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Mitochondria are a subset of extracellular vesicles released by stressed monocytes and induce inflammatory responses in endothelial cells

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Submitted by

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I dedicate this thesis to my late grandfather Erich Brunner, who lived up to the engineers ideal:

„*Dem Ingenieur ist nichts zu schwere / Er lacht und spricht: Wenn dieses nicht, so geht doch das*!“
“*To an engineer nothing is too difficult / He laughs and says: If this does not work, that will!*”

- Ingenieurlied (Heinrich Seidel, 1871)

Declaration

This thesis includes a shared first author publication. I performed and designed all experiments and data analysis included in the manuscript (unless otherwise specified below) at the Medical University of Vienna (Department of Laboratory Medicine) and CeMM (Research Center for Molecular Medicine). I was supervised by Univ. Prof. DDr. Christoph J Binder. Dr. Taras Afonyushkin, who is shared first author on the publication, is currently a Post-doctoral Fellow at the Medical University of Vienna (Department of Laboratory Medicine) and did not or will not use any content of this manuscript for the purpose of a dissertation.

I designed all figures in this thesis (Thesis Schemes 1 to 5). The thesis schemes 1 to 4 do not show new data. Their intention is to illustrate current concepts in extracellular vesicle research (Thesis Scheme 1 and 2), monocyte to endothelial cell communication (Thesis Scheme 3) and mitochondria as a source of damage-associated molecular patterns (DAMPs) (Thesis Scheme 4). Thesis Scheme 5 is a graphical abstract of the thesis/publication arising from the thesis.

The results section consists of the manuscript, published in Circulation Research (DOI: 10.1161/CIRCRESAHA.118.314601), that I wrote on this thesis with the title „Mitochondria are a subset of extracellular vesicles released by activated monocytes and induce Type I IFN and TNF responses in endothelial cells“.

The material and methods sections consists of the online material / supplemental material, published in Circulation Research (DOI: 10.1161/CIRCRESAHA.118.314601), that I wrote for the manuscript with the title „Mitochondria are a subset of extracellular vesicles released by activated monocytes and induce Type I IFN and TNF responses in endothelial cells“.

Individual and shared contributions as follows (this declaration is also included in the publication):
Florian Puhm, Taras Afonyushkin, Ulrike Resch, Manfred Rohde, Thomas Penz, Gabriel Wagner, and Imene Melki conducted experiments and performed data analysis. Florian Puhm, Taras Afonyushkin, and Christoph J Binder designed experiments and wrote the article. Florian Puhm and Taras Afonyushkin handled all in vitro experiments. Ulrike Resch conducted Western Blots and immunocytochemistry. Manfred Rohde conducted electron microscopy. Thomas Penz, Michael Schuster, and Andre F Rendeiro conducted and analyzed RNA sequencing. Gabriel Wagner characterized respiratory activity of cells. Georg Obermayer,

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Abstract

Microvesicles, a major subtype of extracellular vesicles, have recently gained attention as important mediators of cell-to-cell communication in cardiovascular disease. Their biological activity, including inflammatory properties, is considered to be defined by their composition. Furthermore, communication between monocytes and endothelial cells is an essential component to propagate inflammatory responses in the circulation. However, little is known about the role of microvesicles in this context. In this thesis, I studied the content of microvesicles released from stressed monocytic cells and their biological activity towards endothelial cells, as well as what defines this activity.

I could show that LPS (Lipopolysaccharide)-activated monocytic cells release microvesicle-encapsulated and free mitochondria. Notably, mitochondria released in free form or embedded in microvesicles, as well as mitochondria directly isolated from LPS-activated cells and circulating microvesicles isolated from donors receiving low-dose LPS-injections, induced TNF (tumor necrosis factor) and type I IFN (interferon) responses in endothelial cells. RNA from stressed mitochondria and TNFα-associated with mitochondria were identified as the principal components mediating this effect. Finally, the proinflammatory potential of isolated mitochondria and released microvesicles was dramatically reduced when they were released from nonrespiring cells or when these cells had been cultured in presence of a mitochondrial ROS (reactive oxygen species) scavenger or pyruvate.

In conclusion, free mitochondria and microvesicle-encapsulated mitochondria are a biologically active subset of extracellular vesicles released by stressed monocytic cells. Moreover, their potential to induce proinflammatory responses in endothelial cells was determined by the activation status of their parental cells. Therefore, mitochondria released by stressed cells may represent important intercellular mediators in inflammatory diseases associated with TNF and type I IFN signaling.

Zusammenfassung

Mikrovesikel sind eine wichtige Untergruppe extrazellulärer Vesikel, welche vermehrt als wichtige Bestandteile interzellulärer Kommunikation in Herz- und Kreislauferkrankungen wahrgenommen werden. Es wird angenommen, dass ihre biologische Aktivität, einschließlich ihrer Fähigkeit Entzündungen auszulösen, von ihrer Zusammensetzung bestimmt wird. Im Kreislaufsystem ist die Kommunikation zwischen Monozyten und Endothelzellen ein essentieller Bestandteil der Weiterverbreitung von Entzündungssignalen. Welche Rolle Mikrovesikel in diesem Kontext spielen ist jedoch kaum bekannt. In dieser Dissertation habe ich die Beschaffenheit von Mikrovesikel, die von gestressten monozytischen Zellen freigesetzt wurden, untersucht. Weiters, habe ich analysiert welche zellulärer Antwort diese Mikrovesikel in Endothelzellen auslösen und wodurch ihre biologische Aktivität bestimmt wird.

Ich konnte zeigen, dass LPS (Lipopolysaccharid)-aktivierte monozytische Zellen Mitochondrien in freier Form und von Mikrovesikeln umhüllt sezernieren. Diese freigesetzten Mitochondrien und direkt aus aktivierten Monozyten isolierte Mitochondrien, so wie Mikrovesikel, isoliert aus dem Plasma von Individuen, welche niedrig dosierte LPS-Injektionen erhalten hatten, induzierten TNF (Tumornekrosefaktor) und Typ I IFN (Interferon) Signalwege in Endothelzellen. Weiters beobachtete ich, dass zwei Komponenten für diese biologische Aktivität der Mitochondrien und Mikrovesikel verantwortlich waren: RNA gestresster Mitochondrien und mit Mitochondrien assoziierter TNFα, welche jeweils für die induktion der Typ I IFN und TNF Antwort verantwortlich waren. Schließlich konnte ich zeigen, dass das Entzündungspotential isolierter Mitochondrien und freigesetzter Mikrovesikel drastisch verringert war, wenn die Zellatmung in Mutterzellen gehemmt war, oder wenn diese Zellen in Gegenwart eines für Mitochondrien spezifischen Antioxidans oder Pyruvat mit LPS aktiviert wurden.

Diese Beobachtungen ließen folgende Schlüsse zu: Erstens, von gestressten Monozyten freigesetzte Mitochondrien (frei bzw. eingebettet in Mikrovesikeln) stellen eine biologisch aktive Untergruppe extrazellulärer Vesikel dar. Zweitens, ihr Potenzial Entzündungsreaktionen in Endothelzellen auszulösen, wird vom Aktivierungszustand der Monozyten, welche die Mikrovesikel freisetzen, bestimmt. Ausgehend von diesen Befunden können von gestressten Zellen freigesetzte Mitochondrien eine wichtige Rolle in entzündlichen Erkrankungen, die mit TNF und Typ I IFN Signalwegen assoziiert werden, spielen.

Publications arising from this thesis

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Abbreviations

ALIX	ALG-2-interacting protein X
ATP	Adenosine triphosphate
BCL-2	B-cell lymphoma 2
CCL	C-C Motif Chemokine Ligand
CD	Cluster of Differentiation
COXIV	mitochondrial cytochrome c oxidase subunit IV
DAMP	Damage Associated Molecular Pattern
EVs	Extracellular vesicles
HUVEC	Human Umbilical Vein Endothelial Cell
ICAM	Intercellular Adhesion Molecule 1
IL	Interleukin
LPS	Lipopolysaccharide
miRNA	microRNA
$Mito_{Co}$	Mitochondria, isolated from unstimulated THP-1 monocytic cells
$Mito_{Stress}$	Mitochondria, isolated from LPS-stimulated THP-1 monocytic cells
mtDNA	mitochondrial DNA
mtRNA	mitochondrial RNA
MV_{Co}	Microvesicles, derived from unstimulated THP-1 monocytic cells
MV	Microvesicle(s) (a subtype of extracellular vesicles)
MV_{Stress}	Microvesicles, derived from LPS-stimulated THP-1 monocytic cells
NLRP3	NOD-like receptor family, pyrin domain containing-3 protein
PS	Phosphatidylserine
ROS	Reactive Oxygen Species
ShamFT	sham-depleted MV_{Stress}
THP-1	Human Monocytic Leukemia Cell Line, THP-1 monocytic cell
$THP\text{-}1_{co}$	Unstimulated THP-1 monocytic cells
$THP\text{-}1_{stress}$	LPS-stimulated THP-1 monocytic cells
TLR	Toll-Like Receptor
TNF	Tumor necrosis factor
TOM22	Translocase of the Outer Membrane, Subunit 22
TOM22FT	TOM22+ subset depleted MV_{Stress}
VCAM	Vascular Cell Adhesion Molecule-1
ρ0 cell	THP-1 monocytic cells with impaired oxidative phosphorylation

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1. Introduction

1.1 Microvesicles

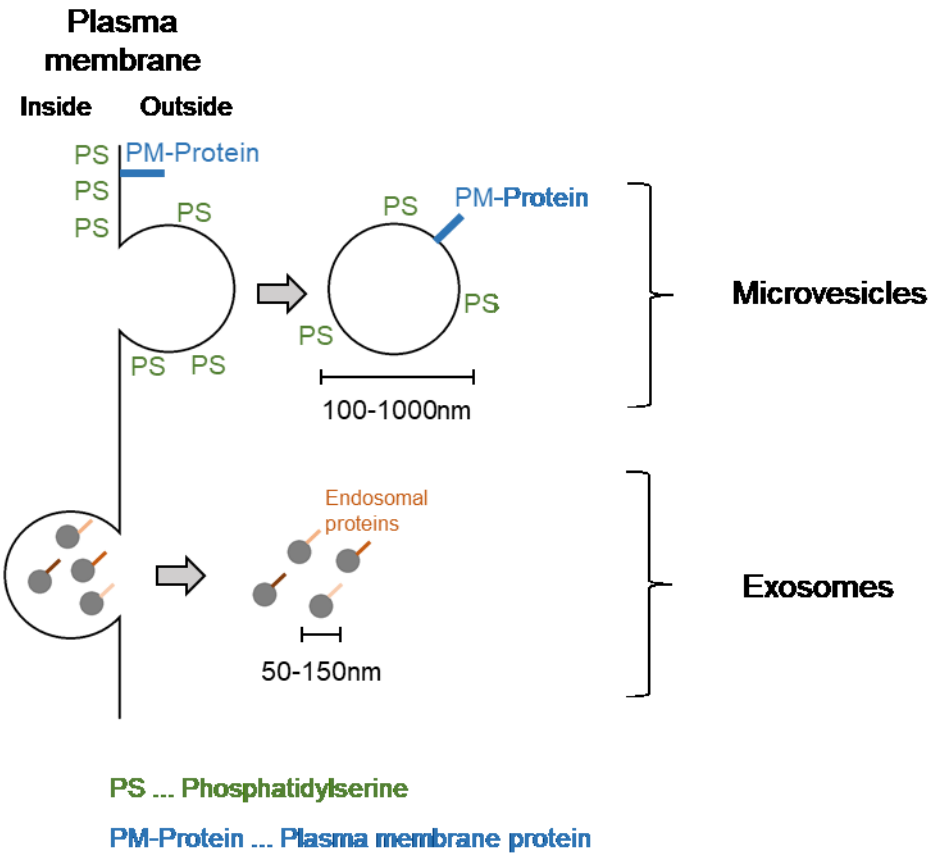
1.1.1 Definition of microvesicles

Extracellular vesicles are membrane vesicles, that include vesicles released from the plasma membrane (microvesicles) or endosomal compartment via exocytosis (exosomes) [1][2]. This thesis is primarily concerned with microvesicles. The first time microvesicles have been described was by Wolf, P. (1967) [3], who coined the term 'platelet dust'. He described that lipid-rich particles could be separated from platelets by centrifugation and imaged by electron microscopy [3]. Later on it was shown that activated platelets release phosphatidylserine-exposing vesicles [4]. Similarly, microvesicles were found to be released by many different cell types [5][6][7][8][9], including endothelial cells [8] and monocytes [9]. Microvesicles are defined by their size, ranging from 100-1000nm diameter [10][11][12][13], and exposure of phosphatidylserine, which can be identified by Annexin-V binding [4] (*see* Thesis Scheme 1). Microvesicles originate from the plasma membrane of cells through outward budding and inherit plasma membrane proteins, which mark their cellular origin [12]. They can be isolated from suspension by centrifugation at 10,000 to 20,000g [14][15][16][17]. Exosomes, another major subtype of extracellular vesicles, are characterized by an on average smaller diameter (30-150nm) and originate from multi vesicular bodies in the form of intraluminal vesicles (ILVs), which are released from cells via exocytosis [12][17] (*see* Thesis Scheme 1). In contrast to microvesicles, they inherit endosome-associated proteins (e.g. ALIX [18]) and if they expose phosphatidylserine on their surface is still a matter of debate [19]. Moreover, exosomes are isolated by centrifugation at around 100,000g [20].

A commonly used technique for microvesicle-analysis is flow cytometry [21]. Achieving reliable results requires the use of beads with known sizes to calibrate laser and detector of the machine, as well as consistency in the isolation protocol. The lack of consensus on isolation and detection techniques of microvesicles – and extracellular vesicles in general – is a persistent problem in the field, although there has been a large effort to define minimal requirements of experimental procedures in extracellular vesicle research [21]. It is important to note that the terminology used to describe extracellular vesicles can be confusing – owed to the heterogeneity of extracellular vesicles and analysis methods - and is another hurdle in the field [22]. For instance, to describe microvesicles that fit the definition above, many different terms have been used, which include: microparticles, neurospheres, migrasomes, ARRMs (arrestin-domain-containing protein 1-mediated microvesicles), oncosomes, shedding vesicles, blebbing vesicles and ectosomes [12][22]. Furthermore, not all extracellular vesicles

that fit the size range of microvesicles are positively labeled with Annexin-V [23]. Additionally, selective sorting of protein cargo of microvesicles has been shown [24], thus not every protein of the plasma membrane of the microvesicle-shedding cell may be equally expressed in released microvesicles. Moreover, lowly expressed proteins, due to the small size of microvesicles, might be overlooked by flow cytometric analysis classifying such microvesicles as false-negative. It can thus be difficult to judge the cellular origin of microvesicles by exclusion of plasma membrane protein expression, which is a commonly employed method for identification of cells by flow cytometry. Taken together, when interpreting results of a study on extracellular vesicles one has to pay special attention to the isolation protocol and the analysis techniques employed.

This thesis is focuses on 'classical' microvesicles, i.e. extracellular vesicles that fit the following criteria: 100-1000nm diameter, isolation by 10,000-20,000g centrifugation and positive staining with Annexin-V.



Thesis Scheme 1: Definition of Microvesicles and Exosomes. The scheme represents the general definition of microvesicles (vesicles shed from the plasma membrane) and exosomes (vesicles secreted by exocytosis).

1.1.2 Biogenesis of microvesicles

To date, the mechanisms of microvesicle-release have not been thoroughly elucidated but certain proteins involved in exosome-biogenesis, for example ESCRT (endosomal sorting complexes required for transport)-proteins, have been shown to be relevant in the biogenesis of extracellular vesicles in general [25]. In principle, the biogenesis of microvesicles is a process involving three steps: i) sorting or concentration of cargo, ii) induction of membrane curvature and budding of the membrane, iii) a fission event resulting in shedding of the membrane bud [12][17][26].

1.1.2.1 Sorting of cargo and contents of microvesicles

Sorting of cargo to sites of microvesicle release has not been clearly described, although it is possible that sorting and concentration of cargo at the microvesicle-release site depends on changes in membrane curvature [27]. Sorting of lipids [28] and proteins [29][30] depending on membrane curvature has been described, but the relevance of this has not been clearly demonstrated for microvesicles. Nevertheless, membrane curvature may be regulated by tetraspanins [31], which were also found in different types of extracellular vesicles [32]. Even though the tetraspanins (CD9, CD63 and CD81) are generally considered to be specific for exosomes [33][34][35], they were also found in microvesicles [36][37]. These data support the view that extracellular vesicles are quite heterogeneous and tetraspanin-dependent sorting mechanisms may also be considered to be relevant for the biogenesis of microvesicles [12].

Most literature on miRNA-association with extracellular vesicles concerns exosomes. However, in a study [38] investigating miRNA-association with breast cancer cell-derived extracellular vesicles the authors describe miRNA-association with ‘large exosomes’ (size range between 100nm-400nm) that express the tetraspanin CD63 and the plasma membrane protein CD44 [39]. Even though CD63 is generally considered to be an exosome marker, expression of a cell surface protein and the large vesicle size suggests that these are similar to microvesicles. Furthermore, Jeppesen et al [13] showed that other nucleic acids (double-stranded DNA) are associated with vesicles with a higher density than exosomes, but with a similar endosomal origin, further adding to the view that extracellular vesicles are heterogeneous.

