV release ($P = 0.004$). Moreover, the WKYMVm peptide, another known ALX/FPR2 agonist (43, 44) potently inhibited TNF-α-induced EMV release (Supplemental Fig. 1*A*). Together, these results indicate that ALX/FPR2 signaling controls EMV release.

LXA_4 selectively modulates miR content in EMVs and HUVECs

Microvesicles contain miRs that exert regulatory effects on cell uptake (22, 23, 38). To determine whether LXA_4 also had an impact on the miR profile of EMV, we evaluated the relative abundance of 86 miRs, selectively expressed by endothelial cells (45), using an miR PCR array. LXA_4 modulated the contents of several miRs in EMVs from TNF-α-stimulated HUVECs. Among these, 18 were significantly down-regulated (**Fig. 2*A***), whereas 54 were up-regulated, although only miR-126-5p reached statistical significance (Supplemental

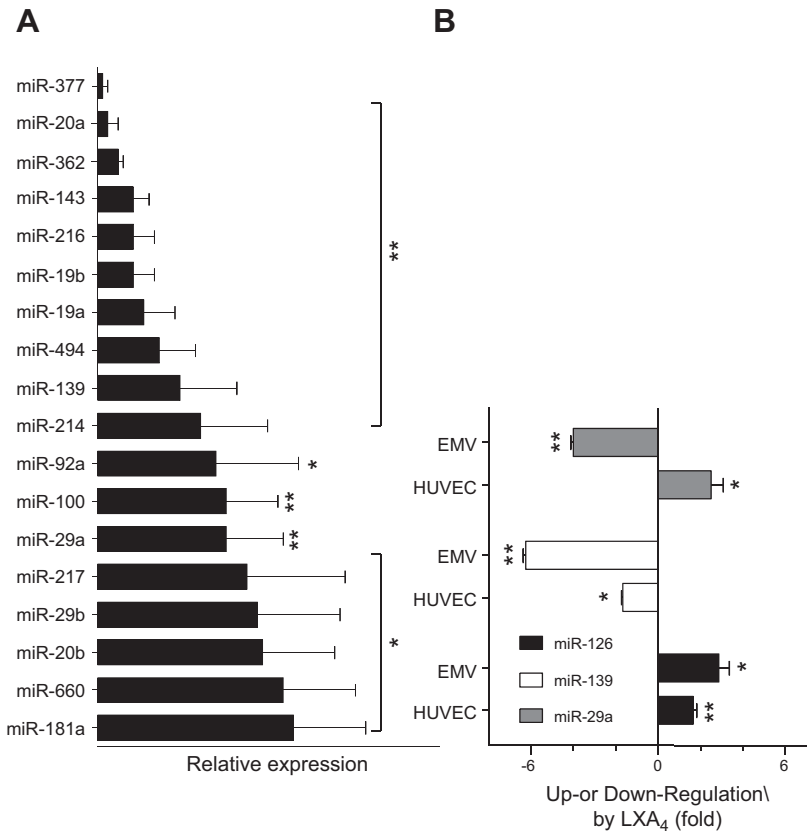

Figure 2. LXA_4 modulates miR profile in HUVECs and EMVs. Cells (1.5×10^6) were incubated with TNF-α (1 ng/ml) in the presence or not of LXA_4 (10 nM) for 24 h. Endothelial-specific miRs were isolated from cells and EMVs and analyzed. (*A*) LXA_4 significantly down-regulated 18 miRs. $^*P < 0.01$, $^{**}P < 0.001$. Results are expressed as means ± SEM of 4 different experiments. (*B*) miR differentially modulated in HUVECs and EMVs by LXA_4. Data are means ± SEM of 4 different experiments. miR-29a: $^*P = 0.05$, $^{**}P = 0.002$; miR-139: $^*P = 0.005$ $^{**}P = 0.001$; miR-126: $^*P = 0.02$, $^{**}P = 0.01$.

Table 1). A comparative analysis of cell-associated *vs.* EMV-containing miRs showed that LXA_4: *1*) down-regulated miR-29a in EMVs ($P = 0.05$), while up-regulating it in cells ($P = 0.02$); *2*) down-regulated miR-139, both in EMVs ($P = 0.001$) and HUVECs ($P = 0.005$); and *3*) up-regulated miR-126-5p in EMVs ($P = 0.02$) and cells ($P = 0.01$) (Fig. 2*B*). These results show for the first time that LXA_4 regulate the relative abundance of miRs and packaging of miR into microvesicles.

