Endothelial cells suppress monocyte activation through secretion of extracellular vesicles containing anti-inflammatory microRNAs

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Key Points:

- Quiescent endothelial cells secrete extracellular vesicles that can be taken up by monocytes to suppress their activation.
- MiR-10a is transferred to monocytic cells and inhibits the activation of the pro-inflammatory NF-κB pathway.

Abstract:

The blood contains high concentrations of circulating extracellular vesicles (EVs), and their levels and contents are altered in several disease states, including cardiovascular disease. However, the function of circulating EVs – especially the microRNAs that they contain – are poorly understood. We sought to determine the effect of secreted vesicles produced by quiescent endothelial cells (ECs) on monocyte inflammatory responses, and to assess whether transfer of microRNAs occurs between these cells. We observed that monocytic cells co-cultured (but not in contact) with ECs were refractory to inflammatory activation. Further characterization revealed that endothelium-derived EVs (EC-EVs) suppressed monocyte activation by enhancing immunomodulatory responses and diminishing pro-inflammatory responses. EVs isolated from mouse plasma also suppressed monocyte activation. Importantly, injection of EC-EVs in vivo repressed monocyte/macrophage activation, confirming our in vitro findings. We found that several anti-inflammatory microRNAs were elevated in EC-EV-treated monocytes. In particular, miR-10a was transferred to monocytic cells from EC-EVs and could repress inflammatory signaling through the targeting of several components of the NF-κB pathway, including IRAK4. Our findings reveal that ECs secrete EVs that can modulate monocyte activation, and suggest that altered EV secretion and/or microRNA content may affect vascular inflammation in the setting of cardiovascular disease.
**Introduction:**

Vascular endothelial cells (ECs) recruit circulating monocytes to regions of vascular injury and/or infection. Following their entry into the vessel wall, monocytes differentiate into macrophages, which drive an inflammatory response to neutralize invading pathogens, repair tissue damage or activate other immune cells. Monocyte and macrophage phenotypes are highly heterogeneous and can be dynamically modulated by the microenvironment. Classical activation promotes a pro-inflammatory (M1-like) response, that includes the secretion of pro-inflammatory cytokines and reactive oxygen and nitrogen species, and is driven by exposure to bacterial lipopolysaccharide (LPS) or Th1 cytokines such as Interferon-γ. Conversely, exposure to Th2 cytokines such as IL-4, IL-10 or IL-13 supports alternative activation, which is an immunomodulatory, pro-angiogenic and tissue reparative (M2-like) response that includes the secretion of IL-10 and transforming growth factor β (TGF-β). The balance of pro-inflammatory vs. immunomodulatory responses appears to play an important, but poorly understood role in cardiovascular pathologies. Both transcriptional pathways (e.g. IRF5, NF-κB, STATs) and post-transcriptional regulators (e.g. microRNAs) play key roles in modulating monocyte/macrophage phenotype.

Extracellular vesicles (EVs) of diverse size, composition and cellular origin are abundant in the circulation. These EVs include exosomes, microparticles (MPs) and apoptotic bodies (ABs). Secreted EVs can be taken up by target cells, and cell-surface and encapsulated EV proteins can modulate cellular signaling pathways in the recipient cell. In addition, microRNAs are packaged into EVs and can alter target gene expression in recipient cells. For example, ECs secrete EV-encapsulated microRNAs that can be taken up by smooth muscle cells in vitro. The abundance and type of EVs as well as their contents can vary during disease progression and this has provided an impetus to measure these parameters as circulating biomarkers. However, the functional consequences of these EV alterations are poorly understood.

MicroRNAs have been implicated as key regulators of inflammatory signaling pathways in ECs and monocytes/macrophages. Several microRNAs that are highly expressed in ECs are known to inhibit the pro-inflammatory NF-κB transcriptional pathway. For example, miR-146a targets adaptor proteins to limit NF-κB signaling and miR-10a represses the expression of proteins that destabilize IκB. Finally, miR-181b represses importin-α3 to inhibit NF-κB nuclear import. These microRNAs likely play a co-operative role in suppressing EC activation. In the current study we have assessed whether ECs can modulate myeloid inflammatory responses through secretion of EVs containing anti-inflammatory microRNAs. We find that EVs secreted from unstimulated (i.e. quiescent) ECs have potent anti-inflammatory properties in vitro and in vivo, and this appears to be due in part to the transfer of anti-inflammatory microRNAs, including miR-10a, to recipient monocytes/macrophages. Our studies suggest that circulating EVs and the microRNAs that they contain may have a significant impact on the responsiveness of monocytes/macrophages to inflammatory mediators, and that alterations to EVs may impact cardiovascular disease progression.
Materials and Methods:

Cell culture, co-culture experiments and treatments: Detailed methodology can be found in the Data Supplement.