The release of extracellular vesicles has been proposed as a mechanism for the secretion of proteins lacking a leader peptide/sequence, most prominently IL-1β [27]. Even though many reports indicate the release of cytokines in association with extracellular vesicles, it is important to note that these studies mostly describe exosomes. In particular the release of IL-32 [40], membrane-bound TNF [41], IL-6 [42], CCL (C-C Motif Chemokine Ligand 2/3/4/5 and 20) [43] and TGFβ (transforming growth factor beta) [44] is attributed to exosome-release

and not microvesicles. Furthermore, while the release of IL-8 associated with microvesicles has been reported [45], extracellular vesicles in that study were isolated by 50,000g centrifugation, which risks co-isolation of microvesicles and exosomes [17]. On a side note, in some studies the terminology is confusing and authors may use the term microvesicles, when isolating extracellular vesicles by centrifugation speeds of 100,000g, at which exosomes, and other small vesicles, are concentrated in the pellet as well [17][20]. Furthermore, the release mechanism of IL-1β in association with extracellular vesicles is still under investigation and it is not clear whether it is primarily released in association with microvesicles [46][47] or exosomes [48][49].

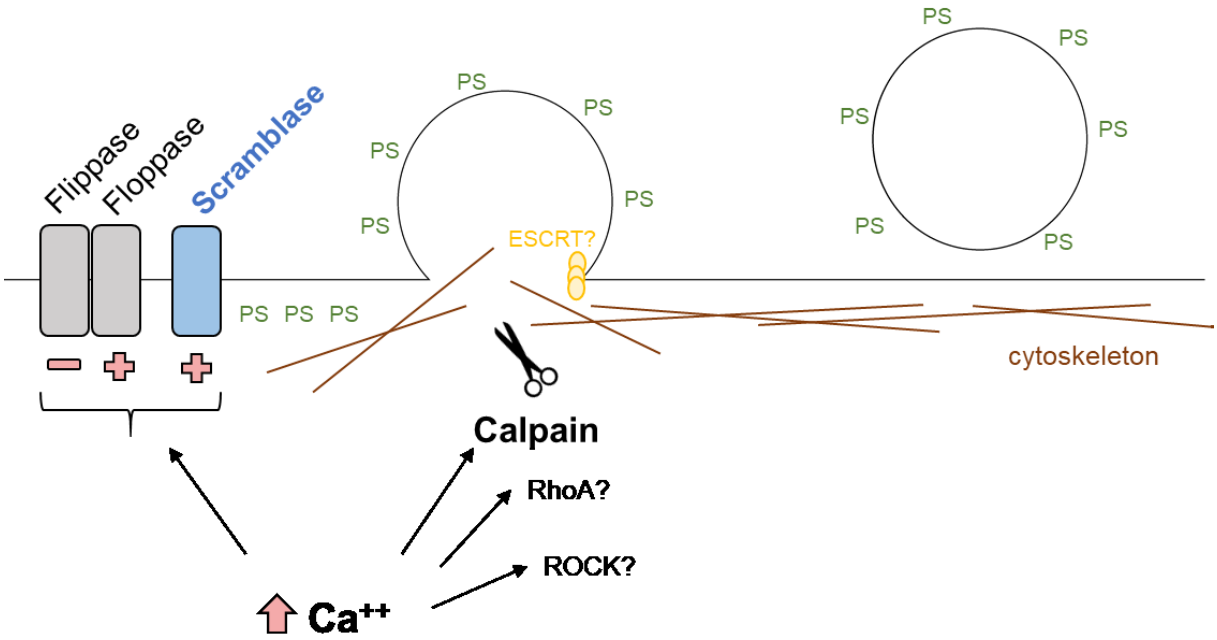
1.1.2.2 Microvesicle budding and fission from the plasma membrane

Outward blebbing of the plasma membrane and consequentially the shedding of phosphatidylserine-exposing microvesicles, is a process that depends on changes in the phospholipid distribution of the membrane (loss of asymmetry) and local restructuring and destabilization of the cytoskeleton [50] (*see* Thesis Scheme 2).

In the resting state, flippases, floppases and scramblases maintain the asymmetrical distribution of phospholipids in the plasma membrane [50]. Specifically, phosphatidylserine, phosphatidylethanolamine and aminophospholipids, of which only phosphatidylserine has a net negative charge, are located on the inside and high amounts of phosphatidylcholine are located on the outside of the plasma membrane [51]. Local increase of intracellular Calcium, via release from mitochondria or the endoplasmic reticulum [50], triggers the activation of Calpains - calcium-dependent proteases involved in cytoskeletal remodeling - [52][53][54], activation of RhoA (Ras homolog gene family, member A) [55] or ROCK (Rho-associated protein kinase) - causing myosin light chain phosphorylation - [56], and activation of scramblases [50] - leading to the loss of membrane asymmetry and exposure of phosphatidylserine on the surface.

The loss of membrane asymmetry induces bending of the membrane, which in turn affects the local structure of the actin-cytoskeleton [50]. While it has been proposed that microvesicle-biogenesis can be independent of loss of lipid asymmetry [12], this interpretation should be taken with caution as the cited studies [23][57][58] either do not test this or do not differentiate circulating microvesicles from exosomes and other lipid vesicles (very-low-density lipoprotein (VLDL) and chylomicrons) which can be co-isolated [17][59] and thus might be co-detected.

Lastly, ESCRT (endosomal sorting complexes required for transport) proteins can become located in the neck region of a budding vesicle and may be involved in the membrane fission process leading to the shedding of microvesicles [27][60][61].



Thesis Scheme 2: Biogenesis of microvesicles. This scheme summarizes the main molecular events leading to the release of microvesicles from cells. Flippase, floppase and scramblase maintain the asymmetric distribution of phospholipids in the plasma membrane. (1) Upon local rise in intracellular calcium (Ca^{++}), the balance shifts from flippase to floppase and scramblase activity, which results in redistribution of phosphatidylserine to the extracellular surface of the plasma membrane. (2) Additionally, this is accompanied with activation of Calpain (and RhoA and/or ROCK) which destabilizes the cytoskeleton. (3) It is possible that the fission leading to the shedding of the microvesicle is mediated by ESCRT proteins.

1.1.3 Signaling and uptake mechanisms of microvesicles

To date, the mechanisms by which microvesicles intercellular signals have not been thoroughly investigated and it is still not clear if signaling and uptake mechanisms differ between different classes of extracellular vesicles. In general, signaling and uptake of extracellular vesicles has been reported via the following mechanisms [12][62]: i) signaling via surface interaction (via surface receptors) of extracellular vesicles with target cells, ii) uptake via clathrin- or caveoline-mediated endocytosis, iii) lipid raft mediated uptake, iv) uptake via phagocytosis, v) uptake via macropinocytosis and vi) uptake by fusion of microvesicles with the plasma membrane. However, in these studies only uptake of either exosomes or mixtures of exosomes and microvesicles was tested.

There is evidence that microvesicles can be taken up by target cells by active endocytosis. For example, Menck et al [63] reported that inhibition of dynamin-dependent endocytosis did prevent uptake of MCF-7 cell-derived microvesicles by primary macrophages but did not affect their signaling. This may depend on surface recognition of the signaling

molecule while it is associated with microvesicles. On the other hand, microvesicles may act as carriers from which attached molecules could be shed.

Furthermore, phagocytosis is a possible uptake mechanism for phosphatidylserine–exposing (PS) extracellular vesicles as PS-exposure is recognized by receptors, such as TIM4, triggering phagocytosis [64]. For example, Feng et al [65] reported that blocking TIM4 reduced uptake of extracellular vesicles. It is important to note that this study [65] does not clearly demonstrate whether these extracellular vesicles were PS-exposing exosomes or an exosome preparation contaminated with PS-exposing microvesicles. On the other hand, it was shown that lactadherin, which may act as an opsonin by binding to PS [66], facilitates the uptake of platelet-derived microvesicles via phagocytosis [67]. Notably, blocking of exposed PS by Annexin-V did inhibit the uptake of monocytic microvesicles by platelets [58], indicating phagocytosis-like mechanisms to be responsible for microvesicle uptake. It is important to mention that the method of extracellular vesicle preparation in this study [58] cannot clearly differentiate between microvesicles and exosomes, as the final vesicle pellet was obtained by 200,000g centrifugation.

1.1.4 Functions of microvesicles in the vascular system

Little is known about the physological and pathological roles of microvesicles *in vivo*, but they have been reported to act proangiogenic, procoagulatory and pro- or anti-inflammatory, depending on their composition.

For example, there are studies showing that extracellular vesicles are able to promote neovascularization of an ischaemic hind limb [68] and pancreatic islets [69]. Even though angiogenic miRNAs (micro RNAs) were found in the vesicles [70], these studies isolated extracellular vesicles by 100,000g centrifugation and detected both microvesicles and exosomes in the pellets [70], making it impossible to know whether the miRNAs and their effects can be attributed to all vesicles or only certain vesicle subtypes. Extracellular vesicles derived from platelets have also been shown to be angiogenic and to promote revascularization [71]. However, in this study it is not possible to discern whether the effect stems from microvesicles or exosomes due to the isolation method used. On the other hand, endothelial cells have been shown to release microvesicles enriched in miR-92a (miRNA-92a) when activated by oxidized LDL (low-density lipoprotein) [72]. These miR-92a rich, endothelial cell-derived microvesicles induced proliferation in target endothelial cells via inhibition of thrombospondin-1 expression [72], demonstrating a mechanism by which microvesicles might support angiogenesis.

Importantly, monocytes have been shown to release microvesicles with tissue-factor (TF) activity [9]. Studies attributing TF-association with microvesicles generally rely on

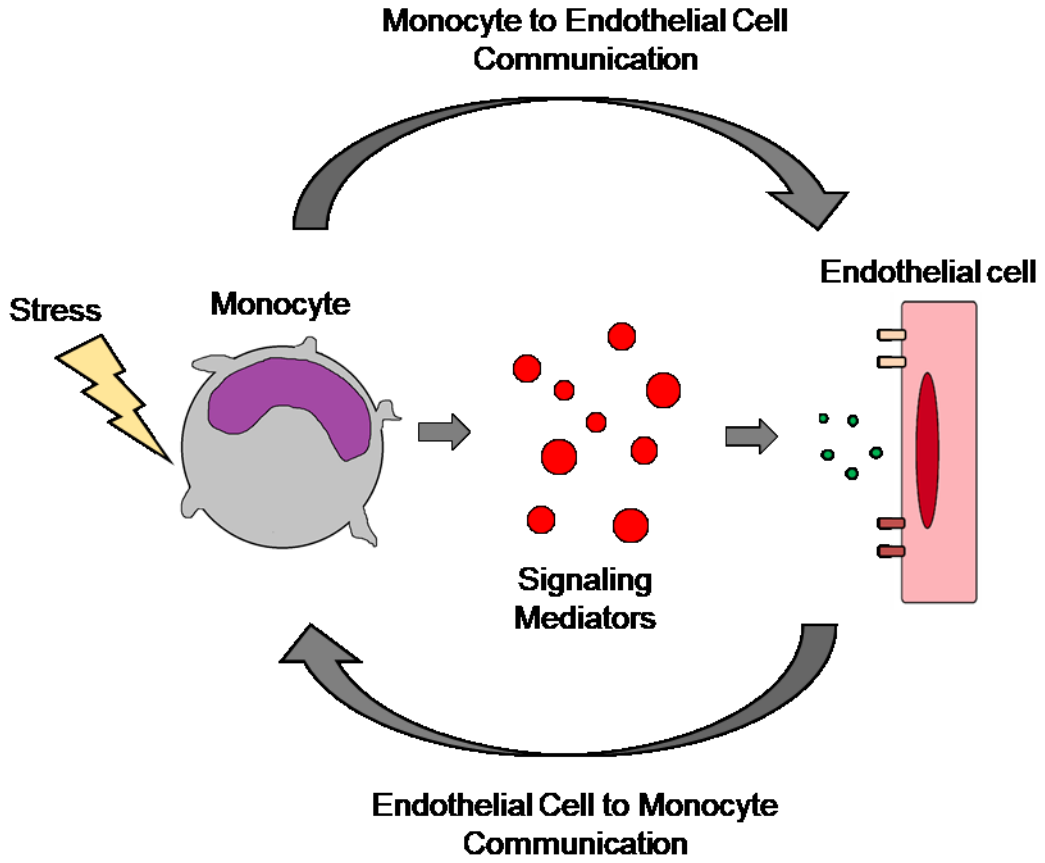
measurement of TF-activity, as the concentration of TF needed to induce coagulation is below the detection limit by ELISA [73] and it can thus be difficult to detect TF-expression on microvesicles. On the other side, microvesicles act procoagulatory as they expose negatively-charged phosphatidylserine on their surface [73]. Interestingly, in Scott syndrome, a rare bleeding disorder, both the ability of platelets to expose phosphatidylserine and the release of microvesicles are impaired [74][75][76].

Furthermore, depending on their origin, microvesicles have been found to act pro- and anti-inflammatory. For example, cardiac microvesicles obtained in a mouse model of induced myocardial infarction have been shown to increase IL-6, CCL-2 and CCL-7 expression by Ly6C+ cardiac monocytes [77]. On the other hand, serum-starved endothelial cells release microvesicles enriched in miR-222, which reduces ICAM-1 expression in targeted endothelial cells, leading to less monocyte adhesion to these cells [78], which may be associated with reduced inflammation. Importantly, it has been documented that the biological effects of circulating microvesicles differ between patients and healthy controls. In particular, circulating microvesicles isolated from patients with metabolic syndrome reduced the production of nitric oxide and superoxide by endothelial cells *in vitro* [79].

1.2 Monocyte – Endothelial Cell signaling in vascular inflammation

Chronic and acute inflammation in the vasculature are characterized by activation of the endothelium [80][81]. In particular, monocytes are early responders to immunological challenge and have been shown to activate endothelial cells [80]. For example, in acute endotoxemia monocytes recognize bacterial endotoxin (LPS) and subsequently release inflammatory mediators, such as TNFα and IL-1β, which in turn signal to endothelial cells [80]. Endothelial cells are poor at sensing LPS, as they lack CD14 expression [82], thus they only recognize LPS when it is bound by soluble CD14 – which in turn is produced by other cells in the circulation, such as monocytes [80]. On the other hand, in sterile inflammation (e.g. atherosclerosis and ischemic cardiovascular disease) monocytes contribute to the disease by producing inflammatory cytokines and by infiltrating the tissue [83][84][85]. Importantly, tissue infiltration by monocytes requires expression of chemokines (IL-8) and adhesion molecules (ICAM-1, VCAM-1, E-Selectin) by endothelial cells [80][86] and sensing of endothelial cell released cytokines and chemokines can activate monocytes [80]. Hence, monocyte – endothelial cell signaling contributes to inflammation in a reciprocal cycle (*see* Thesis Scheme 3).

Interestingly, activated monocytes have been shown to release microvesicles able to induce proinflammatory activation of endothelial cells via IL-1β [47]. However, blocking of IL-1β signaling only partially reduced this effect, suggesting other mechanisms contributing to the proinflammatory activity of these microvesicles. On the other hand, increased release of mitochondrial proteins in association with microvesicles derived from LPS-activated monocytic cells has been reported [87]. It is possible that these contribute to the proinflammatory activity of microvesicles due to the presence of mitochondrial damage-associated molecular patterns (mitoDAMPs).

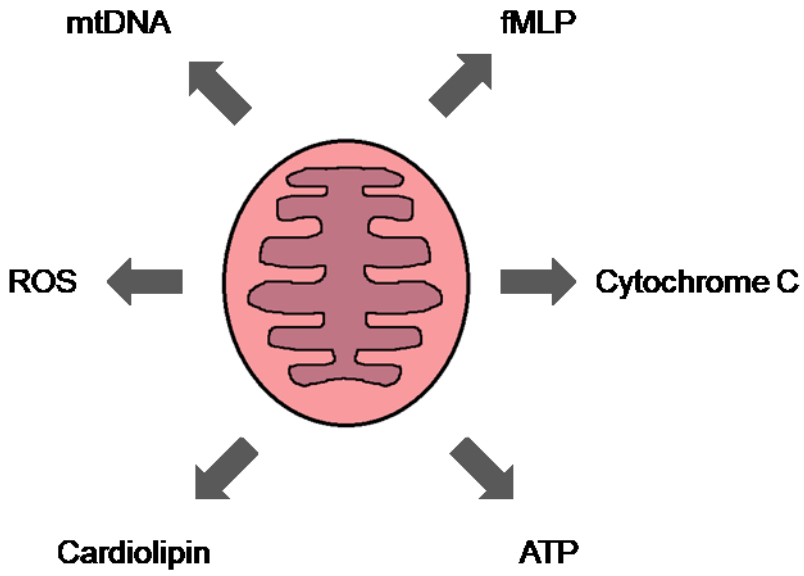


Thesis Scheme 3: Monocyte to endothelial cell communication. This scheme illustrates the crosstalk between monocyte and endothelial cells, with a focus on stress-induced monocyte signaling. It is important to note that stress (eg inflammatory stimuli) may also induce endothelial cell signaling to monocytes. Signaling mediators include proteins (cytokines, chemokines, etc.), miRNAs, lipids and extracellular vesicles. Direct cell-to-cell contact, while important in intercellular communication, is not depicted in this illustration.