Analysis of miR-126-5p up-regulation by LXA_4

Recent evidence indicates that miR-126 plays key roles in vascular endothelial cell repair and inflammation (46, 47).Therefore, we analyzed the direct effects of LXA_4 on this miR. To this end, we evaluated the relative abundance of miR-126 in HUVECs after exposure to increasing LXA_4 concentrations (0.01–100 nM). As illustrated in **Fig. 3*A***, LXA_4 up-regulated miR-126-5p expression in a concentration-dependent manner, with significant increments at 0.1 nM (~3-fold; $P = 0.04$), 1 nM (~5-fold; $P = 0.00003$) and 10 nM (~3-fold; $P = 0.02$). A time course of exposure to 1 nM LXA_4, showed a significant miR-126-5p increase after 18 h (~3-fold; $P = 0.02$) and 24 h (~4-fold; $P = 0.009$; Fig. 3*B*). In contrast, LXA_4 did not change miR-126-3p expression (results not shown). Unlike LXA_4, the WKYMVm peptide did not modify miR-126-5p expression (Supplemental Fig. 1*B*).

Impact of LXA_4 on EMV uptake by HUVECs

EMV uptake by recipient endothelial cells as mechanism of paracrine intercellular signaling has been documented (48).To determine whether LXA_4 has an impact on this process, HUVECs were exposed to 1 nM LXA_4 or vehicle for 24 h, and EMVs were isolated, labeled with PKH26, and coincubated with recipient HUVECs for 4 and 24 h. Image Stream flow cytometry showed a significant uptake of PKH26-labeled EMVs by HUVECs. Notably, after 4-h incubation EMVs from LXA_4-treated HUVECs (LXA_4-EMVs) were incorporated with higher affinity (~1.5-fold; $P = 0.0002$) compared with EMVs from cells exposed to vehicle (CTRL-EMVs; **Fig. 4*C***). This effect was no longer observed after 24 h. miR-126-5p was unexpectedly more abundant in cells exposed for 4 h to LXA_4-EMVs than to either untreated HUVECs (~7-fold increase, $P = 0.0004$) or cells incubated with CTRL-EMVs (~2 fold increase, $P = 0.04$; (Fig. 4*D*). No significant changes in miR-126-3p were instead detected (results not shown). These results indicate that miR-126-5p can be transferred to recipient endothelial cells *via* EMVs and that such transfer can be enhanced by LXA_4.

LXA_4-EMVs down-regulate the expression of miR-126-5p targets and stimulate endothelial migration *in vitro*

Because LXA_4-EMVs enhanced miR-126-5p expression in recipient HUVECs, we next determined whether they also

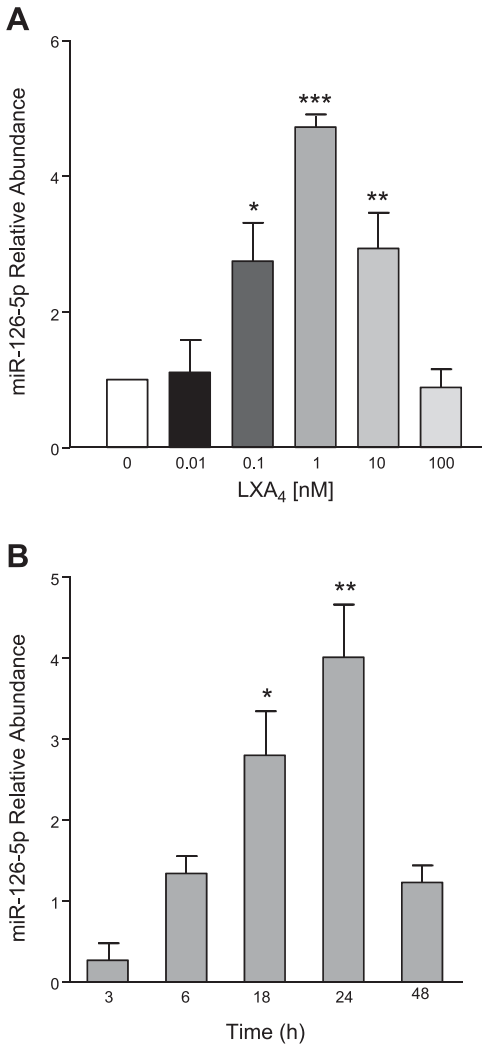

Figure 3. Time- and concentration-dependent miR-126-5p up-regulation by LXA_4. (*A*) HUVECs (1.5×10^5) were incubated with the indicated concentrations of LXA_4 for 24 h. miR-126-5p expression was evaluated by real-time PCR. Results are means ± SEM ($n = 3$). *$P = 0.04$, **$P = 0.02$, ***$P = 0.00003$. (*B*) miR-126-5p expression in HUVEC exposed to 1 nM LXA_4 for the indicated time. Bars represent means ± SEM of 3 different experiments. *$P = 0.02$; **$P = 0.009$.