Isolation and characterization of EVs: After 48 h culture of confluent monolayers of human umbilical vein ECs (HUVEC) or human coronary artery ECs (HCAEC), culture medium was collected and pre-cleared by centrifugation at 400 g for 5 min, then 2,000 g for 20 min to eliminate dead cells and cellular debris. The supernatant was then ultracentrifuged at 120,000 g for 120 minutes at 4°C, followed by an additional washing step of the EV pellet with PBS at 120,000 g for 120 min at 4°C (Optima L-100XP Ultracentrifuge, Beckman Coulter). The EV pellet was resuspended in PBS and stored at -80°C. Protein content of EVs was used to normalize for EV quantity between experiments using Pierce microplate BCA protein assay kit (Thermo Scientific). To isolate circulating EVs, mouse blood was collected via cardiac puncture and transferred to EDTA-containing tubes. Plasma was isolated from the blood by centrifugation at 1,500 g for 10 min at 4°C to remove blood cells, then the supernatant was centrifuged at 3,000 g for 15 minutes at 4°C to remove platelets and cell debris. EVs from 100 µL of plasma were isolated using ExoQuick Precipitation Solution (# EXOQ5A-1, System Biosciences), according to manufacturer’s recommendations, and resuspended in 50 µL of PBS. EVs were characterized by nanoparticle analysis (as in27; see Data Supplement for details).

Transfection of cells with siRNA, microRNA mimics, microRNA inhibitors or plasmids and electroporation of EC-EVs with miR-39 mimic: Detailed methodology can be found in the Data Supplement.

Cloning of luciferase constructs and luciferase assays: Detailed methodology can be found in the Data Supplement.

ELISA: Quantification of IL-12p40 was performed on 50-100 µL (of 1 mL total) of THP-1 cell or primary monocyte culture supernatants using a QuantiKine ELISA kit (DP400, R&D Systems), according to the manufacturer's recommendations.

Western blotting: Western blots were performed as described previously24 using antibodies directed to IRAK4 (Sigma-Aldrich, SAB3500304), IRF5 (Santa Cruz, sc-390364), CD63 (Santa Cruz, sc-5275) or GAPDH (Santa Cruz, sc-47724).

MicroRNA arrays: MicroRNA expression was measured in untreated or EC-EV-treated THP-1 cells (10 µg/mL of EC-EVs, 24 h) using QuantiMir technology (MicroRNA qPCR Array, #RA660A-1) from Systems Biosciences, according to the manufacturer's recommendations.

Real-time quantitative reverse transcriptase PCR (qRT-PCR): qRT-PCR analyses were performed as described before24,28. Detailed methodology and primers used for analyses can be found in the Data Supplement.

In vivo experiments: All animal protocols were approved by the Animal Care Committee at the University Health Network (Toronto) and the Institute for Cardiovascular Prevention (Munich). Peritonitis was induced in C57BL/6 mice (3-4 months of age) with 1 ml of 4% thioglycollate injected i.p.29 (Sigma-Aldrich). On day 3, EC-EVs (60 µg in 500 µL PBS) or PBS were injected into the peritoneum, and 24 h later mice were injected i.p. with LPS (5 mg/kg) for 2 h. Peritoneal leukocytes were harvested by lavage.
**Statistical Analyses:** Unless otherwise indicated, data represent the mean of at least three independent experiments and error bars represent the standard error of the mean. Pair-wise comparisons were made using a Student's t-test. Comparison of three or more groups was performed using a 1-way analysis of variance (ANOVA) with Newman-Keuls post-hoc test. A p-value of less than 0.05 was considered to be statistically significant. In all figures *, ** and *** represent a p-value of <0.05, <0.01 and <0.001, respectively.
Results:

**ECs suppress monocyctic pro-inflammatory responses through a process that does not require direct cell-to-cell contact:** To assess cellular communication between ECs and monocytes we established a co-culture system where human umbilical vein ECs (HUVECs) were cultured together with the human monocytic cell line, THP-1 or primary human monocytes, separated by a 1 μm transwell filter (Fig. 1A). Following mono-culture or co-culture, THP-1 cells or primary monocytes were removed from transwell inserts and activated by lipopolysaccharide (LPS) stimulation. Co-culture suppressed monocyte activation, as assessed by expression of IL-12p40, IL-23p19, IL-1β and TNF-α (Fig. 1B,C). We considered whether ECs secrete EVs that are taken up by monocytes. Confocal analysis revealed that THP-1 cells co-cultured with membrane-labeled ECs took up the label, demonstrating transfer of membranous vesicles from ECs to monocyctic cells (Fig. 1D).