1.3 Mitochondria as immunological organelle

Mitochondria are cellular organelles of bacterial origin, most likely a bacterial species related to *Rickettsia prowazekii* [88]. They are primarily known for their metabolic function as powerhouse of eukaryotic cells and in regulation of apoptosis [51]. Recently, additional roles of mitochondria have been identified, such as modulation of immunological responses and

immune cell functions [89][90][91] marked by changes in cellular metabolism affecting the release of cytokines and inflammasome activation. Specifically, metabolic shift towards glycolysis or oxidative phosphorylation is associated with short-term or long-term immunological responses, respectively [89][90]. Importantly, immunological triggers are able to induce changes in the metabolic status of leukocytes and when sensing LPS, leukocytes switch towards glycolysis [89]. On a side note, mitochondrial morphology is linked to metabolic status and mitochondria fragment when a cell becomes more reliant on glycolysis and oxidative phosphorylation is impaired [92]. Considering the evolutionary origin of mitochondria, these organelles share many molecular features with bacteria, which can be recognized by the immune system as mitochondrial damage associated molecular patterns (DAMPs) [93]. Known mitochondrial DAMPs include mitochondrial DNA, *N*-formyl-methionyl dipeptides (fMLP), Cytochrome C, ATP, Cardiolipin and mitochondrial ROS [93] (*see* Thesis Scheme 4).



Thesis Scheme 4: Major mitochondrial DAMPs.

1.3.1 DAMPs of mitochondrial origin and their activities

Mitochondrial DNA (mtDNA) is thought to be a potential DAMP due to the presence of hypomethylated CpG sequences [94]. It has been shown that high concentrations of mtDNA (10µg/ml) or CpG (10µg/ml) in combination with 10nM N-formyl-Met-Leu-Phe (fMLP), another mitochondrial DAMP, were able to induce IL-8 release by neutrophils [94]. Such high concentrations of free mtDNA can be reached in tissue upon traumatic injury [94]. These data suggested a TLR9-dependent signaling mechanisms. The required amount of mtDNA to induce signaling might be so high as TLR9 is an endosomal receptor [51], hence recognition of mtDNA would require its uptake by phagocytosis. Additionally, the proinflammatory potential

of mtDNA may be influenced by oxidation. A study [95] reported that injection of mtDNA, but not nuclear DNA, induced arthritis when injected into joints. The data suggested that this proinflammatory effected depended on the presence of 8-oxoG as GpC ODN (oligodeoxynucleotide) with 8-oxoG was immunogenic in contrast to a GpC ODN without 8-oxoG [95]. Moreover, oxidized, extracellular mtDNA capable of inducing TLR9 responses was reported in a model of hepatic inflammation [96].

N-formyl-methionyl dipeptides (fMLP) are byproducts of mitochondrial protein translation and sensed by high-affinity formyl peptide receptor-1 (FPR-1) [93]. fMLP acts as a chemoattractant for neutrophils [97], monocytes [98] and thrombin-activated, N-formyl peptide receptor expressing platelets [99].

Cytochrome C is located between the inner and outer mitochondrial membrane and transfers electrons from complex III to complex IV in the respiratory chain [51]. Intracellular release of cytochrome C from mitochondria is a known trigger of apoptosis [51]. On the other hand, extracellular cytochrome C has been reported to induce cell death of lymphocytes [100].

Extracellular ATP can be sensed by $P2X_7$ purinergic receptors and thereby act as a stimulus for NLRP3 (NOD-like receptor family, pyrin domain containing-3 protein) inflammasome activation [101].

Cardiolipin is primarily located on the inner mitochondrial membrane [51]. However, cardiolipin can become exposed on the outer mitochondrial membrane upon stress [102]. This can be the case in apoptosis, in which cardiolipin is exposed on the surface of mitochondria after induction of ROS but before DNA fragmentation and changes in mitochondrial membrane potential [103]. Additionally, cardiolipin can become expressed on the surface of dead cells [104]. Moreover, cardiolipin has been reported to facilitate inflammasome formation possibly by providing a common binding platform for NLRP3 and (pro)caspase-1, which are essential components of the inflammasome [105].

Mitochondria are the primary source for reactive-oxygen species (ROS) in eukaryotic cells [51]. ROS can act as inflammatory signaling molecules themselves [106] or change the immunostimulatory properties of other molecules by oxidative modification. For example, oxidation of phospholipids and nucleic acids has been reported to enhance their inflammatory potential [96][107][108][109][110]. On the other hand, oxidation may also decrease proinflammatory activity, as was shown for oxidation of high-mobility group box-1 (HMGB1) [111].

1.3.2 Biological activity of extracellular mitochondria

The release of mitochondrial components in association with microvesicles has been reported [87][96][112][113][114]. Garcia-Martinez et al [96], showed that oxidized mitochondrial DNA

can be released by hepatocytes and act inflammatory in a model of hepatic inflammation by activating TLR9. Furthermore, increased release of mitochondrial proteins in association with microvesicles has been found for LPS-activated monocytic cells [87]. However, in this study [87], the biological activity of these mitochondrial proteins was not tested. Another study [112] reported that platelets release mitochondria-embedded in microvesicles and free mitochondria upon activation. Interestingly, these mitochondria were not inflammatory *per se*, but rather released inflammatory mediators that were liberated upon secreted phospholipase A_2 IIA ($sPLA_2$-IIA) catalyzed hydrolysis [112]. On the other hand, the studies by Islam et al [113] and Phinney et al [114] reported beneficial, cytoprotective effects of mitochondrial transfer. In particular, transfer of active mitochondria from BMSCs (Bone marrow-derived stromal cells) could protect lung epithelial cells in a model of LPS-induced acute lung injury [113] and MSCs (mesenchymal stem cells) could release mitochondria-embedded in microvesicles, which were taken up by macrophages where they improved bioenergetics [114]. Taken together, these studies demonstrate the release of mitochondria or mitochondrial components from different viable cells and platelets. However, whether these mitochondria and mitochondrial vesicles are inflammatory or not appears to depend on their origin and secondary modifications rather than the mere presence of mitochondrial components.

1.4 Hypothesis and aims

As described above, microvesicles are contributing to cell-to-cell communication. However, little is known about their properties and what defines their biological activity. Monocyte to endothelial cell signaling is important in vascular inflammation [80], but the contribution of microvesicles has not been thoroughly adressed. It has been shown that monocytes release proinflammatory microvesicles capable to induce endothelial cell activation partially by the transfer of IL-1β [115]. Interestingly, microvesicles were also demonstrated to be associated with or act as carriers of mitochondrial proteins [87] or mitochondrial DNA [96] and, in some cases, even whole mitochondria [112][114]. Intriguingly, a switch of cellular metabolism from oxidative phosphorylation to glycolysis and vice versa, shapes immune cell responses and mitochondrial structure and function is tightly linked to these metabolic changes [89][90][91]. Importantly, microvesicles derived from LPS-activated monocytes were found to be enriched in mitochondrial proteins [87], but their biological activity was not assessed. Notably, mitochondria are a potential source of sterile inflammatory mediators (mitoDAMPs) [93]. Additionally, previous studies of the biological activity of extracellular mitochondria suggest that their activity may depend on their cellular origin and secondary modifications s[96][112][113][114]. Therefore, the hypothesis of this dissertation was that mitochondria actively contribute to the content and inflammatory potential of monocyte-derived microvesicles to activate endothelial cells.

This thesis aims to answer the following questions:

1. What is the contribution of mitochondria to the biological activity of microvesicles released by activated monocytes?

2. What defines the inflammatory potential of microvesicles released by activated monocytes?

3. Which cellular responses are induced by such monocytic microvesicles in endothelial cells?

4. Which are the principal components mediating these effects?

2. Results

The following results section consists of the of the manuscript, published in Circulation Research (DOI: 10.1161/CIRCRESAHA.118.314601), that I wrote on this thesis with the title „Mitochondria are a subset of extracellular vesicles released by activated monocytes and induce Type I IFN and TNF responses in endothelial cells“.

Original Research

Mitochondria Are a Subset of Extracellular Vesicles Released by Activated Monocytes and Induce Type I IFN and TNF Responses in Endothelial Cells

Florian Puhm,* Taras Afonyushkin,* Ulrike Resch, Georg Obermayer, Manfred Rohde, Thomas Penz, Michael Schuster, Gabriel Wagner, Andre F. Rendeiro, Imene Melki, Christoph Kaun, Johann Wojta, Christoph Bock, Bernd Jilma, Nigel Mackman, Eric Boilard, Christoph J. Binder

Rationale: **Extracellular vesicles, including microvesicles, are increasingly recognized as important mediators in cardiovascular disease. The cargo and surface proteins they carry are considered to define their biological activity, including their inflammatory properties. Monocyte to endothelial cell signaling is a prerequisite for the propagation of inflammatory responses. However, the contribution of microvesicles in this process is poorly understood.**

Objective: **To elucidate the mechanisms by which microvesicles derived from activated monocytic cells exert inflammatory effects on endothelial cells.**

Methods and Results: **LPS (lipopolysaccharide)-stimulated monocytic cells release free mitochondria and microvesicles with mitochondrial content as demonstrated by flow cytometry, quantitative polymerase chain reaction, Western Blot, and transmission electron microscopy. Using RNAseq analysis and quantitative reverse transcription-polymerase chain reaction, we demonstrated that both mitochondria directly isolated from and microvesicles released by LPS-activated monocytic cells, as well as circulating microvesicles isolated from volunteers receiving low-dose LPS-injections, induce type I IFN (interferon), and TNF (tumor necrosis factor) responses in endothelial cells. Depletion of free mitochondria significantly reduced the ability of these microvesicles to induce type I IFN and TNF-dependent genes. We identified mitochondria-associated TNFα and RNA from stressed mitochondria as major inducers of these responses. Finally, we demonstrated that the proinflammatory potential of microvesicles and directly isolated mitochondria were drastically reduced when they were derived from monocytic cells with nonrespiring mitochondria or monocytic cells cultured in the presence of pyruvate or the mitochondrial reactive oxygen species scavenger MitoTEMPO.**

Conclusions: **Mitochondria and mitochondria embedded in microvesicles constitute a major subset of extracellular vesicles released by activated monocytes, and their proinflammatory activity on endothelial cells is determined by the activation status of their parental cells. Thus, mitochondria may represent critical intercellular mediators in cardiovascular disease and other inflammatory settings associated with type I IFN and TNF signaling. (*Circ Res*. 2019;125:43-52. DOI: 10.1161/CIRCRESAHA.118.314601.)**

Key Words: endothelial cells ■ extracellular vesicles ■ inflammation ■ mitochondria ■ monocytes

Extracellular vesicles are increasingly recognized for their role in intercellular communication and are associated with cardiovascular disease.[1,2] Microvesicles, a major class of extracellular vesicles also known as microparticles, are phosphatidylserine exposing membrane vesicles with a diameter of 0.1 to 1 µm that are released by cells under normal and stressed conditions.[3] Changes in numbers and cell origin of circulating microvesicles have been described in different pathologies where they have been proposed to promote inflammation.[2,4] The cargo (eg, nucleic acids, lipids, and proteins) of microvesicles may reflect the state of activation of the cells they originate from and different mechanisms by which microvesicles activate target cells have been described.[1]

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Novelty and Significance

What Is Known?

- Microvesicles, a subtype of extracellular vesicles, contribute to monocyte-endothelial cell signaling.
- Cargo and surface proteins are considered to define the inflammatory properties of microvesicles.
- Mitochondria are a source of damage-associated molecular patterns.
- Altered mitochondrial activity and cellular metabolism contribute to cardiovascular disease and other pathologies.

What New Information Does This Article Contribute?

- Lipopolysaccharide-activated monocytic cells release mitochondria and mitochondria embedded in microvesicles.
- Both free mitochondria and microvesicle-embedded mitochondria contribute to the ability of microvesicles to activate endothelial cells.
- This proinflammatory capacity is determined by the mitochondrial activity of parental cells rather than the mere presence of mitochondrial content.
- Mitochondria-associated TNF (tumor necrosis factor) and interferonogenic mitochondrial RNA are the major proinflammatory mediators of microvesicles released from activated monocytic cells.

Extracellular vesicles, including microvesicles, are increasingly recognized as important mediators of intercellular communication. Their cargo is considered to define their biological activity, including inflammatory properties. Monocyte to endothelial cell signaling is crucial for the propagation of inflammatory responses. However, the contribution of microvesicles in this process is poorly understood. Here, we show that activated monocytic cells release both free and microvesicle-embedded mitochondria with the capacity to trigger inflammatory responses in endothelial cells. This capacity is predetermined by the mitochondrial activity in parental cells rather than by the mere presence of mitochondrial content released. We identified mitochondria-associated TNF, and interferonogenic mitochondrial RNA as the major proinflammatory mediators of mitochondria released from activated monocytic cells Thus, extracellular mitochondria released by activated monocytic cells may represent critical proinflammatory mediators in diseases associated with TNF- and type I IFN (interferon) signaling pathways, for example, sepsis, SLE (systemic lupus erythematosus), rheumatoid arthritis, and psoriasis. In summary, mitochondria represent a critical component of extracellular vesicles released by activated monocytes, and their inflammatory potential is determined by the activation status of their parental cells.

Nonstandard Abbreviations and Acronyms

ρ0 cell	THP-1 monocytic cells with impaired oxidative phosphorylation
ALIX	ALG-2-interacting protein X
CD	cluster of differentiation
COXIV	mitochondrial cytochrome c oxidase subunit IV
DAMP	damage associated molecular pattern
HUVEC	human umbilical vein endothelial cell
ICAM	intercellular adhesion molecule
IFN	interferon
IL	interleukin
LPS	lipopolysaccharide
$Mito_{Co}$	mitochondria, isolated from unstimulated THP-1 monocytic cells
$Mito_{Stress}$	mitochondria, isolated from LPS-stimulated THP-1 monocytic cells
MV_{Co}	microvesicles, derived from unstimulated THP-1 monocytic cells
MV_{Stress}	microvesicles, derived from LPS-stimulated THP-1 monocytic cells
ROS	reactive oxygen species
THP-1	human monocytic leukemia cell line, THP-1 monocytic cell
TNF	tumor necrosis factor
TOM22	translocase of the outer membrane, subunit 22
VCAM	vascular cell adhesion molecule

Leukocyte to endothelial cell signaling is important for vascular endothelium activation, which is a major part of inflammatory responses. Microvesicles released from THP-1 monocytic cells and peripheral blood mononuclear cells after LPS (lipopolysaccharide)-stimulation have been shown to activate a proinflammatory response in endothelial cells.[5] Therefore, microvesicles are proposed as mediators of monocyte to endothelial cell communication.[2]

Interestingly, the presence of mitochondrial proteins has been reported in microvesicles released by LPS-stimulated monocytes.[6] Intriguingly, it has been recently found that mitochondria or mitochondrial components are associated with platelet-derived microvesicles.[7] In addition, increased circulating levels of microvesicles containing mitochondrial DNA have been reported in mouse models of hepatic inflammation.[8] Nucleic acids and oxidized nucleic acids, in particular, have been shown to induce IFN (interferon) responses.[9] Extracellular mitochondria and mitochondria-derived molecular patterns (mitochondrial DAMPs [damage associated molecular pattern]) are recognized as potent inducers of inflammatory responses.[10] On the other hand, LPS-stimulation alters the activity of mitochondria,[11,12] which themselves are known as important intracellular mediators of the immune response.[11,13] Therefore, we hypothesized that mitochondria may actively contribute to the content and ability of microvesicles shed by activated monocytes to induce a proinflammatory response in endothelial cells.

Methods

All data, analytic methods, and study materials supporting the findings of this study are provided in the article, Online Data Supplement, and available from the corresponding author on reasonable request.

Online Data Supplement contains detailed description of methods.