down-regulated select targets, relevant to inflammation and vascular integrity. To this end, we set 2 experimental conditions: one to mimic endothelial inflammation, whereby HUVECs were exposed to TNF-α in the presence or not of LXA_4; the other to investigate protective LXA_4 bioactions on endothelial cells. For the first condition, we selected VCAM-1 as an miRNA-126-regulated protein (49) and observed that EMVs released by cells exposed to LXA_4, alone or with TNF-α, reduced VCAM-1 expression in recipient cells (**Fig. 5*A***). For the second condition, we focused on SPRED1, which was identified as a key effector of miR-126-dependent stimulation of endothelial cells migration (46). We first validated SPRED1 as the miR-126 target gene. We cotransfected SPRED1 3′UTR (pEZX-MT05) with an miR-126-5p/luciferase-expressing plasmid (pCMV-miR-126-5p) in MDA-MB231 cells and measured luciferase activity 24 h after transfection. We observed a significant (~40%; $P = 0.0002$) reduction in luciferase activity in MDA-MB-231 cells coexpressing pCMV-miR-126-5p and pEZX-MT05 compared with cells transiently transfected with the empty pCMV-miR vector together with pEZX-MT05 (Fig. 5*B*), thus demonstrating direct binding of miR-126-5p to SPRED1 3′UTR. We then examined SPRED1 expression in recipient HUVECs exposed to CTRL- or LXA_4-EMVs. Figure 5C shows that, consistent with previous data (46), CTRL-EMVs reduced SPRED1 expression by ~20% in recipient HUVECs and that this inhibition reached ~40% with LXA_4-EMVs ($P = 0.005$ *vs.* CTRL-EMVs).

Subsequently, we examined the impact of LXA_4-EMVs on HUVEC migration. For this assay, HUVEC monolayers were manually scraped with a 200 μl sterile pipet tip, to obtain a wound of ~80 μm width (as described earlier). Cells were then incubated with CTRL- or LXA_4-EMVs in the presence of 1% FBS, to inhibit cell proliferation, and wound closure was evaluated after 6 and 20 h. At 6 h, both CTRL- and LXA_4-EMVs significantly stimulated wound closure (~1.5-fold; $P = 0.006$ and $P = 0.0094$, respectively; **Fig. 6*A***). At this time point, the effect of the LXA_4-EMVs was not significantly different from that of CTRL-EMVs. However, after 20 h, LXA_4-EMVs stimulated wound closure to a higher extent *vs.* CTRL-EMVs ($P = 0.0072$). To obtain more direct evidence of the involvement of miR-126-5p in these phenomena, we transfected HUVECs with an miR-126-5p inhibitor, which yielded miR-126-5p-depleted EMVs (Fig. 6*B*). miR-126-5p-depleted EMVs, either from untreated (126-5pInh CTRL-EMVs) or LXA_4-treated (126-5pInh LXA_4-EMVs) HUVECs, were unable to stimulate wound closure of the recipient cells (Fig. 6*C*).

DISCUSSION

Starting from a large body of evidence of the role of LXA_4 in vascular biology (8), as well as from previous work showing that proresolving lipid mediators, such as RvD1, control the inflammatory response by modulating miR expression *in vitro* and *in vivo* (50), we tested the hypothesis that LXA_4 controls intercellular pathways of miR delivery. In the present work, we provide evidence that LXA_4 induces transcellular signaling by exploiting microvesicles as carriers and miR as effectors.

EMVs can be regarded as biomarkers of inflammation and endothelial dysfunction, because they may reflect a balance between cell stimulation, proliferation, and death (26). EMVs are released by endothelial cells upon exposure to inflammatory stimuli and increase in the circulation of patients with cardiovascular disease, renal failure, and metabolic disorders (21, 51). Depending on cell and microenvironmental conditions, EMVs can perturb vascular homeostasis by activating endothelial cells with an autocrine loop (52), but they can also promote cell survival, induce anti-inflammatory effects, and stimulate endothelial regeneration (46). Thus, it is likely that in addition to the number of EMVs, their contents can make the difference between damaging or protective effects. Indeed, EMVs are cargo vectors for material selectively acquired from parental cells, including bioactive lipids, integrins,