**Endothelium-derived extracellular vesicles (EC-EVs) suppress monocyte activation and polarize monocyte responses to LPS:** To test whether EC-EVs are involved in suppressing monocyte activation we isolated EVs from EC culture medium using ultracentrifugation. Nanoparticle analysis demonstrated that the isolated EVs were ~50-300 nm in size (Mode = 142 ± 2.6 nm, Fig. 2A), consistent with the size of exosomes or small MPs. Western blot analysis revealed that EC-EVs expressed high levels of CD63, an exosomal marker (Fig. 2B). To determine the effect of EC-EVs on monocyctic cell activation, we added purified EC-EVs to THP-1 monocytic cells for 18 h, followed by stimulation with LPS. EC-EVs dose-dependently suppressed the activation of pro-inflammatory genes (e.g. IL-12p40, IL-23p19, TNF-α, IL-1β), while simultaneously enhancing markers of alternative activation or immunomodulatory responses (e.g. IL-10, MRC1 and TGF-β) (Fig. 2C, S1A, S2A), suggesting that EC-EVs can polarize monocyte responses. Addition of EC-EVs to primary monocytes also suppressed a subset of pro-inflammatory genes, while enhancing expression of MRC-1 and TGF-β (Fig. 2D, S2B). The suppression of IL-12p40 secretion from THP-1 cells and primary monocytes was confirmed by ELISA (Fig. 2E). Although addition of EC-EVs polarized the basal expression of pro-inflammatory/immunomodulatory markers in unactivated THP-1 cells (Fig. S1B), this was less pronounced in primary monocytes (Fig. S1C). EC-EVs isolated from human coronary artery ECs also modulated monocytic cell activation and polarization in response to LPS stimulation, suggesting that this effect is not limited to HUVEC-derived EVs (Fig. 2F).

We next sought to determine whether circulating EVs have anti-inflammatory properties in vivo. To this end we isolated EVs from the plasma of young, healthy mice (Fig. 3A). Plasma EVs have both endothelial and non-endothelial cell origins (e.g. red blood cells, leukocytes and platelets). However, due to the lack of cell-of-origin markers on exosomes, the relative contribution of these diverse cell types to plasma EV pools can not currently be determined. Quantification of EVs in plasma by nanoparticle analysis revealed that there were ~1.8 x 10^{11} ± 0.33 x 10^{11} particles/mL. Importantly, our in vitro experiments utilizing EVs isolated from ECs used approximately ~2.1 x 10^{10} ± 0.29 x 10^{10} particles/mL, suggesting that this is not a supraphysiological dose of EVs. Similar to EC-EVs, addition of plasma EVs to THP-1 cells ex vivo suppressed induction of pro-inflammatory genes while simultaneously activating IL-10 expression in response to LPS (Fig. 3B), but had only a modest effect on these genes in unactivated cells (Fig. S3A).

To determine if HUVEC-derived EVs can suppress inflammation in vivo, we utilized a mouse peritonitis model, where thioglycollate was injected into the peritoneum to induce monocyte recruitment and macrophage differentiation. We then injected PBS or EC-EVs into the peritoneum and assessed the inflammatory response of the recruited leukocytes to LPS challenge (injected i.p.) the following day (Fig. 3C). EVs have previously been used in cross-
species experiments with no obvious immunogenic complications. Importantly, neutrophil and macrophage markers were unchanged in response to EC-EV injection into the peritoneum. In LPS stimulated mice, EC-EVs suppressed the induction of pro-inflammatory genes, such as TNF-α, iNOS and IL-1β, while significantly enhancing the expression of Arg1, a murine marker of immunomodulatory responses. The expression of pro-inflammatory/immunomodulatory markers in non-LPS stimulated mice were largely unaffected and IL-12p40, IL-23p19 and IL-10 were expressed at extremely low levels in unactivated and LPS-injected mice (data not shown), and no changes were observed in their expression.

**EC-EVs inhibit NF-κB signaling and IRF5 expression:** Many pro-inflammatory genes require NF-κB signaling for their induction. We therefore assessed the effect of EC-EVs on activity of an NF-κB-dependent luciferase reporter. Addition of EC-EVs to THP-1 cells suppressed the basal and LPS-stimulated activity of the reporter. We next assessed whether inhibition of NF-κB signaling could recapitulate the effects of EC-EVs. Indeed, the induction of pro-inflammatory genes in LPS-stimulated THP-1 monocytic cells was suppressed by the IKK inhibitor, TPCA-1. However, LPS induction of immunomodulatory genes, such as TGF-β and IL-10 was not enhanced in monocytic cells treated with the NF-κB inhibitor (data not shown). IRF5 is a marker of monocyte/macrophage polarization that has previously been implicated in the induction of pro-inflammatory genes, while simultaneously suppressing immunomodulatory genes. Consistent with the polarization effects of EC-EVs, we found that IRF5 expression was significantly inhibited by EC-EV treatment of THP-1 cells or primary monocytes. Additionally, the expression of IFNγ was reduced in peritoneal leukocytes exposed to EC-EVs in vivo (Fig. 4C). EC-EVs can therefore suppress transcriptional pathways (i.e. NF-κB and IRF5) that are known to be indicative of polarized monocyte/macrophage activation.