Results

LPS-Stimulated THP-1 Monocytic Cells Release Proinflammatory Microvesicles Enriched in Mitochondrial Content and Free Mitochondria

To assess the capacity of monocyte-derived microvesicles to activate endothelial cells, we measured their ability to induce an inflammatory response. THP-1 monocytic cells, stimulated with LPS, released an increased amount of microvesicles (MV_{Stress}) as compared with vehicle-stimulated cells

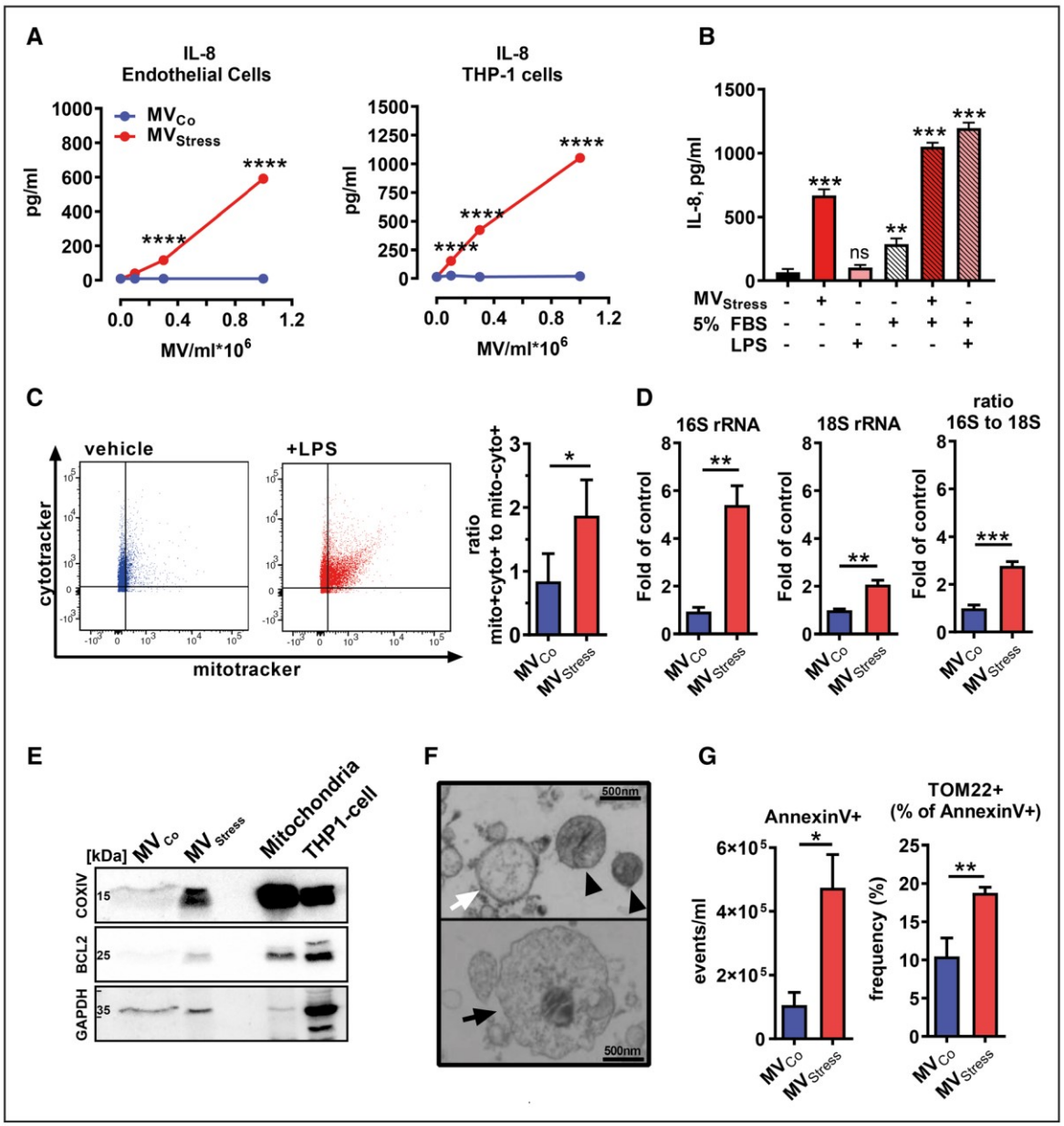

Figure 1. LPS (lipopolysaccharide)-stimulated THP-1 monocytic cells release proinflammatory microvesicles enriched in mitochondrial content and free mitochondria. Microvesicles were isolated from culture media after 16 h stimulation of THP-1 monocytic cells with vehicle (MV_{Co}) or LPS (MV_{Stress}). **A**, Levels of IL (interleukin)-8 protein released by human umbilical vein endothelial cell (HUVEC) or THP-1 monocytic cells, stimulated for 8 h with increasing concentrations of MV_{Co} or MV_{Stress}(n=4). **B**, Levels of IL-8 protein released by HUVECs stimulated for 8 h with MV_{Stress} or LPS in presence or absence of 5% FBS (n=4). **C**, Flow-cytometric analysis of microvesicles released by THP-1 monocytic cells, colabeled with mitotracker and cytotracker, following vehicle-stimulation or LPS-stimulation (n=5). **D**, Analysis of 16S and 18S rRNA content and ratio in microvesicles by reverse transcription-quantitative polymerase chain reaction (n=4). **E**, Western Blot analysis of COXIV, Bcl-2 and GAPDH protein in lysates of equal numbers of MV_{Co} and MV_{Stress}, mitochondria and THP-1 monocytic cells. Complete Western Blot is shown in Online Figure XII. **F**, Representative electron microscopy images of MV_{Stress}. The pictures show microvesicles (white arrow), mitochondria (black arrowhead), and vesicles containing mitochondria (black arrow). Scale bar=500 nm (**G**) flow-cytometric analysis of microvesicles stained with Annexin-V and anti-TOM22 (translocase of the outer membrane 22) antibody. The **left** shows numbers of Annexin-V positive events and the **right** shows the frequency of TOM22-positive events of Annexin-V positive microvesicles (n=4). Data shown as mean±SEM. COXIV indicates cytochrome c oxidase subunit IV.

(MV_{Co}; Online Figure IA and IB). Both, MV_{Co} and MV_{Stress} were obtained after 18 000*g* centrifugation of conditioned media and exposed phosphatidylserine (Online Figure IB), but only contained minute amounts of the exosomal marker ALIX (ALG-2-interacting protein X)[14] as compared with extracellular vesicles (exosomes) obtained after 100 000*g* centrifugation (Online Figure IC). The mean size of MV_{Stress} obtained after 18 000*g* was 206.6 nm (SD±89.8; Online Figure ID). MV_{Stress} induced IL (interleukin)-8 secretion by endothelial cells and THP-1 monocytic cells in a concentration-dependent manner (Figure 1A). In contrast, even high numbers of MV_{Co} were not able to induce IL-8 secretion (Figure 1A). This suggested that specific components, rather than only quantity of microvesicles, define their biological activity. Importantly, MV_{Stress} were able to activate endothelial cells in the absence of serum, while the response to LPS

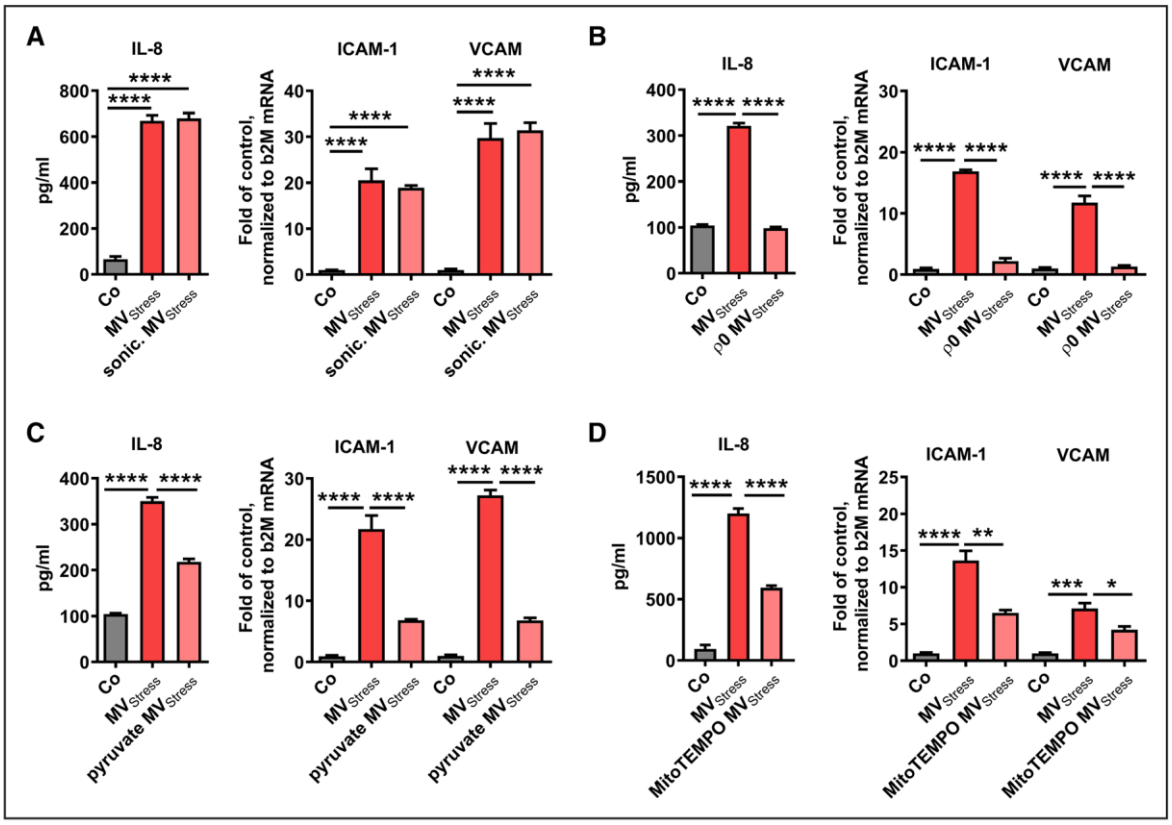

Figure 2. Mitochondrial activity of parental cells defines proinflammatory potential of microvesicles. Secretion of IL (interleukin)-8 protein (8 h) and expression of ICAM (intercellular adhesion molecule)-1 and VCAM (vascular cell adhesion molecule) mRNA (6 h) in stimulated human umbilical vein endothelial cell (HUVEC). Stimulation with (**A**) either intact or sonicated MV_{Stress}; (**B**) MV_{Stress} released by THP-1 monocytic cells or ρ0 THP-1 monocytic cells; (**C**) MV_{Stress} released by THP-1 monocytic cells in presence or absence of 1 mmol/L pyruvate; (**D**) MV_{Stress} released by THP-1 monocytic cells in presence or absence of 50 µmol/L MitoTEMPO. Co=unstimulated; Data shown as mean ±SEM, n=4.

was serum-dependent (Figure 1B). This excludes a significant contribution of LPS-carryover to the effect of MV_{Stress} as endothelial cells require serum-derived soluble CD14 (cluster of differentiation 14) for the recognition of LPS.[15] Because activated leukocytes have been shown to release mitochondria-derived DAMPs with robust proinflammatory properties,[9] we hypothesized that mitochondria contribute to the content and activity of MV_{Stress}. Indeed, THP-1 monocytic cells, prelabeled with mitochondria- and cytoplasm-specific dyes, released microvesicles particularly enriched in mitochondria-specific dye following stimulation with LPS (Figure 1C). Furthermore, MV_{Stress} were particularly enriched in mitochondrial 16S rRNA over cytosolic 18S rRNA as compared with MV_{Co} (Figure 1D). Consistent with this, high amounts of COXIV (mitochondrial cytochrome c oxidase subunit IV) and mitochondria-associated protein Bcl-2 were detected in MV_{Stress} compared with MV_{Co} (Figure 1E, Online Figure XII). Electron-microscopy identified the presence of free mitochondria and mitochondria within MV_{Stress} (Figure 1F). Additionally, LPS-stimulation of THP-1 monocytic cells resulted in an increased release of vesicles presenting the mitochondria outer membrane protein TOM22 (translocase of the outer membrane 22; Figure 1G). Thus, LPS induces the release of free mitochondria and microvesicles enriched in mitochondrial content by THP-1 monocytic cells. Of note, MV_{Co} did not induce IL-8 expression by target cells even at high concentrations (Figure 1A), despite the fact that TOM22+ vesicles were detectable. Thus the biological activity of MV_{Stress} cannot be explained simply by an increased presence of mitochondrial content.

Mitochondrial Activity of Parental Cells Defines the Proinflammatory Potential of Microvesicles

We next tested whether the vesicular integrity of MV_{Stress} is required for their proinflammatory properties. Disintegration of MV_{Stress} by sonication (Online Figure IIIA) did not alter their capacity to induce IL-8 production and ICAM-1 (intercellular adhesion molecule)/VCAM (vascular cell adhesion molecule) mRNA expression by endothelial cells (Figure 2A). We next checked the ability of MV_{stress} to activate inflammasome using LPS-primed THP-1 derived macrophages as model system, because a role of mitochondrial DAMPs (eg, ATP) in inflammasome activation has been reported.[12,16] However, in contrast to ATP, MV_{stress} did not induce inflammasome-dependent IL-1β release (Online Figure IIA and IIB). Microvesicle-associated IL-1β has been reported to activate endothelial cells.[5] We tested the contribution of MV_{stress}-associated IL-1β to endothelial cell activation and observed that blocking of IL-1 receptor on endothelial cells only partially reduced the ability of MV_{Stress} to induce IL-8 production (Online Figure IIC through IIE).

LPS stimulation has been shown to alter mitochondrial activity.[11] Therefore, we hypothesized that this defines the proinflammatory potential of MV_{Stress}. First, we generated

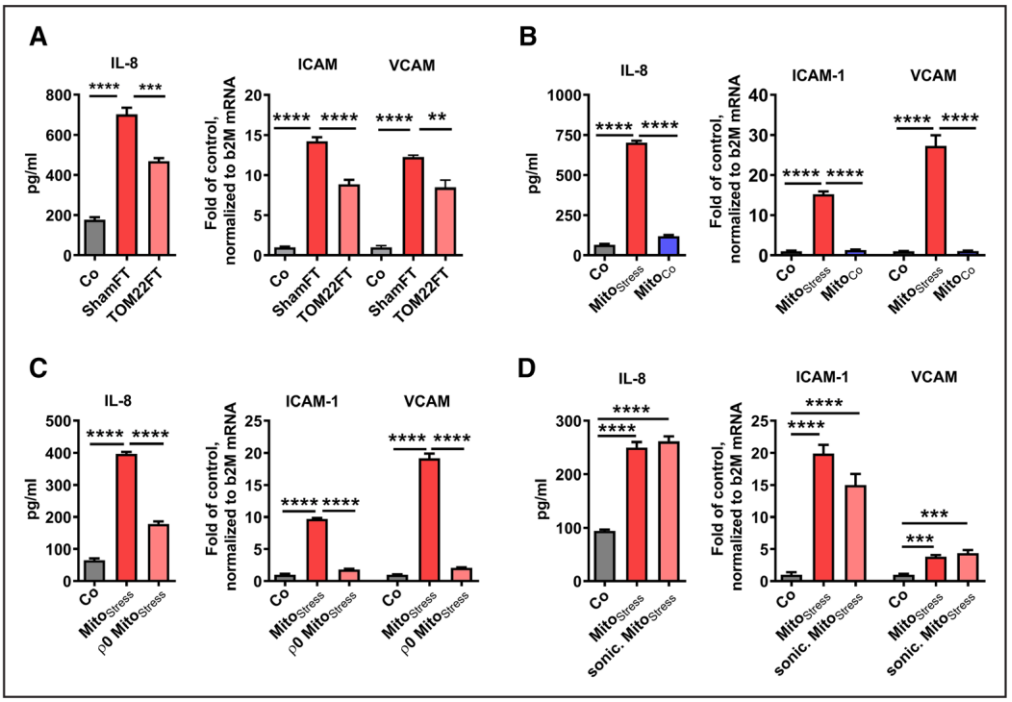

Figure 3. Mitochondria released by or isolated from LPS (lipopolysaccharide)-activated THP-1 monocytic cells activate human umbilical vein endothelial cells (HUVECs). Secretion of IL (interleukin)-8 protein (8 h) and expression of ICAM-1 (intercellular adhesion molecule) and VCAM (vascular cell adhesion molecule) mRNA (6 h) in stimulated endothelial cells HUVEC. **A**, Stimulation with sham-depleted MV_{Stress} (ShamFT) or TOM22+ (translocase of the outer membrane 22) subset depleted MV_{Stress} (TOM22FT). MV_{Stress} were subjected to immune-affinity based depletion by TOM22 antibody-coupled magnetic beads. Stimulation with (**B**) mitochondria isolated from LPS- ($Mito_{Stress}$) or vehicle-treated ($Mito_{Co}$) THP-1 monocytic cells; (**C**) $Mito_{Stress}$ isolated from THP-1 monocytic cells or ρ0 THP-1 monocytic cells; and (**D**) sonicated or nonsonicated $Mito_{Stress}$. Co=unstimulated; Data shown as mean±SEM, n=4.

THP-1 monocytic cells (ρ0 cells) with impaired oxidative phosphorylation by depleting mitochondrial DNA using extended low-dose Ethidium-Bromide treatment (Online Figure IIIB). LPS-stimulation of ρ0THP-1 monocytic cells resulted in the release of similar numbers of microvesicles (Online Figure IIIC). However, ρ0 cell-derived MV_{Stress} displayed a dramatically reduced ability to induce IL-8 protein, as well as ICAM-1 and VCAM mRNA expression in endothelial cells compared with equivalent numbers of MV_{Stress} derived from intact cells (Figure 2B). Second, we stimulated THP-1 monocytic cells with LPS in the presence of pyruvate, which has been reported to preserve mitochondrial membrane potential.[17] Pyruvate treatment did not affect the number of microvesicles released by THP-1 monocytic cells after LPS stimulation (Online Figure IIID). However, MV_{Stress} derived from pyruvate-treated cells displayed a reduced capacity to induce proinflammatory responses in endothelial cells (Figure 2C). Thus, mitochondrial activity of parental cells contributes to the proinflammatory activity of MV_{Stress} released by them. Because of the critical role of mitochondrial reactive oxygen species (ROS) in innate immune responses,[12] we tested if the treatment of parental cells with the mitochondria-specific ROS scavenger MitoTEMPO could affect the proinflammatory capacity of MV_{Stress}. LPS stimulation of THP-1 monocytic cells in presence of MitoTEMPO resulted in the release of similar quantities of microvesicles (Online Figure IIE) but with significantly reduced proinflammatory potential (Figure 2D). Moreover, pyruvate supplementation and mitoTEMPO both reduced LPS-induced mitochondrial ROS production in THP-1 cells (Online Figure IIIF and IIIG). These data indicate that LPS-induced changes in mitochondrial activity of parental cells determine specific proinflammatory constituents of MV_{Stress} released by them.