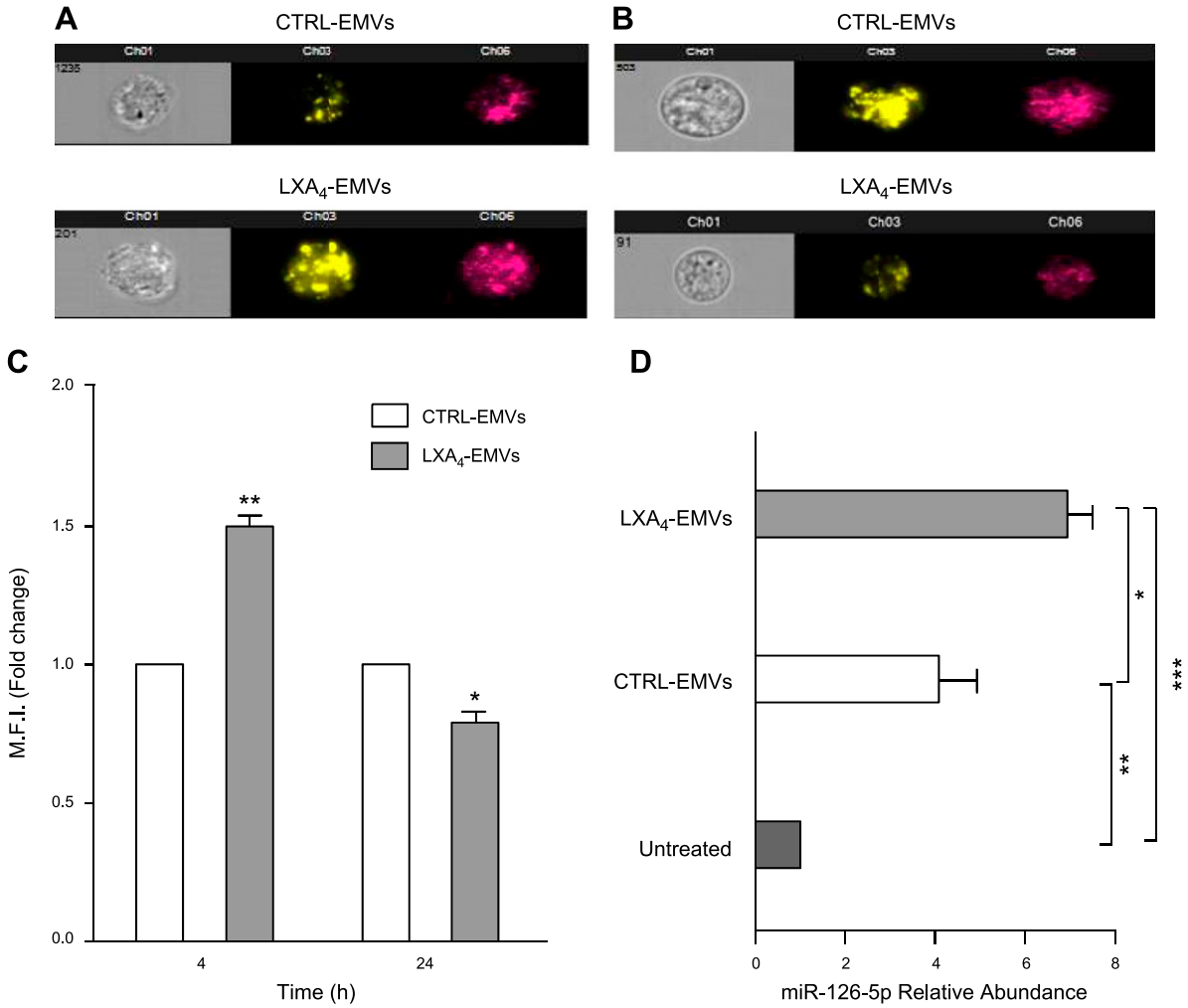

Figure 4. LXA_4 affect EMV uptake by HUVECs. Cells (1.5×10^5) were incubated with CTRL-EMVs or LXA_4-EMVs for 4 (*A*) or 24 h (*B*). EMV uptake was monitored by flow cytometry. Representative bright-field (Ch01), PE-fluorescence (Ch03) and side scatter (Ch06) images are shown. Magnification, ×40. (*C*) EMV uptake was quantitated using a specific algorithm to decode fluorescence data. Results are expressed as means ± SEM of 3 different experiments. $*P = 0.008$; $**P = 0.0002$. (*D*) HUVECs were exposed to CTRL-EMVs or LXA_4-EMVs for 4 h and relative abundance of miR-126 was determined by real-time PCR. MFI, mean fluorescence intensity. Data are means ± SEM ($n = 3$). $*P = 0.04$; $**P = 0.02$; $***P = 0.0004$.

cytokines, enzymes, mRNA and miR, which can reprogram recipient cells (53). Moreover, they can carry oxygen-reactive species, which are more abundant in EMVs generated by HUVECs subjected to reperfusion injury and promote cardiomyocyte apoptosis (54).

We tested the hypothesis that one of the mechanisms of LXA_4 bioactions on vascular homeostasis was the regulation of EMV release and packaging, with consequences on EMV functional impact on recipient cells. Although LXA_4 *per se* did not alter the number of EMVs released under standard culturing conditions, it antagonized TNF-α, significantly reducing the number of microvesicles released by the HUVECs exposed to this proinflammatory cytokine (Fig. 1*B*). This result is consistent with early data showing that LXA_4 can counteract proinflammatory responses triggered by cytokines (18, 55) and indicates that modulation of microvesicle biogenesis represents an unappreciated antagonist function of LXA_4. This effect occurs through the activation of ALX/FPR2 (Fig. 1C), a proresolving receptor that intercepts multiple pro-resolution circuits regulated by RvD1 and AnnA1 in addition to LXA_4 (9). We obtained further evidence of the involvement of ALX/FPR2 in this setting by using the WKYMVm peptide, which binds and activates this receptor (43, 44). This peptide blocked TNF-α-induced EMV release (Fig. 1C), but unlike LXA_4, it did not up-regulate miR-126-5p expression (Supplemental Fig. 1). These results are consistent with different interaction domains and signaling of ALX/FPR2 agonists (56).