**EC-EVs transfer microRNAs to recipient monocytic cells:** Since EVs can transfer their microRNA contents to recipient cells in other cellular contexts, we tested whether microRNAs could be transferred from ECs to monocytic cells in the absence of cell-cell contact. Indeed, transfection of the C. elegans-specific microRNA, miR-39, into ECs resulted in a time-dependent accumulation of miR-39 in co-cultured THP-1 cells. To establish that microRNAs can be transferred from EC-EVs to peritoneal leukocytes in vivo we electroporated miR-39 into EC-EVs and injected these miR-39-loaded EVs into the peritoneum three days following thioglycollate challenge. Isolation of peritoneal leukocytes, spleen, bone marrow and peripheral blood 24 h after EV injection revealed that EVs (as assessed by miR-39 expression) were primarily taken up by peritoneal cells (Fig. S3D). To identify the repertoire of microRNAs that might be transferred from ECs to monocytic cells we performed qPCR microRNA arrays on THP-1 cells treated with EC-EVs and compared this to untreated cells (data not shown). qRT-PCR was utilized to validate several of the microRNAs identified as differentially expressed. As anticipated, the EC-enriched microRNA, miR-126-3p, which has been implicated in the suppression of inflammation, was elevated in EC-EV-treated monocytic cells. Interestingly, several other microRNAs with a known role in the repression of inflammatory signaling (i.e. miR-10a, miR-146a/b, miR-147a, miR-181b, miR-24, miR-26, miR-37) were also increased in the recipient THP-1 cells: most notably miR-10a. The majority of these microRNAs were also elevated in EC-EV-treated primary monocytes and EVs isolated from coronary artery ECs (CAECs).
(Fig. S4E), and these microRNAs were also present in EVs isolated from mouse plasma (Fig. S4F).

To determine whether miR-10a was directly transferred to monocytic cells we utilized several independent approaches. Addition of increasing concentrations of EC-EVs resulted in a dose-dependent increase of miR-10a in THP-1 cells (Fig. 5C). Importantly, miR-10a levels rapidly increased in monocytic cells treated with EC-EVs, but the primary miR-10a transcript was not induced, suggesting a lack of transcriptional induction (Fig. 5D). Co-culture of THP-1 monocytic cells with ECs resulted in increased levels of miR-10a and miR-126-3p in monocytic cells (Fig. 5E), suggesting a transfer from ECs, where these microRNAs are highly expressed. In addition, transfection of miR-10a mimic into ECs resulted in an increase in miR-10a in co-cultured monocytic cells (Fig. 5F). Furthermore, miR-10a levels were elevated in peritoneal cells isolated from mice injected i.p. with EC-EVs compared to PBS injection (Fig. 5G). To establish that transcription is indeed not required for miR-10a induction we pre-treated THP-1 cells with the transcriptional inhibitor Actinomycin D. EC-EV-mediated induction of miR-10a was not affected by Actinomycin D treatment (Fig. 5H). However, expression of the short-lived transcript, TNF-α was reduced in Actinomycin D treated cells, demonstrating the effectiveness of the dose used (Fig. 5H). Taken together, these data provide evidence that miR-10a is directly transferred from EC-EVs to monocytic cells.

**MiR-10a, miR-126 and miR-181b can suppress pro-inflammatory monocytic cell activation:** Since several microRNAs were elevated in EC-EV-treated monocytic cells (Fig. 5B), we sought to determine whether over-expression of any one of these microRNAs could recapitulate the EC-EV effects on the polarization of monocyte activation. We individually over-expressed miR-10a, miR-126, miR-146a, miR-146b, miR-181b or miR-340 in THP-1 monocytic cells (Fig. 6A and Fig. S5A) or in ECs co-cultured with THP-1 cells (Fig. 6B and Fig. S5B). Interestingly, over-expression of miR-10a, miR-126 or miR-181b could suppress the induction of pro-inflammatory genes in THP-1 cells stimulated with LPS, while miR-146a/b and miR-340 did not have significant effects. Modest elevation of immunomodulatory genes (which did not consistently reach statistical significance) was observed with miR-10a transfection. Over-expression of miR-10a in primary bone marrow-derived macrophages (BMDMs) also suppressed pro-inflammatory gene expression in both unstimulated and LPS/IFN-γ-stimulated cells (Fig. 6C). To further assess the role of endogenous and EC-EV-derived miR-10a in monocyte activation, we inhibited miR-10a in THP-1 cells prior to treating them with EC-EVs. While EC-EVs were able to suppress the induction of pro-inflammatory genes in control inhibitor-treated monocytic cells, the effect of EC-EVs on these genes was attenuated in miR-10a inhibited monocytic cells (Fig. 6D). In addition, the induction of the immunomodulatory marker, MRC1, was abrogated in miR-10a inhibited monocytic cells, although the induction of IL-10 and TGF-β1 was unaffected (Fig. 6D). Taken together, these results suggest that miR-10a contributes to the suppression of pro-inflammatory genes and may modestly affect immunomodulatory responses.