Mitochondria Released by or Isolated From LPS-Activated THP-1 Monocytic Cells Activate Endothelial Cells

To evaluate the contribution of free mitochondria within isolated MV_{Stress} to activate endothelial cells, we depleted MV_{Stress} of TOM22+ vesicles (free mitochondria) using the antibody specific for mitochondrial protein TOM22. This led to complete reduction of TOM22+ vesicles and significant decrease in mitochondrial 16S rRNA within the depleted MV_{Stress} fraction (Online Figure IVA and IVB). MV_{Stress} depleted of the TOM22+ vesicles (free mitochondria) displayed a significantly reduced ability to induce IL-8, ICAM-1, and VCAM mRNA in endothelial cells compared with sham-depleted MV_{Stress} (Figure 3A). Thus free mitochondria significantly contribute to the proinflammatory activity of MV_{Stress}. The remaining proinflammatory activity of the depleted MV_{stress} fraction can be explained by the presence of microvesicle-encapsulated mitochondria. Indeed, MV_{Stress} depleted of TOM22+ vesicles were still significantly enriched for 16S rRNA as compared with parental cells (Online Figure IVC). Moreover, these depleted MV_{stress} were still positive for the mitochondria-specific dye inherited from parental cells (Online Figure IVD).

To test whether LPS-induced cellular activation alters the proinflammatory capacity of mitochondria, we directly compared the ability of mitochondria isolated from LPS-activated

($Mito_{Stress}$) and vehicle-stimulated cells ($Mito_{Co}$) to activate endothelial cells. Similar to MV_{Stress}, only $Mito_{Stress}$ but not $Mito_{Co}$ were able to induce IL-8 production, ICAM-1, and VCAM mRNA expression by endothelial cells (Figure 3B). Moreover, $Mito_{Stress}$ isolated from ρ0 THP-1 monocytic cells had greatly reduced proinflammatory potential (Figure 3C). Importantly, disintegration of $Mito_{Stress}$ sonication (Online Figure IVE) did not change their ability to induce IL-8 production, ICAM-1/VCAM mRNA in endothelial cells (Figure 3D). These results further support that the presence of proinflammatory constituents in extracellular mitochondria and mitochondria-containing microvesicles is influenced by activation of the parental cells and predetermined before their release.

Isolated Mitochondria and Microvesicles From LPS-Activated THP-1 Monocytic Cells Induce Type I IFN and TNF Signaling Pathways in Human Umbilical Vein Endothelial Cells

To obtain insights into proinflammatory constituents of $Mito_{stress}$, we performed transcriptomic profiling of endothelial cells stimulated with $Mito_{Stress}$ and $Mito_{Co}$ using RNA-sequencing. Kyoto Encyclopedia of Genes and Genomes pathway and gene ontology analysis of the transcripts differentially regulated by $Mito_{Stress}$ compared with $Mito_{Co}$ identified type I IFN and TNF (tumor necrosis factor) signaling as the pathways with the highest enrichment (Figure 4A). From the 144 differentially expressed genes, we selected 11 genes previously shown to be regulated either by one or both of the identified pathways. These include OAS2 (2'-5'-oligoadenylate synthetase 2), MX1 (interferon-induced GTP-binding protein Mx1), IFIT1 (interferon-induced protein with tetratricopeptide repeats 1), RSAD2 (radical S-adenosyl methionine domain-containing protein 2) (type I IFN pathway),[18] CCL2 (C-C motif chemokine 2), IL-8, VCAM, ICAM-1 (TNF pathway),[5] and CXCL10 (C-X-C motif chemokine 10), CXCL11 (C-X-C motif chemokine 11; TNF and type I IFN).[19] Their induction by $Mito_{Stress}$ was confirmed by reverse transcription-polymerase chain reaction (Figure 4B, Online Figure VA). To elucidate the role of IFN and TNF signaling in the expression of selected genes, we stimulated endothelial cells with $Mito_{Stress}$ either in presence of a type I IFN decoy receptor (B18R) or a TNFα-blocking antibody (Infliximab). Induction of IFN-dependent genes by $Mito_{Stress}$ was reduced in the presence of B18R (Figure 4C, Online Figure VB), while TNFα-blocking reduced induction of TNF-dependent genes (Figure 4C, Online Figure VC). Induction of CXCL10 and CXCL11 mRNA was only affected by blocking of TNFα (Figure 4C, Online Figure VB and VC), although both TNF and type I IFN pathways have been implicated in expression of these genes.[19] These data point to a primary role of TNFα in immediate activation of endothelial cells by $Mito_{Stress}$. Notably, TNFα was already present in the supernatant of endothelial cells immediately after addition of $Mito_{Stress}$ but did not further increase over time (Online Figure VD). In contrast, production of IL-8 by endothelial cells was significantly increased only 4 hours after stimulation (Online Figure VD). This suggested that TNFα is already associated with $Mito_{Stress}$ rather than produced by $Mito_{Stress}$ stimulated cells. Preincubation of $Mito_{Stress}$ with TNFα-blocking antibody was sufficient to inhibit the induction of IL-8 protein, ICAM-1, and VCAM mRNA in stimulated endothelial cells, even by removing of unbound antibodies by washing and centrifugation (Online Figure VE). Notably, TNFα protein was only detectable in $Mito_{Stress}$ and MV_{Stress} preparation but not $Mito_{Co}$ or MV_{Co} (Online Figure VF). To identify the interferonogenic component, we tested the ability of mitochondrial RNA and DNA isolated from $Mito_{Stress}$ to induce type I IFN response. $Mito_{Stress}$ RNA, but not $Mito_{Stress}$ DNA was able to induce expression of IFN-dependent genes in endothelial cells (Online Figure VIA). Moreover, neither $Mito_{Co}$ RNA nor total cellular RNA from LPS-activated THP-1 ($THP\text{-}1_{Stress}$) showed this effect (Online Figure VIB and VIC). Furthermore, we tested whether elevated mitochondrial ROS generation in LPS-activated THP-1 cells (Online Figure IIIF and IIIG) leads to increased oxidative modification of mitochondrial RNA. To assess this, we performed an immunoprecipitation-quantitative reverse transcription-polymerase chain reaction assay of $Mito_{Co}$ and $Mito_{Stress}$ RNA using an antibody against 8-hydryoxyguanine (8-oxoG)[20] (Online Figure VID). We found significantly increased levels of 8-oxoG in mitochondrial 12S and 16S RNAs isolated from $Mito_{Stress}$ compared with $Mito_{Co}$ (Online Figure VID).

Next, we tested if mitochondrial activity affects the capacity of $Mito_{Stress}$ to induce type I IFN and TNF signaling. $Mito_{Stress}$ isolated from ρ0 THP-1 monocytic cells or THP-1 monocytic cells pretreated with pyruvate or MitoTEMPO had a reduced potential to induce the expression of IFN-signaling– and TNF-signaling–dependent genes in endothelial cells (Figure 4D, Online Figure VIIA through VIIC).

To confirm that MV_{Stress} also induce IFN and TNF signaling, we analyzed the expression of the same set of genes in microvesicle-stimulated endothelial cells. Stimulation with MV_{Stress} resulted in a similar gene expression profile as incubation with $Mito_{Stress}$ (Figure 4E, Online Figure VIIIA). Preparations of MV_{Stress}, which were immunodepleted of free mitochondria, had a significantly decreased potential to activate IFN- and TNF-dependent genes compared with sham-treated MV_{Stress} (Figure 4F, Online Figure VIIIB). Moreover, Infliximab and B18R treatment also significantly reduced the remaining capacity of depleted MV_{Stress} to induce type I IFN and TNF responses in endothelial cells (Online Figure IXA and IXB). This indicates that the microvesicle-encapsulated mitochondria trigger the same inflammatory pathways as free mitochondria released by activated THP-1 monocytic cells.

To investigate whether uptake of mitochondrial content by endothelial cells is required for induction of type I IFN and TNF responses, we stimulated endothelial cells with intact and MV_{Stress} disintegrated by sonication (Online Figure XA). Uptake of mitotracker-positive MV_{Stress} by endothelial cells was dramatically reduced when these microvesicles had been disintegrated by sonication (Online Figure XB and XC). The induction of IFN-dependent genes, but not TNF-dependent genes, was significantly reduced when human umbilical vein endothelial cells were stimulated with disintegrated MV_{Stress} compared with intact MV_{Stress} (Online Figure XD). Thus, uptake of MV_{Stress} is a prerequisite for the induction of IFN-dependent genes.

To test the in vivo relevance of our findings, we performed a low-grade endotoxemia model in humans. Fifteen healthy volunteers received 2 ng/kg LPS intravenously. Blood was

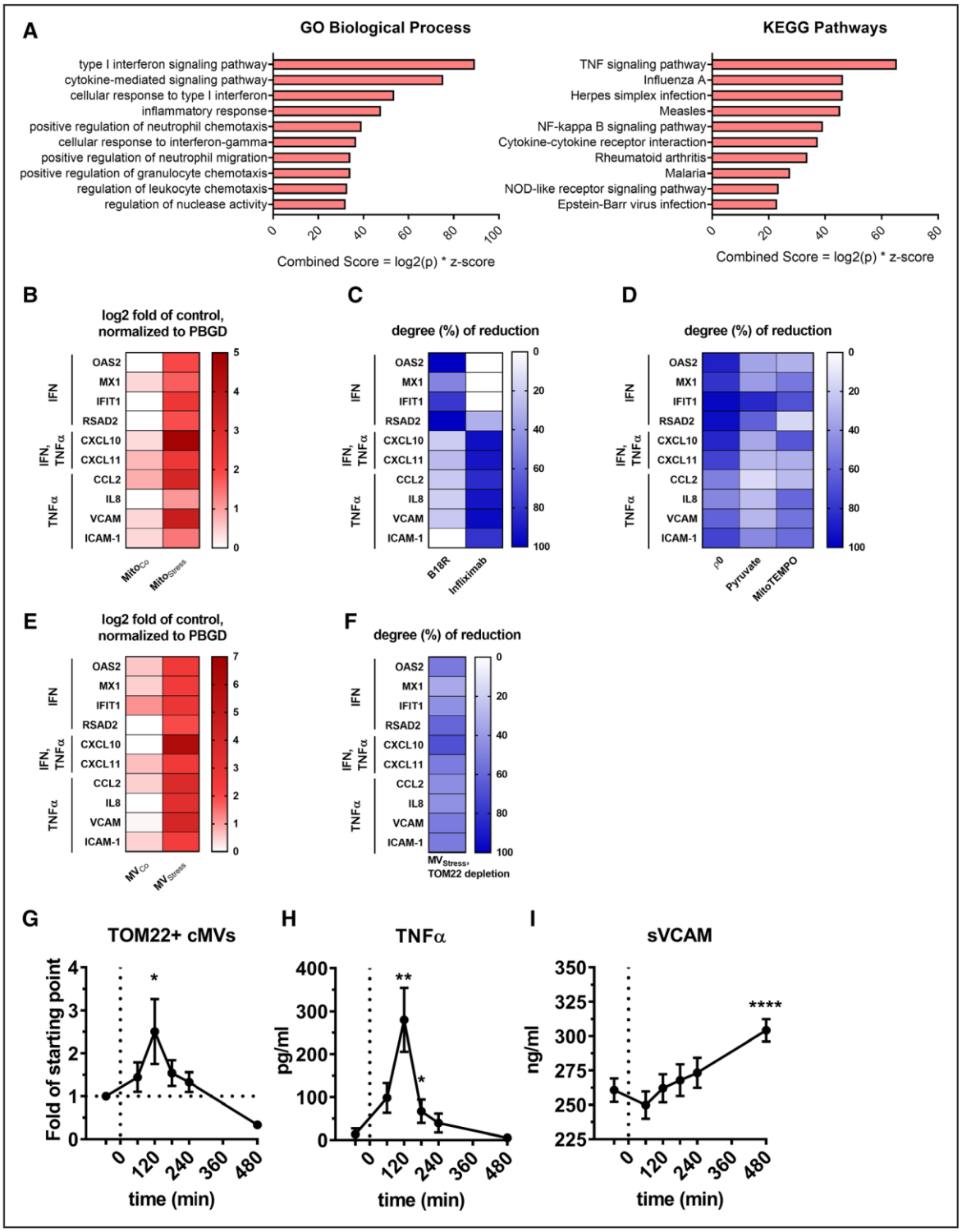

Figure 4. Isolated mitochondria and microvesicles from LPS (lipopolysaccharide)-activated THP-1 monocytic cells induce Type I IFN (interferon) and TNF (tumor necrosis factor) signaling pathways in human umbilical vein endothelial cells (HUVECs). **A**, HUVECs were stimulated for 6 h with $Mito_{Stress}$ or $Mito_{Co}$ isolated from THP-1 monocytic cells and differentially expressed genes were identified by RNAseq analysis. Bar graphs show 10 most enriched pathways based on analysis of gene ontology (GO) biological processes and Kyoto Encyclopedia of Genes and Genomes (KEGG). **B**–**F**, Analysis of mRNA expression of selected genes in stimulated HUVECs by quantitative reverse transcription-polymerase chain reaction. Detailed results of individual mRNA level comparisons presented in online Figures V, VII, and VIII. **B**, Fold induction of mRNA expression in cells stimulated with $Mito_{Co}$ and $Mito_{Stress}$ as compared with untreated cells. **C**–**D**, Percentage of reduction of mRNA expression in cells stimulated with (**C**) $Mito_{Stress}$ in the presence of B18R or infliximab, (**D**) $Mito_{Stress}$ obtained from ρ0 THP-1 monocytic cells/pyruvate-treated THP-1 monocytic cells, and MitoTEMPO-treated THP-1 monocytic cells. mRNA levels induced by stimulation of cells with $Mito_{Stress}$ were set as 100%. **E**, Fold induction of mRNA expression in cells stimulated with Co-microvesicle and MV_{Stress} as compared with untreated cells. **F**, Percentage of reduction of mRNA expression in cells stimulated with sham-depleted MV_{Stress} (ShamFT) or TOM22+ (translocase of the outer membrane 22) subset depleted MV_{Stress} (TOM22FT). mRNA levels induced by stimulation of cells with sham-depleted MV_{Stress} were set as 100%. MV_{Stress} were preincubated with magnetic beads-coupled with anti-TOM22 antibodies and passed through paramagnetic columns to obtain (*Continued*)

collected before and at indicated time-points after the injection. LPS injection resulted in increased levels of TOM22+ vesicles, which peaked 2-hour post injection (Figure 4G). Interestingly, the induction of TNFα in plasma coincided with the occurrence of TOM22+ vesicles (Figure 4H), while the concentration of sVCAM-1 (soluble vascular cell adhesion molecule-1), reflecting endothelial cell activation, was increased only 8 hours after LPS-injection (Figure 4I). Stimulation of human umbilical vein endothelial cells with circulating microvesicles, isolated from plasma obtained after LPS-injection, induced the expression of TNF and IFN-dependent genes, consistent with our findings obtained with in vitro generated MV_{Stress} (Online Figure XI).

Discussion

The activation of vascular endothelium is a hallmark of chronic and acute inflammation, and the intercellular communication between monocytes and endothelial cells represents a critical aspect of this process.[21,22] Extracellular vesicles are increasingly recognized as important mediators of this process.[1,5] Interestingly, the release of microvesicles carrying mitochondrial content and even mitochondria by several cell types after activation has been shown.[6–8] This is important as mitochondria-derived DAMPs are potent inducers of inflammation.[9,10,23] We here demonstrate that activation of monocytic cells by LPS triggers the release of microvesicles enriched in mitochondrial content and free mitochondria that have the capacity to activate endothelial cells. The proinflammatory capacity of the microvesicles released by monocytic cells was significantly reduced after immunodepletion of free mitochondria. Thus, proinflammatory constituents of free mitochondria released by activated cells are important mediators of proinflammatory communication between monocytic and endothelial cells. Moreover, the demonstration of increased levels of TOM22+ vesicles (free mitochondria) in the circulation of humans receiving low-dose LPS-injections, supports our in vitro findings and suggests a role for the release of free mitochondria during inflammation in vivo.

Mitochondria are also recognized for their role in inflammasome assembly and IL-1β production.[24] Monocytic microvesicles have been demonstrated to carry inflammasome components and IL-1β.[5] We found that MV_{Stress} did not induce IL-1β production in primed macrophages, while they had the capacity to induce IL-8 production in both primed and nonprimed macrophages. Thus, MV_{Stress} do not present a secondary stimulus of inflammasome activation at these concentrations. Moreover, the ability of MV_{Stress} to activated endothelial cells was only partially blocked by neutralizing microvesicle-associated IL-1β. This suggested a significant contribution of other components to the biological activity of MV_{Stress}.