Together with biogenesis, the analysis of content is key to understanding the functional significance of EMVs. We focused on miR in light of the emerging role of these small RNA sequences in vascular biology and inflammation (32, 57). We used a top-down approach by analyzing changes in 84 endothelium-specific miRs in EMVs. LXA_4 significantly down-regulated 18 miRs (Fig. 2*A*) and up-regulated 54, among which only the increase in miR-126-5p reached

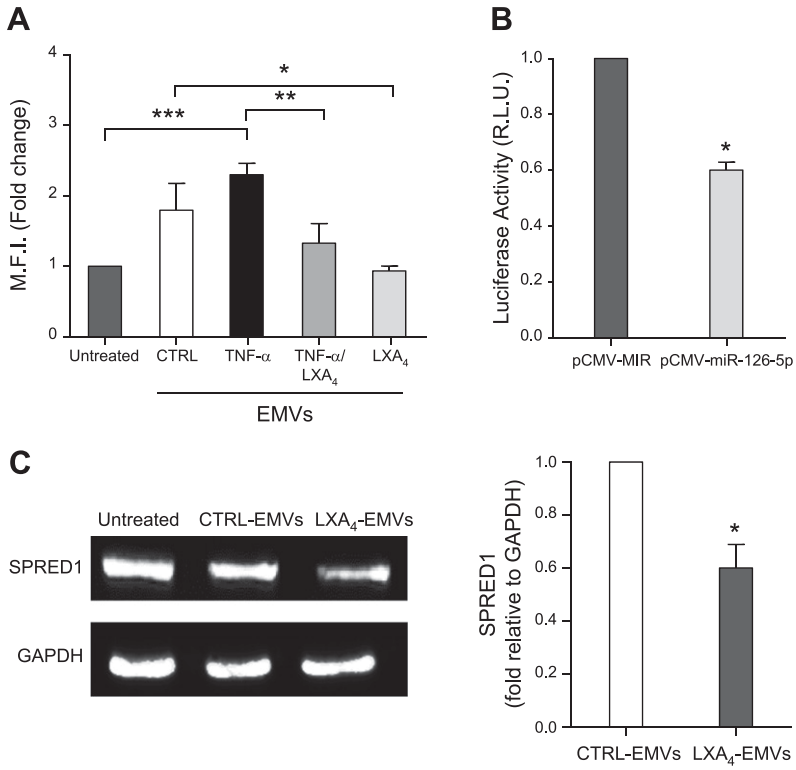

Figure 5. miR-126-5p targets and EMV functions regulated by LXA_4. (*A*) HUVECs were seeded at 1.2×10^5 and treated with CTRL-EMVs, TNFα-EMVs, TNFα+LXA_4-EMVs, or LXA_4-EMVs for 24 h. VCAM-1 was evaluated by flow cytometry. Data are means ± SEM ($n = 3$). *$P = 0.04$, **$P = 0.02$, ***$P = 0.0005$. (*B*) MDA-MB-231 cells were transfected with pEZX-MT05 plus pCMV-MIR empty vector or a miR-126-5p-expressing pCMV-MIR vector (pCMV-miR-126-5p). The luciferase activity was normalized for total proteins in cell lysates. Means ± SEM of 3 independent experiments performed in duplicate. *$P = 0.0002$. (*C*) HUVECs (2×10^5) were exposed to CTRL- or LXA_4-EMVs for 20 h, and SPRED1 expression was evaluated by Western blot analysis. Left: a representative blot; right: densitometric analysis. MFI, mean fluorescence intensity; RLU, relative luminance units. Data are means ± SEM of 5 different experiments. *$P = 0.005$.

statistical significance (Fig. 2*B* and Supplemental Table 1). Notably, comparing data from measurements of miR content in cells *vs.* microvesicles, it was clear that LXA_4 regulated both miR generation and packaging into EMVs (Fig. 2*B*), providing evidence that endogenous proresolution lipid mediators selectively regulate miR packaging. Among the LXA_4–down-regulated miRs, miR-92a may have negative outcomes on vascular integrity as its inhibition prevents endothelial dysfunction and atherosclerosis (58), whereas miR-19a and miR-20a may be relevant in angiogenesis (59). Thus, overall LXA_4 appears to antagonize TNF-α-triggered proinflammatory miR signaling, although further studies are required to support this hypothesis.