**MiR-10a represses NF-κB signaling:** MiR-10a was previously shown to suppress NF-κB signaling in ECs through the repression of β-TRC and MAP3K7/TAK1: effector molecules required for IκB degradation, and hence NF-κB activity. Importantly, monocytic cells treated with EC-EVs have reduced NF-κB activity (Fig. 4A). We identified IRAK4, a known regulator of NF-κB signaling, and latent TGF-β binding protein (LTBP1), a suppressor of TGF-β signaling, as novel potential miR-10a target genes (Fig. S6). We first assessed whether IRAK4 or LTBP1 were targets of miR-10a by cloning a portion of the 3’ UTRs of these genes downstream of a luciferase open reading frame (Fig. 7A). As previously demonstrated, luciferase constructs containing the BTRC 3’ UTR were repressed by miR-10a over-expression. While LTBP1 was not regulated by miR-10a, the 3’ UTRs of both mouse and human IRAK4 were regulated by miR-10a (Fig. 7A).
We next assessed whether EC-EVs were able to suppress miR-10a targets in recipient monocytic cells. BTRC, MAP3K7 and IRAK4 mRNA levels and IRAK4 protein levels were decreased in EC-EV-treated THP-1 cells. However, targets of miR-146a/b (i.e. TRAF6) and miR-181b (i.e. Importin-A3) were unaffected, at least at the mRNA level (Fig. 7B,C). In addition, over-expression of miR-10a in THP-1 monocytic cells repressed BTRC, MAP3K7 and IRAK4 mRNA and IRAK4 protein expression (Fig. 7D,E). Since IRAK4, β-TRC and MAP3K7 act upstream of NF-κB activation, we assessed NF-κB reporter activity in monocytic cells transfected with miR-10a mimic. As anticipated, miR-10a over-expression suppressed NF-κB activity (Fig. 7F). Taken together, these findings delineate a pathway whereby transfer of miR-10a from EC-EVs to monocytes can repress a network of genes involved in NF-κB signaling to suppress the activation of pro-inflammatory genes (Fig. 7G).
Discussion:

Here we demonstrate that quiescent ECs secrete extracellular vesicles (EC-EVs) that have potent anti-inflammatory activities. Primary monocytes and THP-1 monocytic cells treated with EC-EVs in vitro and peritoneal leukocytes exposed to EC-EVs in vivo have reduced pro-inflammatory responses and enhanced immunomodulatory responses, and this is accompanied by suppressed NF-κB signaling and reduced levels of IRF5. The anti-inflammatory effects of EC-EVs appear to be attributable in part to the transfer of miR-10a from EC-EVs to monocytes/macrophages. Exogenous miR-10a suppresses NF-κB activity by targeting several components of the NF-κB pathway, including IRAK4, β-TRC and MAP3K7. In addition, we find that miR-126 and miR-181b are also increased in EC-EV-treated monocytic cells, and over-expression of these microRNAs can suppress monocyte activation. These anti-inflammatory microRNAs may act together to suppress monocyte activation. Importantly, these microRNAs are conserved between mouse and human, which may explain why human EC-EVs can potently inhibit the activation of mouse leukocytes. It is likely that proteins or lipids contained in EC-EVs also contribute to their anti-inflammatory and immunomodulatory effects.

Monocytes are continually exposed to EVs in the circulation, but little is known regarding the functional consequences of EV uptake. Prior studies have revealed that endothelium-derived MPs (EMPs), which are present at elevated levels in chronic inflammatory states, are preferentially taken up by monocytes compared to neutrophils and lymphocytes, and that exposure to EMPs leads to monocyte activation. In contrast, our studies suggest that healthy, quiescent endothelium secretes EVs (exosomes and small MPs) that reduce the magnitude of pro-inflammatory responses and shifts monocyte activation towards an immunomodulatory response. We have additionally found that these EC-EVs can suppress EC activation (our unpublished results), suggesting that their protective effects may extend beyond the regulation of monocyte activation. Endothelial dysfunction and activation occur in various chronic cardiovascular disease states, and this likely contributes to alterations in the levels and types of EVs (i.e. exosomes vs. MPs vs. apoptotic bodies) in circulation, as well as modulating their contents; ultimately influencing their function. For example, MP secretion is enhanced from ECs and leukocytes exposed to inflammatory mediators, and these vesicles have been shown to promote monocyte activation, thrombosis and the dysfunction/activation of ECs during atherogenesis. Interestingly, we have found that EVs isolated from acutely activated ECs lose some of their anti-inflammatory properties (Fig. S7), suggesting that altered EV function may be an early event in vascular inflammatory disease.