Several types of DAMPs of mitochondrial origin, such as FMLP, nucleic acids, ATP, and cardiolipin, have been identified.[10] Nucleic acids, oxidized nucleic acids, in particular, have been shown to induce IFN production.[9] Consistent with this, we also identified that stressed mitochondria, but not mitochondria from nonstressed cells, induce type I IFN signaling in endothelial cells. Here, we identified RNA, but not DNA, as the major interferonogenic component of $Mito_{Stress}$. Moreover, our data showing high interferonogenic potential of $Mito_{Stress}$ RNA as compared with total cellular RNA from LPS-activated THP-1 cells points to the major role of mitochondrial RNA in induction of type I IFN genes by $Mito_{Stress}$. Importantly, our results demonstrate that RNA isolated from $Mito_{Stress}$, but not $Mito_{Co}$, induce a type I IFN response, which suggests that modification of RNA in mitochondria of parental during LPS-induced stress may promote the interferonogenic potential of released mitochondria and microvesicles. Thus, the interferonogenic potential of extracellular mitochondria is critically determined by alterations of mitochondrial components during cell activation before their release. Unexpectedly, we also found that $Mito_{Stress}$, but not $Mito_{Co}$, induce TNF-dependent signaling in endothelial cells, in particular, the expression of adhesion molecules and IL-8. Indeed, we could demonstrate that TNFα is directly associated with $Mito_{Stress}$. Thus, next to the classical mitochondrial DAMPs such as nucleic acids, TNFα itself represents a major proinflammatory trigger associated with $Mito_{Stress}$. Thus, the association of TNFα with $Mito_{Stress}$ may preferentially target interferonogenic content to cells expressing high levels of TNF receptors. Altogether, we show that mitochondria isolated or released from activated monocytes are manifold stronger inducers of specific inflammatory responses as compared with nonstressed mitochondria.

Importantly, depletion of TOM22+ vesicles (free mitochondria) significantly reduced the capacity of MV_{Stress} to induce TNF and type I IFN responses in endothelial cells. Thus, extracellular mitochondria are important contributors to the proinflammatory activity of extracellular vesicles released from activated monocytic cells. Of note, MV_{Stress} depleted of free mitochondria (TOM22+ vesicles) were still enriched in mitochondrial content and had the capacity to induce TNF and type I IFN responses, which was specifically inhibited by targeting either TNF or IFN sensing. Thus, free mitochondria and mitochondria embedded within vesicles induce the same inflammatory pathways in endothelial cells. Moreover, we show that LPS-administration to humans results in an increase of TOM22+ vesicles in the circulation, which coincides with elevated levels of TNFα. Notably, circulating microvesicles isolated from these volunteers after LPS-administration were able to induce TNF and type I IFN responses in endothelial cells.

In addition to their central role in cellular metabolism, mitochondria and mitochondria generated ROS are recognized as regulators of immune responses.[25] In particular, the degree of oxidative phosphorylation is known to define the proinflammatory/anti-inflammatory status of monocytes.[26] Our data demonstrate that the capacity of mitochondria from LPS-stimulated monocytic cells to activate endothelial cells was drastically reduced when mitochondria were derived from nonrespiring cells. Similarly, mitochondria from cells treated with inhibitors of mitochondrial stress or mitochondrial ROS generation had a reduced proinflammatory

Figure 4 Continued. TOM22-depleted vesicles (TOM22-FT). MV_{Stress} were passed through paramagnetic columns without preincubation with antibodies to obtain sham-depleted microvesicles (ShamFT). **G–I**, Characterization of the plasma from human low-grade endotoxinemia study (n=15). **G**, Fold increase over the time after LPS injection of the numbers of TOM22 positive vesicles assessed by flow cytometry. Level of TNFα (**H**), and soluble VCAM (**I**) after LPS injection assessed by ELISA. Data shown as mean±SEM. IL indicates interleukin.

potential. Increased mitochondrial ROS production induced by LPS may result in the oxidative modification of mitochondrial lipids (eg, Cardiolipin) and nucleic acids, and thereby enhance their proinflammatory activity.[8,9,27–30] Our recent finding that lipid peroxidation-derived structures, such as malondialdehyde (MDA)-adducts, mark a subset of microvesicles supports a role for oxidative stress in microvesicle biology.[31] Our new data point to an important role of the oxidation of mitochondrial RNA in enhancing the proinflammatory potential of mitochondria and microvesicle-encapsulated mitochondria. Indeed, we show increased oxidative modification of mitochondrial 12S and 16S RNA isolated from $Mito_{Stress}$ as compared with $Mito_{Co}$. These data further support the major contribution of modified $Mito_{Stress}$ RNA to the induction of Type I IFN response in endothelial cells. We show that the biological activity of MV_{Stress} is predefined by the activation status of their parental cells. Thus, MV_{Stress} have the ability to communicate cellular stress between microvesicle-shedding and microvesicle-sensing cells.

Importantly, disintegration of MV_{Stress} dramatically reduced their uptake by endothelial cells and their ability to induce type I IFN-responses. These data, together with the identification of mitochondrial RNA as interferonogenic component of $Mito_{Stress}$, suggests that uptake of vesicle-embedded RNA by endothelial cells is required to induce type I IFN-responses. This is in agreement with the cytoplasmic sensing of nucleic acids.[32] In contrast, disintegration of MV_{Stress} did not change their capacity to induce a TNF-response in endothelial cells, which is consistent with the surface expression of TNF-receptors.

In summary, our work provides new insights to the contribution of mitochondria to the content and biological activity of extracellular vesicles. Mitochondrial stress, mitochondrial DAMPs, TNF, and IFN signaling have been demonstrated to play important roles in acute and chronic inflammation, such as trauma, cardiovascular diseases, rheumatoid arthritis, neurodegeneration, and psoriasis.[23,25,33–37] Our data show that free mitochondria and microvesicle-encapsulated mitochondria released by activated monocytic cells serve as a carrier of both TNFα and interferonogenic mitochondrial RNA, with the capacity to trigger the simultaneous induction of 2 critical pathways of inflammation in target cells. Moreover, we demonstrate that the biological activity of released mitochondria reflects the activation status of their parental cells. Thus, approaches targeting mitochondria and their release may represent novel points for therapeutic intervention in several pathologies.

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Disclosures

None.

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3. Material and Methods

The following material and methods sections consists of the online material / supplemental material, published in Circulation Research (DOI: 10.1161/CIRCRESAHA.118.314601), that I wrote for the manuscript with the title „Mitochondria are a subset of extracellular vesicles released by activated monocytes and induce Type I IFN and TNF responses in endothelial cells“.

SUPPLEMENTAL MATERIAL

Data Disclosure Statement

All data, analytic methods and study materials supporting the findings of this study are provided in the manuscript, supplemental material and available from the corresponding author upon reasonable request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Cell lines

THP-1 monocytic cells were maintained in RPMI Media (Invitrogen) supplemented with 10 % FBS. EBM-2 complete media (Lonza) containing 2% FBS was used for cultivation of human umbilical vein cells (HUVECs). HUVECs were used up to the 5th passage. All cells were incubated in a humidity-controlled environment at 37°C, 5% CO2 (*Thermo Scientific Heraeus Cytoperm* 2) THP-1 monocytic cells and HUVEC were purchased from ATCC and Lonza, respectively.

Human Endotoxemia Model

This study was approved by the Ethical Committee of the Medical University of Vienna and complies with the Declaration of Helsinki. Informed consent was obtained from all participants before the beginning of the study. Blood samples were obtained from 15 healthy male volunteers (mean ± SD age, 28.5 years ± 4.5 years, mean ± SD body weight, 78.9 ± 6.4 kg, mean ± SD body mass index, 23.5 ± 2 kg/m^2) 50 min before and 2h, 3h, 4h and 8h after bolus infusion of LPS injection (2 ng/kg; CCRE lot from NIH). See also [1].

METHOD DETAILS

Cell culture, ELISA, qPCR and flow cytometry of cells.

THP-1 cells were maintained in RPMI Media (Invitrogen) supplemented with 10 % FBS. EBM-2 complete media containing 2% FBS was used for cultivation of HUVECs. HUVECs were used up to the 5th passage. For stimulation experiments 100*10^3 cells were seeded per well in 96-well plates (Nunclon Delta Surface, Thermo Fisher Scientific). To obtain THP-1-derived

macrophages, cells were stimulated for 3 hours with 100 nM phorbol-12-myristate-13-acetate (PMA) and experiments were performed after 48 hours. Before stimulations with MVs, culture media was changed to FBS-free media supplemented with 200 µg/ml BSA, unless indicated otherwise in the figure legends. Cells were stimulated for time-points indicated in figure legends and the conditioned media was centrifuged for 5 min at 400g at the end of the stimulation. Cell-free supernatants were collected for ELISA measurements of IL-8 and IL-1β using commercial ELISA kits (Becton Dickinson and RD Systems, respectively) according to the manufacturer's instructions. Levels of TNFα in human plasma, MVs and mitochondria suspensions were measured by using commercial ELISA kit (RD Systems). RNA was isolated from cells and MVs by using peqGOLD Total RNA kit (VWR International). Afterwards, cDNA synthesis was performed using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). The CFX96 system (Bio-Rad Laboratories) and KAPA SYBR® FAST qPCR Kit (Kapa Biosystems) were used for real-time PCR-based quantifications. Expression of mRNAs of interest was analyzed using the deltaCt method and normalized to b2M or PBGD mRNA level, as indicated in the figure legends. Level of 16S and 18S ribosomal RNAs was represented as fold of control or the level of 16S RNA was normalized to 18S RNA level. Flow-cytometric analysis of cells was performed with two different flow cytometers (BD FACScalibur and BD LSRFortessa II). Acquisition was stopped when 10,000 events were acquired.

Labelling of cells with Mito- and Cytotracker

In selected experiments, THP-1 cells were labeled with cytoplasm- and mitochondria-specific dyes (Cell Trace CFSE 1µM and Mitotracker Deep Red 100nM, Invitrogen) for 30 min at 37°C in protein-free media. Afterwards, cells were recovered in complete media for 1 hour before stimulations.

Generation and Isolation of MVs

For isolation of microvesicles *in vitro*, THP-1 cells were set in fresh media (10% FBS, P/S) at a density of $1*10^6$ cells per ml. After 16 hours stimulation with vehicle or LPS (500ng/ml), conditioned media was harvested and centrifuged at 400g for 5min to pellet non-adherent cells. The supernatant was centrifuged at 1,500g for 10min to pellet cellular debris. Following this step, the supernatant was centrifuged at 18,000g for 30min to pellet microvesicles, which were then taken for FACS analysis and functional studies. To study circulating microvesicles, blood samples were collected into Sodium-Citrate containing collection tubes and spun twice at room temperature for 10min at 2,000g (with transfer of supernatant between centrifugation steps) to obtain platelet-free plasma. The supernatants contained microvesicles and were transferred to fresh micro centrifuge tubes.

Depletion of free mitochondria from isolated MVs

Free mitochondria were depleted from MV_{stress} with anti-TOM22 microbeads kit (Miltenyi Biotec). MV suspensions were preincubated for 1 hour with or without anti-TOM22 antibody coupled magnetic beads. Thereafter samples were applied onto magnetic columns. Flow through and column retained MVs were characterized by Flow cytometry and RT-PCR (Online Figure IV).

Analysis of MVs and isolated mitochondria

Measurement of MVs was done by BD FACScalibur and BD LSRFortessa II. To calibrate the flow cytometer for detection of MVs and isolated mitochondria, Megamix-Plus SSC beads (0.16 µm, 0.20 µm, 0.24 µm and 0.5 µm) and latex beads (1.1µm) were measured (Online Figure I). The detection gate was limited by 0.24µm and 1.1µm beads as detected by SSC and FSC as this gating strategy enabled reproducible quantification when measuring samples with two different flow cytometers (BD FACScalibur and BD LSRFortessa II). For detection of MVs and isolated mitochondria with the BD FACScalibur the following settings were used: FSC (Voltage: E02; Amplifier Gain: 1.0; Mode: log), SSC (Voltage: 335; Amplifier Gain: 1.00; Mode: log), primary parameter: SSC. For detection of MVs and isolated mitochondria with the BD LSRFortessa II the following settings were used: FSC-H (Voltage: 500), SSC-H (Voltage: 700), primary parameter: SSC. For both flow cytometers the SSC-threshold was set to exclude 0.16µm and 0.2µm mega mix beads to reduce the influence of background/electronic noise. Samples were acquired for 30 sec at low-speed (12µl/min) and the sample dilution was adjusted to keep the acquisition rate between 250 and 5,000 events/sec. Quantification of MVs was performed by 3 different methods: staining with primary antibody and subsequent Annexin-V or Calcein-AM staining; pre-labeling of cells with cytotracker/mitotracker and detection of released, labeled material; direct staining of cMVs by anti-TOM22-FITC antibody. MVs released by or mitochondria isolated from unlabeled cells were acquired to define unstained populations or non-specific staining by using isotype controls. Additionally, control measurements were also performed with MV-free/depleted material, such as 0.2µm filtered buffer (PBS or 10mM HEPES with 2.5mM CaCl2), or MV-depleted media (cell culture media obtained after 18,000g, 30min centrifugation). For quantification of MVs and isolated mitochondria, serial dilutions of the samples were prepared and measured to identify the appropriate dilutions within the linear range.

Probing of MVs released by cytotracker/mitotracker-labeled cells

Cells were pre-labeled and stimulated as described above. Conditioned media was centrifuged at 400g for 5min, supernatant transferred to fresh tubes and subsequently centrifuged at 1,500g for 10min. The supernatant of the final centrifugation was directly analyzed by using BD LSRFortessa II to detect labeled MVs.

Probing of MVs by Annexin-V or Calcein-AM

MV pellets were resuspended in staining buffer (10mM HEPES, 2.5mM CaCl2, filtered with 0.2µm filters) containing 5µM Calcein-AM (Thermo Fisher Scientific) or Annexin-V PE (BioLegend) and incubated for 20 min at room-temperature, protected from light. Specificity of Annexin-V binding to MVs was tested by staining of MVs in presence or absence of a calcium-chelator (2.5mM EDTA), as Annexin-V requires calcium to bind to phosphatidylserine. Analysis of MVs was performed using BD FACScalibur or BD LSRFortessa II.

Probing of cMVs by direct labeling with anti-TOM22-FITC antibody

10µl of plasma were labeled with TOM22-FITC antibody (clone 1C9-2) for 20 min at 4°C. Analysis of MVs was performed using BD LSRFortessa II.

Analysis of Extracellular Vesicles by NTA

Concentration and size distribution of particles in samples were measured with Nanoparticle Tracking Analysis (NTA) (LM10; Nanosight, Amesbury, UK). Silica beads (200-nm diameter; Microspheres-Nanospheres**,** Cold Spring, NY) were used for instrument calibration. Samples were diluted 10- to 20- fold in PBS. For each sample 9x30 second videos were taken. The same detection threshold was set to measure different samples. Data were analyzed using the Nanosight Tracking Analysis software (NTA 2.3).

Sonication

MVs or isolated mitochondria were resuspended and diluted in EBM-2 media (Lonza) to the desired concentrations. Sonication of samples was performed using a Sonopuls Instrument (Bandelin) at 100% power and 6 30-second sonication rounds with 1 min breaks in between. Efficiency of MVs and mitochondria disintegration was analyzed by FACS measurements using Calcein-AM staining (See above). Calcein leaks out of vesicles if membrane integrity is lost. The sonicated samples were used for the stimulation of HUVECs, as described above.

Mitochondria isolation

Mitochondria were isolated from the THP-1 cells after 16h of stimulation either with vehicle or LPS using Mitochondria Isolation Kit (Thermo Fisher Scientific) according to the manufacturer's protocol. The purity of mitochondrial preparation was assessed by Western blot (Figure 1E). Serial dilution of mitochondria preparations were counted by flow cytometry, as described above.

Generation and characterization of mitochondrial DNA-depleted (ρ0) cells

THP-1 monocytes were grown in RPMI medium supplemented with 10% FBS, uridine (50 µg/ml), pyruvate (100 µg/ml) and EtBr (100 ng/ml) for 2 to 3 weeks [2]. Loss of oxidative phosphorylation was documented by measuring cell oxygen consumption rates using the XF 24 Flux Analyzer (Seahorse Bioscience). THP-1 cell ($100x10^3$) were seeded on CellTec pretreated XF 24-well cell culture microplates. Cells were washed after a 4-hour recovery period and a final volume of 630 µl buffer-free Assay Medium (Seahorse Bioscience), supplemented with 5 mM glucose (Sigma) and 1 mM sodium pyruvate (Gibco) was added to each well. Cells were then kept in a CO2-free incubator at 37°C for 1 hour. After instrument calibration, cells were transferred to the XF 24 Flux Analyzer to record cellular oxygen consumption rates. Measurements were performed with repetitive cycles of 2 min mixture, 2 min wait and 4 min OCR measurement times. For the mitochondrial stress test, the following compounds were injected: Oligomycin (2 µM working concentration) to inhibit ATP synthase, then FCCP (1 µM working concentration) to induce mitochondrial uncoupling and rotenone/antimycin A (2 µM working concentration each) to block the mitochondrial respiratory chain.