Among the 84 miRs evaluated in this study, only miR-126-5p was significantly up-regulated by LXA_4, either by itself or in combination with TNF-α, both in HUVECs and EMVs (Fig. 2). Because no changes in miR-126-3p were observed, it is likely that LXA_4 acts on premiR-126 maturation. Both the 3p and 5p strands of miR-126 exert protective actions on the vasculature, although they may control different pathways. In fact, miR-126-3p delivery *via* microvesicles released by apoptotic endothelial cells, reduces experimental atherosclerosis (60), whereas miR-126-5p, but not miR-126-3p, promotes endothelial cell proliferation and vascular integrity (61). Along these lines, deletion of the gene encoding premiR-126 impairs postischemic neovascularization in mice (62). Together with previous work showing LXA_4 attenuation of renal fibrosis by the induction of let-7c in human renal epithelial cells (34), our present data indicate that the regulation of selected miR is a relevant mechanism of action of LXA_4, which can be helpful to unveil organ protective pathways relevant in pathobiology and pharmacology.

LXA_4 also modulated properties and paracrine functions of EMVs. We observed that LXA_4-EMVs had a faster kinetic of uptake by recipient HUVECs compared with the EMVs released in the absence of LXA_4. Although an uptake enhancement at 4 h (Fig. 4*C*) appears to be consistent with an amplification of microvesicle-transferred miR bioactivity (48), the mechanisms underlying this phenomenon are elusive. LXA_4 did not influence EMV expression of adhesion molecules (*i.e.*, PECAM-1, M-CAM, VCAM-1 and VE-cadherin), which can potentially control EMV uptake by recipient HUVECs, indicating that other mechanisms may be involved. On the other hand, the LXA_4-EMV–induced increase in miR-126-5p expression in recipient HUVECs (Fig. 4*D*) is likely to be related to the higher miR-126-5p content in LXA_4-EMV, although the stimulation of *de novo* miR-126-5p formation cannot be excluded. This point also should be studied further.

However determined, miR-126-5p up-regulation by LXA_4-EMV in recipient HUVECs correlated with the suppression of EMV- or TNF-α-induced EMV up-regulation of VCAM-1, a miR-126 regulated gene and main component of the inflammatory cascade (49) (Fig. 5*A*). It was also associated with enhanced down-regulation of SPRED1 (Fig. 5*C*), which we validated as miR-126-5p target (Fig. 5*B*), as well as with the stimulation of wound closure (Fig. 6*A*). This last effect was lost when EMVs were depleted in miR-126-5p (Figs. 6*B*, *C*), emphasizing the role of this miR in EMV-dependent intercellular communication routes.