Our findings provide a new paradigm for the role of ECs in orchestrating vascular inflammation in cardiovascular disease. We propose that the anti-inflammatory activities of EVs that we have observed in healthy mice may be lost during disease pathogenesis, and we anticipate that this can influence the activation state of circulating monocytes. This may in turn influence the type of macrophage response (i.e. pro-inflammatory or immunomodulatory) that occurs in the setting of vascular inflammation. Interestingly, recent studies have revealed that MPs isolated from patients with coronary artery disease contain less microRNA and have defects in their ability to transfer these microRNAs to recipient cells. Our findings show that EVs that are constitutively secreted by ECs harbor protective microRNAs that can be transferred to monocytes. We speculate that MPs present in chronic inflammatory conditions may lack this repertoire of microRNAs or they may contain inflammatory mediators (i.e. protein or lipid) that over-ride this anti-inflammatory effect.

MiR-10a appears to play a critical role in the inflammatory response. For example, circulating levels of miR-10a are reduced in patients suffering from acute pancreatitis, and exposure to microbiota can down-regulate miR-10a in dendritic cells in an NF-κB-dependent manner. Importantly, miR-10a levels are also decreased in atherosclerotic plaque. In contrast
to the down-regulation of miR-10a by inflammatory stimuli, elegant studies have shown that laminar shear stress enhances EC miR-10a expression, while disturbed flow (which is accompanied by NF-κB activation) represses miR-10a expression\textsuperscript{25}. Fang et al demonstrate that miR-10a inhibits NF-κB signaling and EC activation, and speculate that atherosclerosis may develop preferentially in regions of disturbed flow because of a reduction in miR-10a expression\textsuperscript{29}. In addition to targeting several components of the NF-κB pathway, miR-10a can also directly target the pro-inflammatory cytokine IL-12p40 in mouse dendritic cells\textsuperscript{44}, and we provide evidence that IL-12p40 is also suppressed by miR-10a over-expression in monocyctic cells. Our findings suggest that miR-10a secreted from ECs inhibits monocyte activation, and therefore loss of miR-10a in ECs during atherogenesis\textsuperscript{45} may in turn influence monocyte activation. Taken together, our studies reveal a novel cross-talk between the endothelium and monocytes/macrophages that is mediated via secreted EVs that contain anti-inflammatory microRNAs.

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**Authorship Contributions:** M-S.N. designed and performed experiments, analyzed data and wrote the manuscript. H.C., L.T.D., M.N-J, A.L., M.R. and E.B. designed and performed experiments and analyzed data. A.S. supervised M.N-J and designed experiments. M.I.C. supervised A.L. and M.R. and designed experiments. J.E.F. supervised M-S.N., H.C., L.T.D. and E.B., designed experiments, analyzed data and wrote the manuscript.

**Disclosure of Conflicts of Interest:** The authors have no disclosures.
References:


Figure Legend:

Figure 1: Co-culture with endothelial cells (ECs) suppresses monocyte activation in a contact-independent manner
(A) Schematic of co-culture of monocytes (THP-1 or primary monocytes) with ECs (human umbilical vein ECs, HUVEC) using a transwell apparatus (1 μm pore size).
(B) Co-culture of THP-1 monocytic cells with ECs for 48 h results in a suppressed responsiveness to a 2 h LPS stimulation, as assessed by qRT-PCR analysis of pro-inflammatory genes (normalized to GAPDH). Data is relative to gene expression in THP-1 cells grown in EC medium in mono-culture. Shown is the mean ± SEM of 6 independent experiments.
(C) Co-culture of primary monocytes with ECs for 24 h results in suppressed LPS responsiveness, as assessed by qRT-PCR analysis of pro-inflammatory genes (normalized to GAPDH). Shown is the mean ± SEM of 8 independent experiments.
(D) THP-1 monocytic cells co-cultured with membrane-labeled ECs for 24 h take up the label. Shown is a representative confocal image. The cell nucleus is stained with DAPI. Scale bar = 10 μm.

Figure 2: ECs secrete extracellular vesicles (EC-EVs) that inhibit monocyte activation and modulate polarization
(A) Nanoparticle analysis of EVs isolated from HUVEC media by ultracentrifugation. A representative experiment of 3 is shown. The mode particle size is 142 ± 2.6 nm.
(B) Western blotting of the exosomal marker, CD63, in lysates from ECs and isolated EC-EVs (two independent preparations).
(C) EC-EVs (10 μg/mL) isolated from HUVEC suppress the activation of THP-1 monocytic cells by LPS (as assessed by qRT-PCR of pro-inflammatory genes), and promote their polarization (as assessed by qRT-PCR of immunomodulatory markers). n=5.
(D) HUVEC-derived EC-EVs (10 μg/mL) polarize the response of primary monocytes to LPS treatment. ND; not detected. n=5.
(E) Protein levels of IL-12p40 in the medium of EC-EV-treated THP-1 cells and primary monocytes, as measured by ELISA. Cells were either unstimulated or treated with LPS for 8 h. n=5.
(F) EC-EVs isolated from human coronary artery ECs (CAECs) polarize monocyte activation in response to LPS treatment. n=3.