Measurement of reactive-oxygen species (ROS)

THP-1 cells were seeded in fresh RPMI medium supplemented with 10% FBS at a density of 1*10^6 per well. After pre-stimulation for 60min with vehicle, mitoTEMPO (50µM) or pyruvate (1mM), cells were stimulated for 60min with vehicle or LPS (0.1µg/ml). Thereafter, the cell suspension was centrifuged for 3min at 300g and the cell pellet was washed once with warm PBS. After subsequent centrifugation (3min, 300g), the cell pellet was resuspended in PBS containing 1% BSA and mitoSOX (0.5µM) and incubated for 10min at 37°C protected from light. Afterwards, the cell suspension was centrifuged (3min, 300g), the supernatant removed and the cell pellet resuspended in PBS (1% BSA) and then analyzed by flow-cytometry.

Measurement of mitotracker-positive MV uptake by immunocytochemistry and flow-cytometry

Immunocytochemistry

HUVECs were seeded onto fibronectin-coated 12mm coverslips placed in 24-well plates. HUVECs were incubated with MVs for 60min at 37°C. Cells were washed twice with warm PBS and fixed with 4% PFA pH 7.4 in PBS for 15min at 37°C, quenched with 150mM Tris pH 8.0 for 5min and washed 3 times with PBS. Nuclei were stained by Hoechst (10mM T in PBS) for 3min. Coverslips were mounted on aqueous mounting media (Thermo, FA-030-FM), sealed and images were acquired on a Olympus IX71 microscope equipped with a coolLED pE4000 light source (Optoteam) and a Andor iXonLife EMCCD-camera with implemented ImageJ based post processing (Oxford instruments) at a 36x fold magnification. Mitotracker-positive MVs were detected in the RFP-channel (550nm) at 350ms exposure time (70% LED-power) and nuclei were detected at 385nm for 20ms (20% LED-power) in a multi-wavelength mode and at a constant gain of 200. Brightfield pictures were acquired separately at 200ms exposure.

Flow-cytometry

HUVECs were seeded on gelatin-coated 6-well plates. MVs were obtained from LPS-stimulated mitotracker-labeled THP-1 cells. Before incubation of HUVECs with MVs, culture media was removed and cells were washed with sterile PBS. MVs were suspended in FBS-free media supplemented with 200µg/ml BSA and added to HUVECs at different concentrations. HUVECs were incubated with MVs for 60min at 37°C or 4°C. Afterwards, cells were washed with warm PBS. Subsequently, cells were detached by trypsinization. Cells were then centrifuged (300xg, 3min). Finally, the cell pellets were resuspended in PBS (1%BSA) and analyzed by flow-cytometry.

Isolation of RNA and DNA for stimulation of HUVECs and assessment of mitochondrial RNA oxidation status.

DNA and RNA were extracted from $Mito_{Co}$, $Mito_{Stress}$, $THP\text{-}1_{co}$ and $THP\text{-}1_{stress}$ using the AllPrep DNA/RNA kit (Qiagen).

The Dynabeads Protein G Immunoprecipitation Kit (Invitrogen) was used for RNA immunoprecipitation. 0.1 µg of $Mito_{Co}$ or $Mito_{Stress}$ RNA were incubated with either anti-DNA/RNA damage antibody [15A3] (StressMarq), which recognizes 8-hydryoxyguanine (8-oxoG), or isotype control mouse IgG2b Antibody (BioLegend) bound to Dynabeads Protein G

(Invitrogen) for 2 hours at room temperature. After 3 washing steps antibody-bound RNA and input RNA was extracted using RNeasyMini Kit (Qiagen) and quantified by qRT-PCR as described above.

Embedding and ultrathin sections

MVs were fixed with 5% formaldehyde and 2% glutaraldehyde in HEPES buffer (HEPES 0.1M, 0.09 M sucrose, 10 mM CaCl2, 10 mM MgCl2, pH 6.9) for overnight at 7°C. After washing with HEPES buffer samples were further fixed with 1% osmiumtetroxide in HEPES for 1 h at room temperature. After washing with HEPES buffer samples were dehydrated with 10%, 30% and 50% acetone on ice before incubation in 70% acetone with 2% uranylacetate for overnight at 7°C. Samples were further dehydrated with 90% and 100% acetone on ice, allowed to reach room temperature and further dehydrated with 100% acetone. Subsequently, samples were infiltrated with the epoxy resin Low Viscosity resin (Agar Scientific, Stansted, UK) applying the hard mixture formula (LV resin 48 g, VH2 hardener 52 g, accelerator 2,5 ml). After polymerisation for 2 days at 75°C ultrathin sections were cut with a diamond knife, collected onto butvar-coated 300 mesh grids, and counterstained with 4% aqueous uranylacetate for 3 min and lead citrate for 15 sec. Samples were imaged in a Zeiss TEM 910 transmission electron microscope at an acceleration voltage of 80 kV and at calibrated magnifications. Images were recorded digitally at calibrated magnifications with a Slow-Scan CCD-Camera (ProScan, 1024x1024, Scheuring, Germany) with ITEM-Software (Olympus Soft Imaging Solutions, Münster, Germany). Contrast and brightness were adjusted with Adobe Photoshop CS5.

RNA-sequencing

NGS Library Preparation

RNA was isolated from HUVECs stimulated for 6h with either $Mito_{Co}$ or $Mito_{Stress}$ by using RNeasy Mini Kit (Qiagen). The amount of total RNA was quantified using the Qubit Fluorometric Quantitation system (Life Technologies) and the RNA integrity number (RIN) was determined using the Experion Automated Electrophoresis System (Bio-Rad). RNA-seq libraries were prepared with the TruSeq Stranded mRNA LT sample preparation kit (Illumina) using both, Sciclone and Zephyr liquid handling robotics (PerkinElmer). Library concentrations were quantified with the Qubit Fluorometric Quantitation system (Life Technologies) and the size distribution was assessed using the Experion Automated Electrophoresis System (Bio-Rad). For sequencing, samples were diluted and pooled into NGS libraries in equimolar amounts.

Sequencing and Raw Data Processing

Expression profiling libraries were sequenced on Illumina HiSeq 3000/4000 instruments in 50-base-pair-single-end mode and base calls provided by the Illumina Real-Time Analysis (RTA) software were subsequently converted into BAM format (Illumina2bam) before de-multiplexing (BamIndexDecoder) into individual, sample-specific BAM files via Illumina2bam tools (1.17.3 https://github.com/wtsi-npg/illumina2bam).

Transcriptome Analysis

[Tuxedo Suite]

Transcriptome analysis was performed with the Tuxedo suite. For each sample, NGS reads passing vendor quality filtering were aligned to the [hg38/mm10] reference genome assembly provided by the UCSC Genome Browser based on Genome Reference Consortium [GRCh38/GRCm38] with the TopHat2 (v2.1.1, http://genomebiology.com/2013/14/4/R36/abstract), a splice junction mapper utilising the Bowtie2 short read aligner (v2.2.9 http://www.nature.com/nmeth/journal/v9/n4/full/nmeth.1923.html). Thereby, "basic" Ensembl transcript annotation from version e87 (December 2016) served as reference transcriptome. Cufflinks (v2.1.1, http://www.nature.com/nbt/journal/v31/n1/full/nbt.2450.html) allowed for transcriptome assembly, customary including novel transcript structures, on the basis of the reference transcriptome and spliced read alignments, as well as raw transcript quantification. Before differential expression calling with Cuffdiff (included in Cufflinks v2.1.1, http://www.nature.com/nbt/journal/v28/n5/full/nbt.1621.html), transcriptome sets of each sample of each group to be compared were combined via the Cuffmerge algorithm. Finally, the cummeRbund (https://bioconductor.org/packages/release/bioc/html/cummeRbund.html), biomaRt (https://bioconductor.org/packages/release/bioc/html/biomaRt.html) and rtracklayer (https://bioconductor.org/packages/release/bioc/html/rtracklayer.html) Bioconductor packages were utilised in custom R scripts to perform quality assessment and further refine analysis results.

[STAR Aligner and DESeq2]

NGS reads were trimmed based on quality and adapter sequence content with Trimmomatic in single-end (ILLUMINACLIP:TruSeq3-SE.fa:2:30:10:1:true, SLIDINGWINDOW:4:15, MINLEN:20) or paired-end (ILLUMINACLIP:TruSeq3-PE-2.fa:2:30:10:1:true, SLIDINGWINDOW:4:15, MINLEN:20) mode. The resulting reads were aligned with the “Spliced Transcripts Alignment to a Reference” (STAR) aligner (https://www.ncbi.nlm.nih.gov/pubmed/23104886) to the [hg38/mm10] reference genome assembly provided by the UCSC Genome Browser resembling the Genome Reference

Consortium [GRCh38/GRCm38] assembly. Thereby, "basic" Ensembl transcript annotation from version e87 (December 2016) served as reference transcriptome. Reads overlapping transcript features were counted with the summarizeOverlaps function of the Bioconductor GenomicAlignments (https://bioconductor.org/packages/release/bioc/html/GenomicAlignments.html) package, taking into account that the Illumina TruSeq stranded mRNA protocol leads to sequencing of the second strand so that all reads needed inverting before counting. The Bioconductor DESeq2 (https://bioconductor.org/packages/release/bioc/html/DESeq2.html) library was then used to model the data set and call differentially expressed genes.

Enrichment Analysis

GO and KEGG pathway analysis were performed using EnrichR (http://amp.pharm.mssm.edu/Enrichr/).

Western blotting

MVs, mitochondria and cells were denatured in Laemmli buffer. Proteins were separated on 10 or 12% SDS-PAGE (Bis-Tris) and semidry-blotted onto nitrocellulose membrane (GE-Healthcare, # 10600003) in Towbin Buffer (0.025M Tris-HCl, 0.192M Glycine, 20% MeOH). Membranes were probed with antibodies against Bcl-2 (DAKO), GAPDH (Santa-Cruz), COXIV (Cell Signaling Technology) and ALIX (BIO-RAD). Horseradish peroxidase conjugated anti-mouse or anti-rabbit IgG (GE Healthcare) were used for detection of bound primary antibodies. Binding of secondary antibodies was detected by using AlphaImager System (ProteinSimple) and WesternBright Chemiluminescence Substrate Sirius (Biozym Scientific GmbH).

Quantification and Statistical analysis

Graph Pad Prism 8 for Windows (Graph Pad Software) software was used for statistical analyses. Student´s unpaired or paired t-test was used for comparing data from two groups. One-way ANOVA test with subsequent Bonferroni's multiple comparison tests were used for multiple group data analysis. Data were tested for normal distribution by Shapiro-Wilk test. Data that did not fit the assumption of normal distribution were compared using non-parametric analysis (Mann-Whitney) indicated in the respective figure legends. Data are presented as mean ± SEM. A *P* value of <0.05 was considered significant. ****P< 0.0001, ***P < 0.001, **P < 0.01, *P < 0.05

Data and software availability

The raw sequencing data have been deposited to the NCBI Gene Expression Omnibus (GEO). Accession number: GSE130225.
https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE130225

References

1. Matzneller P, Strommer S, Drucker C, Petroczi K, Schörgenhofer C, Lackner E, Jilma B, Zeitlinger M. Colistin Reduces LPS-Triggered Inflammation in a Human Sepsis Model In Vivo: A Randomized Controlled Trial. *Clin Pharmacol Ther*. 2017;101:773–781.
2. Hashiguchi K, Zhang-Akiyama QM. Establishment of human cell lines lacking mitochondrial DNA. *Methods Mol Biol*. 2009;554:383–391.

Resources Table

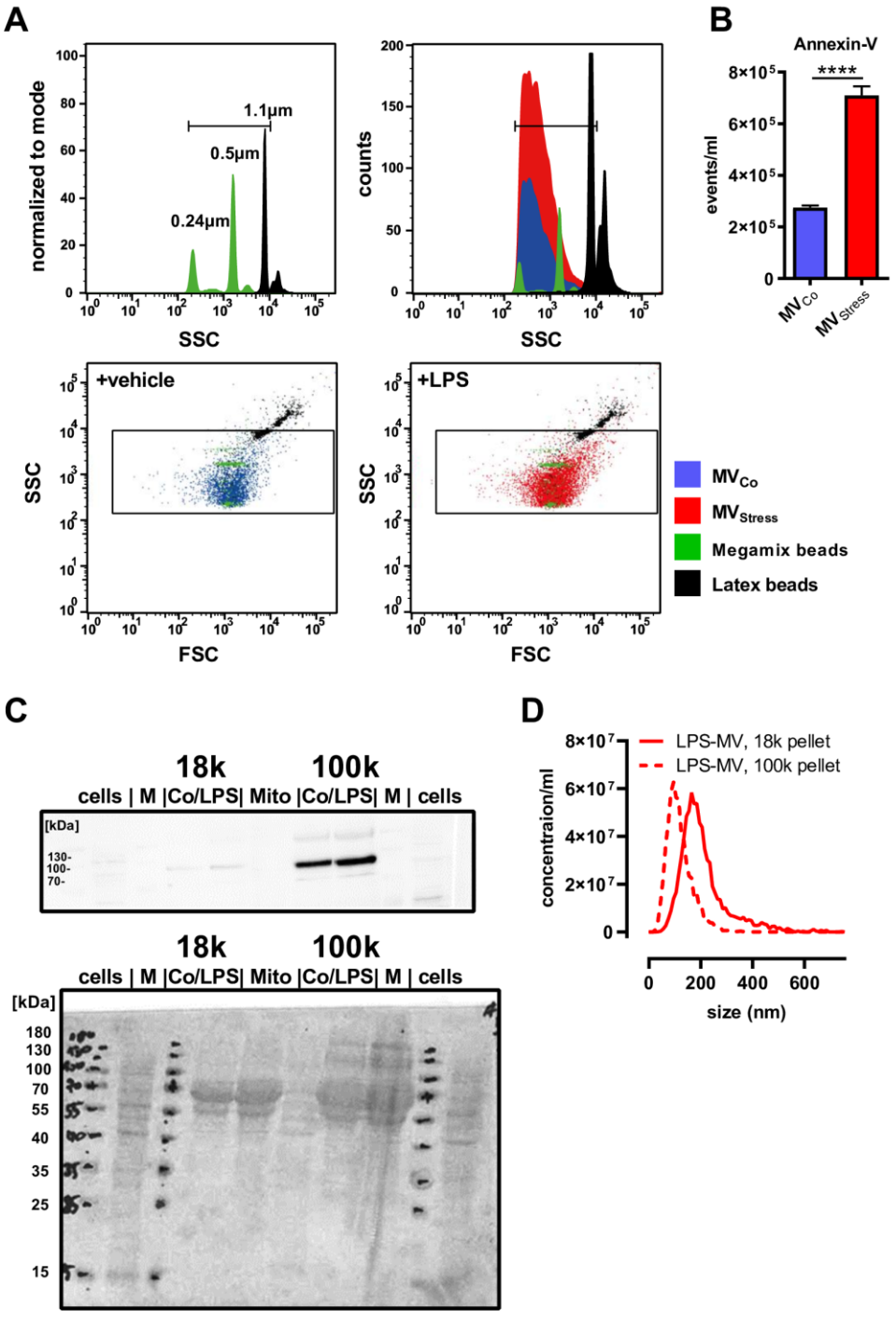
Reagent or Resource	Source	Identifier
Antibodies		
Mouse Anti-TOM22-FITC	Miltenyi Biotec	Cat# 130-107-696; RRID: AB_2654217
Mouse Anti-TOM22-APC	Miltenyi Biotec	Cat# 130-107-698; RRID: AB_2654221
Rabbit Anti-GAPDH	Santa-Cruz	Cat# 2118; RRID: AB_561053
Mouse Anti-BCL2	DAKO	Cat# M0887; RRID: AB_2064429
Mouse Anti-ALIX	BIO-RAD	Cat# VMA00273
Rabbit Anti-COXIV	Cell Signaling	Cat# 4844; RRID: AB_2085427
Sheep Anti-mouse-HRP	GE Healthcare	Cat# NA931; RRID: AB_772210
Donkey Anti-rabbit-HRP	GE Healthcare	Cat# NA934; RRID: AB_772206
Chemicals, Peptides, and Recombinant Proteins		
Bovine Serum Albumin (BSA) Fatty acid free	Pan Biotech	Cat# P06-139450
MitoTracker ® Deep Red FM	Invitrogen	Cat# 8778S
Cell Tracker CFSE	Invitrogen	Cat# 423801
MitoSOX Red	Invitrogen	Cat# M36008
Annexin-V PE-conjugated	BioLegend	Cat# 640908
Calcein-AM	BD Biosciences	Cat# 564061
Anakinra	Amgen	N/A
Infliximab	Janssen	N/A
Interleukin-1β human	Sigma-Aldrich	Cat# I9401
Lipopolysaccharides from Escherichia coli 0111:B4,gamma-irradiated, BioXtra, suitable for cell culture	Sigma-Aldrich	Cat# L4391
MitoTEMPO	Sigma-Aldrich	Cat# SML0737
Commercial Assays		
BD optEIA human IL-8 ELISA	Becton Dickinson	Cat# 555244