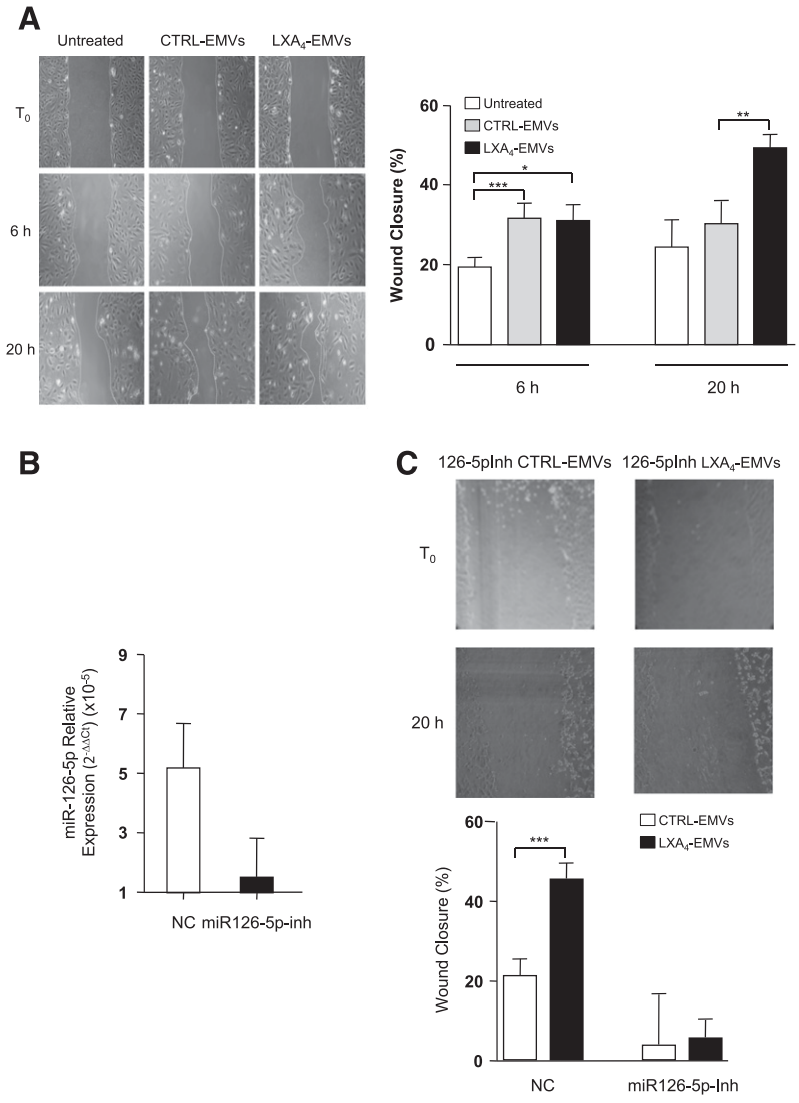

Figure 6. (*A*) HUVECs were seeded at 2×10^5 and maintained in 1% FBS for 24 h. Monolayers were scraped with a 200-μl pipet tip, and cells were either left untreated or exposed to CTRL- or LXA_4-EMVs. Wound closure was quantified at 6 and 20 h after wounding, by measuring the area not filled by cells with image analysis software. Left: representative images obtained by phase-contrast microscopy. Magnification ×10. Right: quantitative analysis. Results are expressed as percentage above T_0 and represent means ± SEM of 4 different experiments. 6 h: $*P = 0.0094$, $***P = 0.006$; 20 h: $**P = 0.0072$. (*B*) miR-126-5p expression in EMVs from HUVECs transfected with the miR-126-inhibitor, as compared with EMVs from cells transfected with the scrambled negative control miRNA (NC). Results are means ± SE from 2 representative experiments. (*C*) Wound closure of HUVECs exposed to EMVs released by cells transfected with an NC miRNA or the miR-125-5p inhibitor and treated or not with LXA_4. Results are means ± SE from 4 separate experiments performed in duplicate. $***P = 0.0008$.

Together, these results are consistent with early reports on the protective functions of miR-126 on the vasculature (46, 47, 59, 61) and indicate that LXA_4 anti-inflammatory signaling can propagate by intercellular pathways involving the modulation of microvesicle biogenesis, content, and function. Along these lines, reduced miR-126 content in microvesicles released by endothelial cells exposed to high glucose as well as in peripheral blood microvesicles from diabetic patients has been reported (46), suggesting that the observed LXA_4 actions on miR-126 could be relevant in disease states.

In summary, our present results highlight the biologic significance of EMVs as autocrine modulators of endothelial repair functions. This is a relevant concept that supports the view that cell-to-cell communication is achieved by multiple circuits, involving soluble mediators, as well as signals delivered with microvesicles. In this respect, our findings showing that the endogenous pro-resolution lipid mediator LXA_4 can regulate these mechanisms open new perspectives for the understanding of protective circuits that may be relevant in vascular biology and more generally during the immune–inflammatory response. FJ

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AUTHOR CONTRIBUTIONS

M. Codagnone designed and performed the research and wrote the paper; A. Recchiuti performed the research, analyzed the data, and wrote the paper; P. Lanuti, A. M. Pierdomenico, E. Cianci, S. Patruno, V. C. Mari, F. Simiele and P. Di Tomo performed the research and analyzed the data; A. Pandolfi analyzed the data; and M. Romano designed the research and wrote the paper.

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Lipoxin A_4 stimulates endothelial miR-126-5p expression and its transfer *via* microvesicles

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Supplementary Table 1 miRNA that are expressed at higher levels (Relative expression ≥ 0,1) in Microvesicles derived from HUVEC treated with TNFα+LXA_4 than those from HUVEC treated with TNFα after 24h.