Figure 3: EVs isolated from mouse plasma suppress monocyctic cell activation and EC-EVs suppress peritoneal leukocyte activation in vivo
(A) Nanoparticle analysis of EVs isolated from mouse plasma by ExoQuick isolation. A representative experiment of 4 is shown. The mode particle size is 112 ± 5.5 nm.
(B) EVs isolated from mouse plasma suppress THP-1 monocytic cell activation in response to LPS treatment. n=9.
(C) Peritonitis was established by i.p. injection of thioglycollate for 3 days. PBS or HUVEC-derived EC-EVs (60 μg) were injected into the peritoneum and the response to LPS treatment (i.p., 2 h) was assessed in peritoneal leukocytes the following day by qRT-PCR analysis.
(D) The levels of pro-inflammatory genes (Tnf-α, Il-1β, iNOS) are suppressed by EC-EV injection, while immunomodulatory genes are elevated. n=7.

Figure 4: EC-EVs suppress the induction of NF-κB and repress the expression of IRF5
(A) EC-EV-treated THP-1 monocytic cells have reduced basal and LPS-stimulated NF-κB reporter activity. n=4.
(B) THP-1 cells pretreated with the NF-κB inhibitor, TPCA-1 (10 μM), have reduced induction of pro-inflammatory genes in response to LPS stimulation. n=4.
(C) EC-EV-treated THP-1 cells or primary monocytes have reduced levels of IRF5 mRNA as assessed by qRT-PCR. n=5. Peritoneal leukocytes isolated from EC-EV-injected mice have reduced levels of Irf5 mRNA compared to PBS-injected controls. n=7.
(D) Western blot of IRF5 in EC-EV-treated THP-1 cells. Densitometry is indicated above. Shown is a representative result of 4 independent experiments.

Figure 5: MiR-10a is transferred from EC-EVs to monocytic cells
(A) Over-expression of the C. elegans-specific microRNA, miR-39, in ECs results in a time-dependent accumulation of this microRNA in co-cultured THP-1 cells (in the absence of cell-cell contact). MiR-39 expression level after 1 h co-culture was set to 1 since miR-39 was not detectable (ND) by qRT-PCR in THP-1 monoculture. Data is normalized to U6 expression. n=3.
(B) qRT-PCR was used to assess the expression of several microRNAs, including the EC-enriched microRNA, miR-126-3p, and several anti-inflammatory microRNAs (miR-10a, miR-146a/b, miR-147a, miR-181b) in THP-1 monocytic cells treated with EC-EVs for 24 h. Data is normalized to U6 expression. n=5.
(C) Addition of increasing concentrations of EC-EVs results in a dose-dependent increase in miR-10a in treated THP-1 cells. n=2.
(D) Kinetics of mature miR-10a and pri-miR-10a expression after treatment of THP-1 cells with EC-EVs (10 μg/mL). n=2.
(E) Levels of miR-10a and miR-126-3p are increased in THP-1 cells co-cultured with ECs for 24 h. n=4.
(F) Over-expression of miR-10a in ECs results in an increase in miR-10a expression in co-cultured THP-1 cells. n=4.
(G) miR-10a expression is enhanced in peritoneal leukocytes isolated from LPS-stimulated mice injected with EC-EVs compared to PBS injection control. n=5.
(H) EC-EV-dependent induction of miR-10a is resistant to Actinomycin D pre-treatment of THP-1 cells, suggesting a transfer from EVs. Expression of TNF-α, a short-lived transcript, is reduced in Actinomycin D-treated cells. n=3.

Figure 6: miR-10a suppresses monocyte/macrophage activation
(A) MiR-10a over-expression in THP-1 monocytic cells suppresses the induction of pro-inflammatory genes in response to LPS stimulation, as assessed by qRT-PCR. n=6.
(B) MiR-10a over-expression in ECs suppresses the induction of pro-inflammatory genes in co-cultured THP-1 cells stimulated with LPS. Values are expressed relative to co-cultured cells transfected with control mimic, and the suppressive effective of miR-10a over-expression is therefore in addition to the effect of EC co-culture on monocytic cell activation. n=6.
(C) Over-expression of miR-10a in mouse bone marrow-derived macrophages (BMDM) suppresses the expression of pro-inflammatory genes. Shown are unstimulated BMDMs (left) or BMDMs treated with LPS/IFN-γ (right). n=6 and n=5, respectively.
(D) Inhibiting miR-10a in THP-1 monocytic cells negates the EC-EV-dependent down-regulation of pro-inflammatory genes and affects the expression of the immunomodulatory marker MRC1 in LPS stimulated cells. Data is expressed relative to cells transfected with control inhibitor with no EC-EVs. n=6.

Figure 7: MiR-10a negatively regulates NF-κB signaling
(A) Luciferase assays in bovine aortic endothelial cells. BTRC, a known miR-10a target, is included as a positive control. Over-expression of miR-10a suppresses the activity of mouse and
human IRAK4 3'UTR-containing luciferase constructs, but LTBP1 (another miR-10a predicted target gene) is not suppressed. n=3.

(B) EC-EV treatment suppresses BTRC and MAP3K7 (known miR-10a target genes), as well as IRAK4 mRNA expression in LPS-stimulated (2 h) THP-1 cells, while effects on known miR-146a (TRAF6) or miR-181b (Importin-A3) targets are not observed. n=4.

(C) Western blotting confirms that IRAK4 protein is suppressed by EC-EV treatment. Densitometry is indicated above. A representative experiment of 4 is shown.

(D) MiR-10a over-expression (OE) in THP-1 monocytic cells represses BTRC, MAP3K7 and IRAK4 mRNA levels. n=4.

(E) MiR-10a over-expression in THP-1 cells represses IRAK4 protein as assessed by western blot. Densitometry is indicated above. A representative experiment of 3 is shown.

(F) NF-κB activity is repressed in THP-1 cells over-expressing miR-10a. Shown is a representative experiment of 4 with triplicate determinations.

(G) Model of how ECs suppress monocytic inflammatory responses and promote an immunomodulatory phenotype through the secretion of EC-EVs that contain anti-inflammatory microRNAs, including miR-10a. Suppression of NF-κB signaling is mediated in part by the targeting of IRAK4, TAK1/MAP3K7 and β-TRC by miR-10a, while IRF5 is down-regulated by EC-EVs in a miR-10a-independent manner (data not shown). MiR-126 and miR-181b (which are present in EC-EVs) can suppress pro-inflammatory responses when over-expressed in monocytic cells.
Figure 1

**A**

Monocytes

Endothelial Cells

**B**

**THP-1 monocyctic cells (48 h co-culture)**

<table>
<thead>
<tr>
<th></th>
<th>IL-12p40</th>
<th>IL-23p19</th>
<th>TNF-α</th>
<th>IL-1β</th>
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<tbody>
<tr>
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<td><img src="image3" alt="Graph" /></td>
<td><img src="image4" alt="Graph" /></td>
</tr>
</tbody>
</table>

**C**

**Primary human monocytes (24 h co-culture)**

<table>
<thead>
<tr>
<th></th>
<th>IL-12p40</th>
<th>IL-23p19</th>
<th>TNF-α</th>
<th>IL-1β</th>
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<tr>
<td>Relative Expression</td>
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<td><img src="image7" alt="Graph" /></td>
<td><img src="image8" alt="Graph" /></td>
</tr>
</tbody>
</table>
A  Endothelial extracellular vesicles (EC-EVs)

![Size distribution graph](imageA)

B  EC  EC-EVs

![Western blot images](imageB)

C  THP-1 treated with EC-EVs

![Relative expression graph](imageC)

D  Monocytes treated with EC-EVs

![Relative expression graph](imageD)

E  THP-1 cells  Monocytes

![Protein concentration graphs](imageE)

F  THP-1 treated with EC-EVs (CAEC)

![Relative expression graph](imageF)
Mouse plasma extracellular vesicles (plasma EVs)

THP-1 treated with plasma EVs

Relative Expression (Plasma EVs vs Control)

EC-EVs injected into the peritoneum

Relative Expression (EC-EVs vs Control)

Figure 3
Figure 5
Figure 6
Figure 7

**A** Relative Luciferase Activity (miR-10a vs Control).

**B** Relative Expression (EC-EVs vs Control).

**C** Western Blot for IRAK4 and GAPDH.

**D** Relative Expression (miR-10a mimic vs Control).

**E** Western Blot for IRAK4 and GAPDH.

**F** Relative NF-κB Activity.

**G** Schematic representation of the interaction between miR-10a-enriched extracellular vesicles and immune cells, showing the regulation of immunomodulatory gene expression.

**Immunomodulatory Phenotype**

- **Pro-Inflammatory Gene Expression**
- **Immunomodulatory Gene Expression**
Endothelial cells suppress monocyte activation through secretion of extracellular vesicles containing anti-inflammatory microRNAs

Makon-Sébastien Njock, Henry S. Cheng, Lan T. Dang, Maliheh Nazari-Jahantigh, Andrew C. Lau, Emilie Boudreau, Mark Roufaiel, Myron I. Cybulsky, Andreas Schober and Jason E. Fish

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