Mature Sanger ID	Mature Sanger Accession	Relative expression	P Value
hsa-miR-363-5p	MIMAT0003385	1,53	0,60
hsa-miR-19b-3p	MIMAT0000074	0,08	8,91E-07
hsa-miR-92a-2-5p	MIMAT0004508	0,23	0,01
hsa-miR-18b-5p	MIMAT0001412	2,07	0,51
hsa-miR-20b-5p	MIMAT0001413	0,96	0,97
hsa-miR-127-3p	MIMAT0000446	1,78	0,19
hsa-miR-1290	MIMAT0005880	4,01	0,38
hsa-miR-132-3p	MIMAT0000426	3,07	0,33
hsa-miR-139-5p	MIMAT0000250	0,16	1,76E-03
hsa-miR-193a-5p	MIMAT0004614	4,92	0,26
hsa-miR-214-3p	MIMAT0000271	0,21	1,04E-03
hsa-miR-216a-5p	MIMAT0000273	0,08	2,66E-05
hsa-miR-217	MIMAT0000274	0,30	0,01
hsa-miR-299-5p	MIMAT0002890	0,86	0,85
hsa-miR-100-5p	MIMAT0000098	0,25	4,68E-04
hsa-miR-10b-5p	MIMAT0000254	1,62	0,52
hsa-miR-1181	MIMAT0005826	4,36	0,84
hsa-miR-125a-5p	MIMAT0000443	4,58	0,26
hsa-miR-193a-3p	MIMAT0000459	3,57	0,59
hsa-miR-204-5p	MIMAT0000265	6,00	0,79
hsa-miR-224-5p	MIMAT0000281	1,21	0,80
hsa-miR-99b-5p	MIMAT0000689	1,04	0,96
hsa-miR-1246	MIMAT0005898	2,68	0,43
hsa-miR-155-5p	MIMAT0000646	10,57	0,29
hsa-miR-126-5p	MIMAT0000444	2,16	0,02
hsa-miR-410	MIMAT0002171	3,96	0,48
hsa-miR-409-3p	MIMAT0001639	3,64	0,34
hsa-miR-377-3p	MIMAT0000730	0,02	1,02E-09
hsa-miR-134	MIMAT0000447	1,41	0,76
hsa-miR-382-5p	MIMAT0000737	1,58	0,64
hsa-miR-381-3p	MIMAT0000736	10,11	0,64
hsa-miR-654-3p	MIMAT0004814	1,11	0,92
hsa-miR-376c-3p	MIMAT0000720	0,36	0,06
hsa-miR-495-3p	MIMAT0002817	1,81	0,42
hsa-miR-543	MIMAT0004954	2,39	0,28
hsa-miR-494	MIMAT0002816	0,13	2,75E-05
hsa-miR-379-5p	MIMAT0000733	3,37	0,08
hsa-miR-532-3p	MIMAT0004780	1,55	0,57
hsa-miR-502-3p	MIMAT0004775	3,91	0,46
hsa-miR-660-5p	MIMAT0003338	0,76	0,01
hsa-miR-362-3p	MIMAT0004683	0,18	1,16E-06
hsa-miR-497-5p	MIMAT0002820	0,58	0,50
hsa-miR-195-5p	MIMAT0000461	0,76	0,66
hsa-miR-365a-3p	MIMAT0000710	5,58	0,35
hsa-miR-24-3p	MIMAT0000080	7,62	0,39
hsa-miR-132-3p	MIMAT0000426	11,22	0,38
hsa-miR-93-5p	MIMAT0000093	6,39	0,40
hsa-miR-1908	MIMAT0007881	4,11	0,36

hsa-miR-1246	MIMAT0005898	2,68	0,37
hsa-miR-3168	MIMAT0015043	5,83	0,38
hsa-miR-1290	MIMAT0005880	3,37	0,22
hsa-miR-659-3p	MIMAT0003337	1,03	0,97
hsa-miR-1273a	MIMAT0005926	1,26	0,78
hsa-miR-3665	MIMAT0018087	1,31	0,77
hsa-miR-486-5p	MIMAT0002177	1,34	0,72
hsa-let-7d-5p	MIMAT0000065	1,57	0,58
hsa-miR-17-5p	MIMAT0000070	0,31	8,85E-07
hsa-miR-19a-3p	MIMAT0000073	0,09	1,14E-05
hsa-miR-20a-5p	MIMAT0000075	0,30	1,11E-06
hsa-miR-21-5p	MIMAT0000076	6,63	0,34
hsa-miR-27a-3p	MIMAT0000084	6,06	0,39
hsa-miR-92a-1-5p	MIMAT0004507	1,79	0,36
hsa-miR-130a-5p	MIMAT0004593	0,22	0,41
hsa-miR-143-3p	MIMAT0000435	0,33	1,59E-05
hsa-miR-146a-5p	MIMAT0000449	3,38	0,14
hsa-miR-155-5p	MIMAT0000646	5,77	0,19
hsa-miR-221-3p	MIMAT0000278	1,09	0,87
hsa-miR-423-3p	MIMAT0001340	12,63	0,81
hsa-miR-15a-5p	MIMAT0000068	1,66	0,67
hsa-miR-29b-3p	MIMAT0000100	0,32	0,01
hsa-miR-223-3p	MIMAT0000280	6,82	0,37
hsa-miR-150-5p	MIMAT0000451	2,00	0,57
hsa-miR-197-3p	MIMAT0000227	0,65	0,36
hsa-miR-20b-5p	MIMAT0001413	0,32	3,50E-03
hsa-miR-191-5p	MIMAT0000440	3,15	0,40
hsa-miR-24-3p	MIMAT0000080	3,85	0,43
hsa-miR-28-3p	MIMAT0004502	1,33	0,61
hsa-miR-486-5p	MIMAT0002177	2,81	0,42
hsa-miR-181b-5p	MIMAT0000257	5,98	0,33
hsa-miR-375	MIMAT0000728	1,44	0,69
hsa-miR-181a-5p	MIMAT0000256	0,38	4,78E-03
hsa-miR-25-3p	MIMAT0000081	2,09	0,23
hsa-miR-320a	MIMAT0000510	1,95	0,49
hsa-miR-29a-3p	MIMAT0000086	0,51	2,93E-03