Human IL-1 beta/IL-1F2 DuoSet ELISA	R&D Systems	Cat# DY201-05
Human VCAM-1/CD106 DuoSet ELISA	R&D Systems	Cat# DY809
Human TNF-alpha DuoSet ELISA	R&D Systems	Cat# DY210-05
peqGOLD TOTAL RNA KIT (C-LINE)	VWR International	Cat# 732-2868
RNaesy Mini Kit	Qiagen	Cat# 74104
High Capacity cDNA Reverse Transcription Kit	Thermo Fisher Scientific	Cat# 10186954
KAPA SYBR FAST BIORAD	Sigma-Aldrich	Cat# KK4608
Mitochondria Isolation Kit, human	Miltenyi Biotec	Cat# 130-094-532
Cell Lines		
THP 1, human monocytic leukemia cells	ATCC	Cat# ATCC® TIB-202™
HUVECs, endothelial cells	Lonza	Cat# C2519A
Software and Algorithms		
FlowJo 10.0.8r1	TreeStar, FlowJo, Ashland, Oregon	https://www.flowjo.com/solutions/flowjo/ downloads
GraphPad Prism 8	GraphPad Software, California	https://www.graphpad.com/scientific-software/prism/
ITEM-Software	Olympus Soft Imaging Solutions, Münster, Germany	https://www.olympus-sis.com/
Adobe Photoshop CS5	Adobe, San José, California, US	https://www.adobe.com/de/#
EnrichR	Icahn School of Medicine at Mount Sinai, Ma'ayan Lab	http://amp.pharm.mssm.edu/Enrichr/
ImageJ	National Institutes of Health and the Laboratory for Optical and Computational Instrumentation	https://imagej.nih.gov/ij/
AlphaImager System	ProteinSimple	https://www.proteinsimple.com/alphaimager_hp.html
Other		
MegaMix-Plus SSC	Biocytex	Cat# 1078
Latex beads, polystyrene, 1.1µm	Sigma	Cat# LB11
Epoxy resin Low viscosity resin	Agar Scientific, Stansted, UK	Cat# 1370
VH2 hardener (EM protocol)	Agar Scientific, Stansted, UK	Cat# R1376
Accelerator (EM protocol)	Agar Scientific, Stansted, UK	Cat# 1078D
Butyar-coated 300 mesh grids	Agar Scientific, Stansted, UK	Cat# G2300N
Nitrocellulose membrane	GE Healthcare	Cat# 10600003
SuperSignal West Femto Substrate	Thermo Scientific	Cat# 34095

WesternBright Chemiluminescence Substrat Sirius	Biozym Scientific GmbH	Cat# K-12043-D20
RPMI 1640 Media	Invitrogen	Cat# 11879020
EBM-2 media	Lonza	Cat# CC-3124
Buffer-free Assay Medium	Agilent	Cat# 103193-100

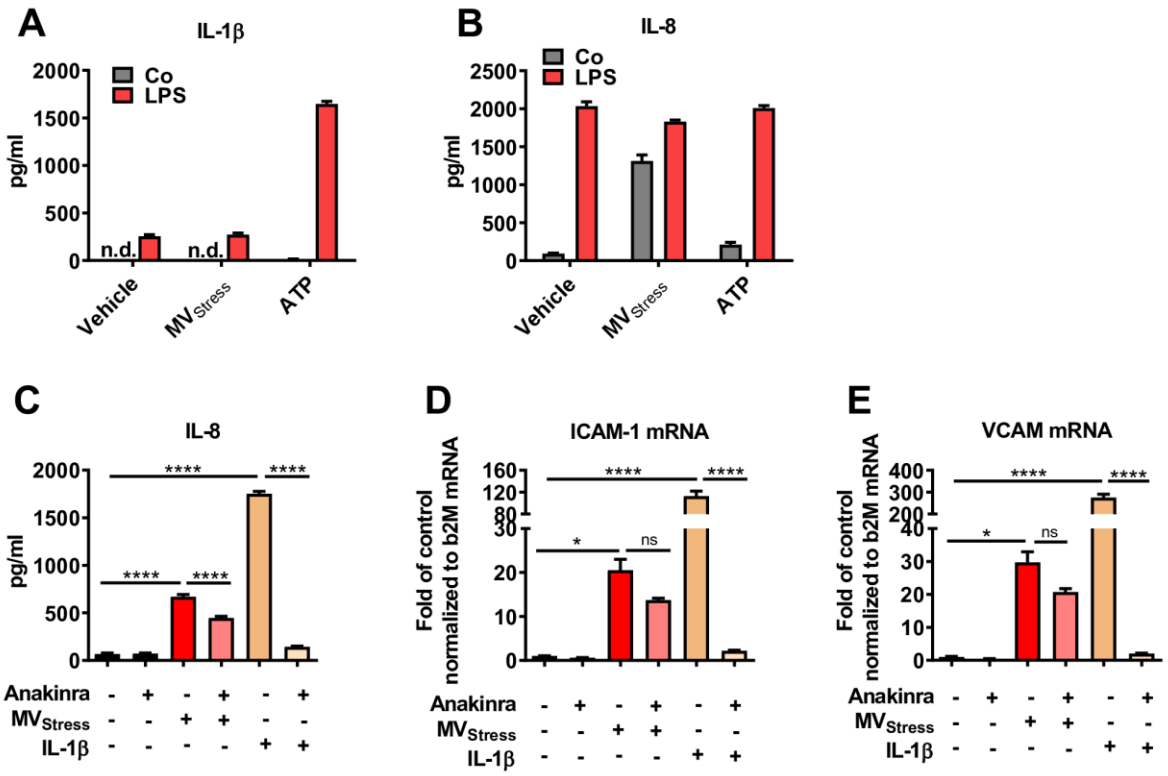
Oligonucleotides		
OAS2 fwd	Sigma	ACGTGACATCCTCGATAAAACTG
OAS2 rev	Sigma	GAACCCATCAAGGGACTTCTG
MX1 fwd	Sigma	GCCACCATTCCAAGCTTACTTTG C
MX1 rev	Sigma	AATGAGGTCGATGCAGGGGCG
IFIT1 fwd	Sigma	TTGATGACGATGAAATGCCTGA
IFIT1 rev	Sigma	CAGGTCACCAGACTCCTCAC
VPR fwd	Sigma	CTTTGTGCTGCCCCTTGAGGAA
VPR rev	Sigma	CTCTCCCGGATCAGGCTTCCA
CXCL10 fwd	Sigma	GTGGCATTCAAGGAGTACCTC
CXCL10 rev	Sigma	TGATGGCCTTCGATTCTGGATT
CXCL11 fwd	Sigma	GACGCTGTCTTTGCATAGGC
CXCL11 rev	Sigma	GGATTTAGGCATCGTTGTCCTTT
CCL2 fwd	Sigma	GAGAGGCTGAGACTAACCCAGA
CCL2 rev	Sigma	ATCACAGCTTCTTTGGGACACT
IL8 fwd	Sigma	CTCTTGGCAGCCTTCCTGATT
IL8 rev	Sigma	TATGCACTGACATCTAAGTTCTTT AGCA
VCAM fwd	Sigma	AGGGGGGTACACGCTAGGAAC
VCAM rev	Sigma	AGGAGTGAGGGGACCAATTC
ICAM fwd	Sigma	CATAGAGACCCCGTTGCCTA
ICAM rev	Sigma	GGGTAAGGTTCTTGCCCACT
h16s fwd	Sigma	CCAAGCATAATATAGCAAGGAC
h16s rev	Sigma	CTTAGCTTTGGCTCTCCTTG
h18s fwd	Sigma	GTAACCCGTTGAACCCCATT
h18s rev	Sigma	CCATCCAATCGGTAGTAGCG

Online Figure I



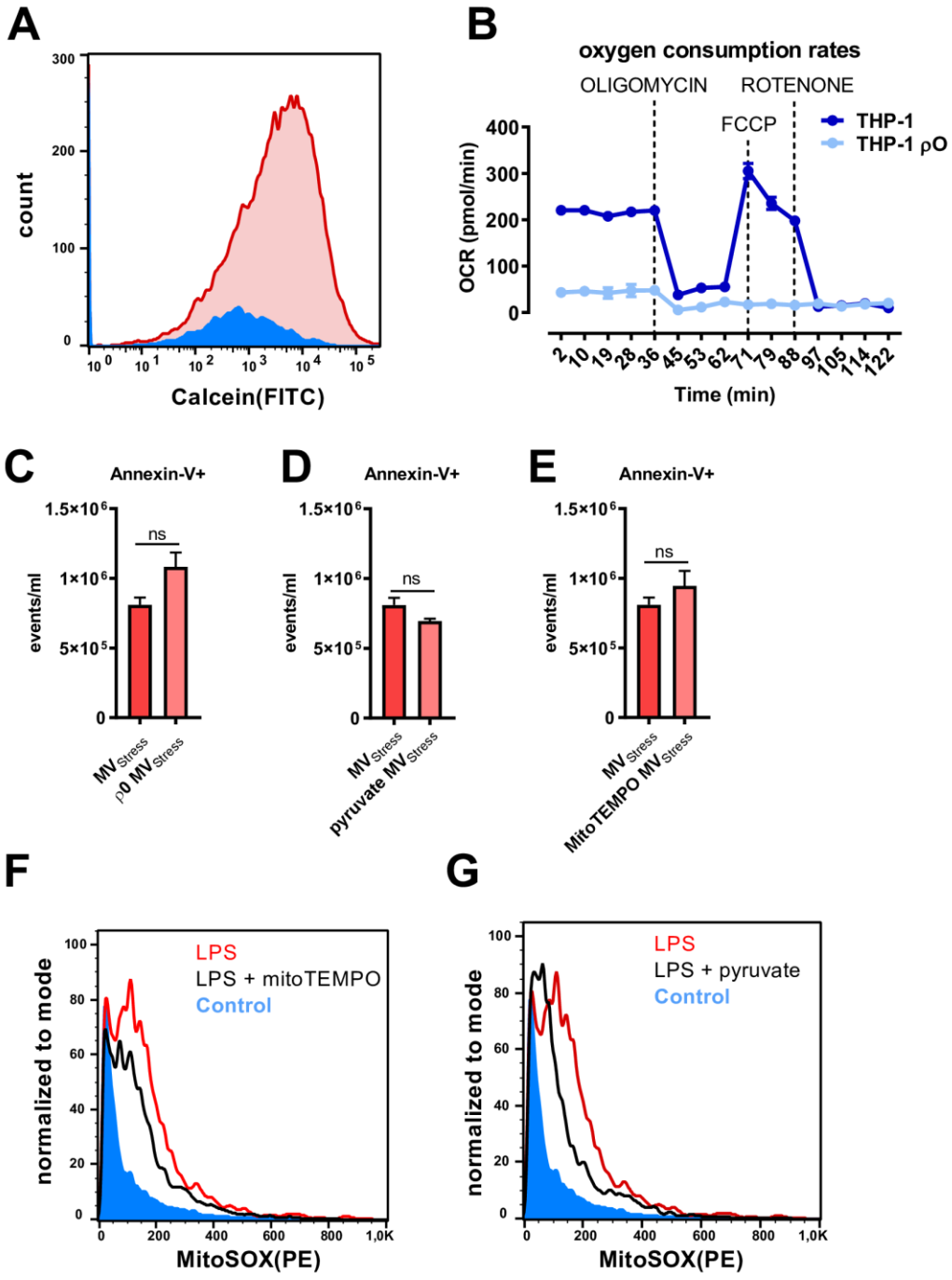
Online Figure I. Gating strategy for MV characterization and quantification by flow cytometry. **(A)** The detection gate was defined by MegaMix-plus SSC beads (green) and latex beads (black). Blue and red populations are MV_{Co} and MV_{Stress}, respectively. **(B)** The bar graph shows quantification of MVs isolated from supernatants of vehicle or LPS (500ng/ml)-treated THP-1 monocytic cells (16h). MVs were labeled with Annexin-V for detection. Data shown as mean ±SEM, n=4. Unpaired t-test;****P< 0.0001 **(C)** Western Blot analysis of ALIX protein in lysates of equal numbers of MV_{Co} and MV_{Stress}, mitochondria and THP-1 monocytic cells. Vesicle pellets were isolated from conditioned media of vehicle or LPS-stimulated THP1 cells by 18,000 g (18k pellet) and 100,000 g (100k pellet) centrifugation. Ponceau staining of Western blot is shown below. **(D)** Size distribution profiles of vesicles isolated from conditioned media of LPS-stimulated THP-1 cells by 18,000 g and 100,000 g centrifugation. Data were obtained by Nanoparticle Tracking Analysis (NTA).

Online Figure II



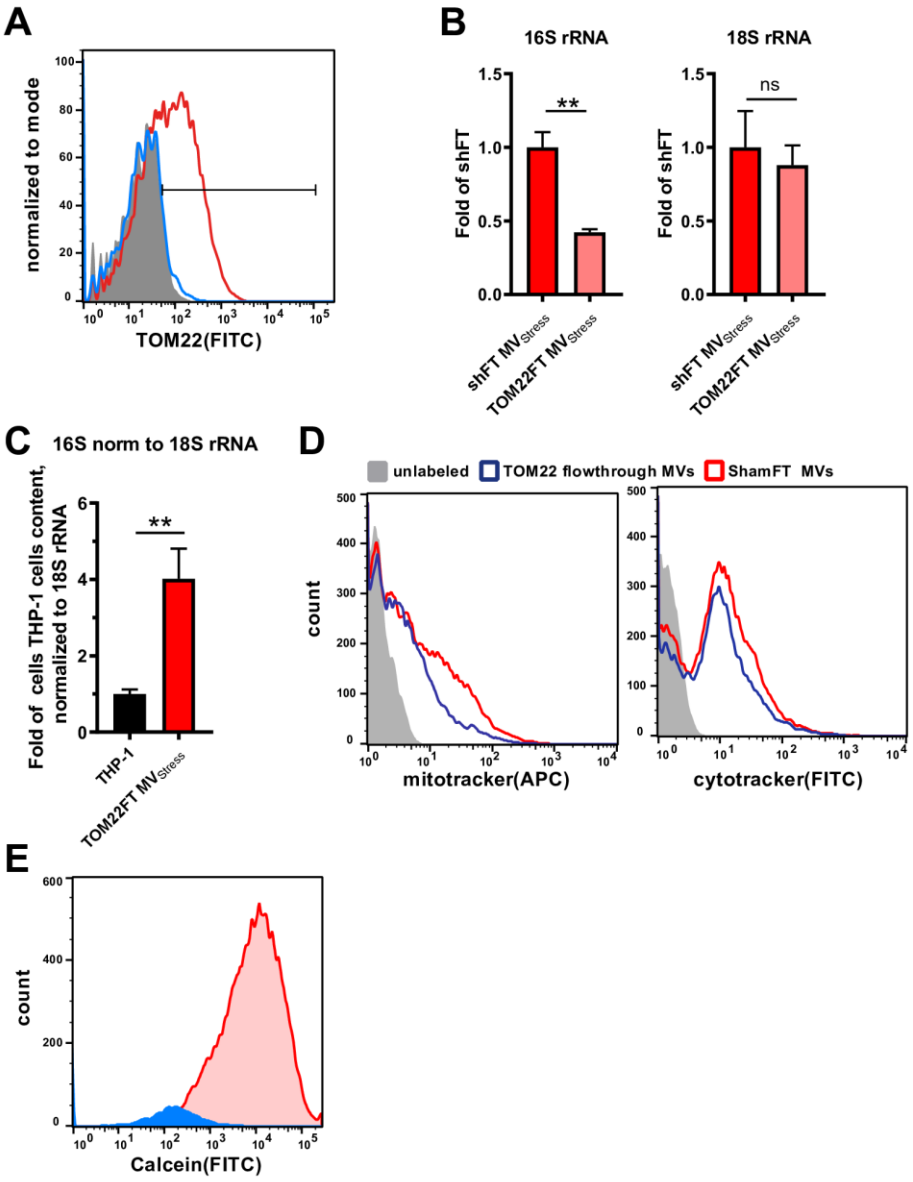
Online Figure II. Characterization of MV_{Stress} ability to serve as secondary stimulus for inflammasome activation and contribution of MV-associated IL-1β to endothelial cell activation
(A)(B) PMA-differentiated THP-1 macrophages were incubated in presence or absence of LPS (0.1µg/ml) for 4h. Afterwards either ATP (1mM) or MV_{Stress} (1*10^6/ml) were added to LPS-primed cells for 2h. IL-1β **(A)** and IL-8 **(B)** protein levels in cell supernatants were determined by ELISA. **(C-E)** HUVECs were stimulated with MV_{Stress} or IL-1β 10 ng/ml in the presence or absence of interleukin 1 receptor antagonist Anakinra (100 µg/ml). Levels of IL-8 in cell supernatants **(C)** were quantified by ELISA 8h after stimulation. VCAM **(D)** and ICAM **(E)** mRNA expression was assessed by RT-qPCR 6h after stimulation. Data shown as mean ±SEM, n=4. One-way ANOVA test; ****P< 0.0001, ***P < 0.001, **P < 0.01, *P < 0.05

Online Figure III



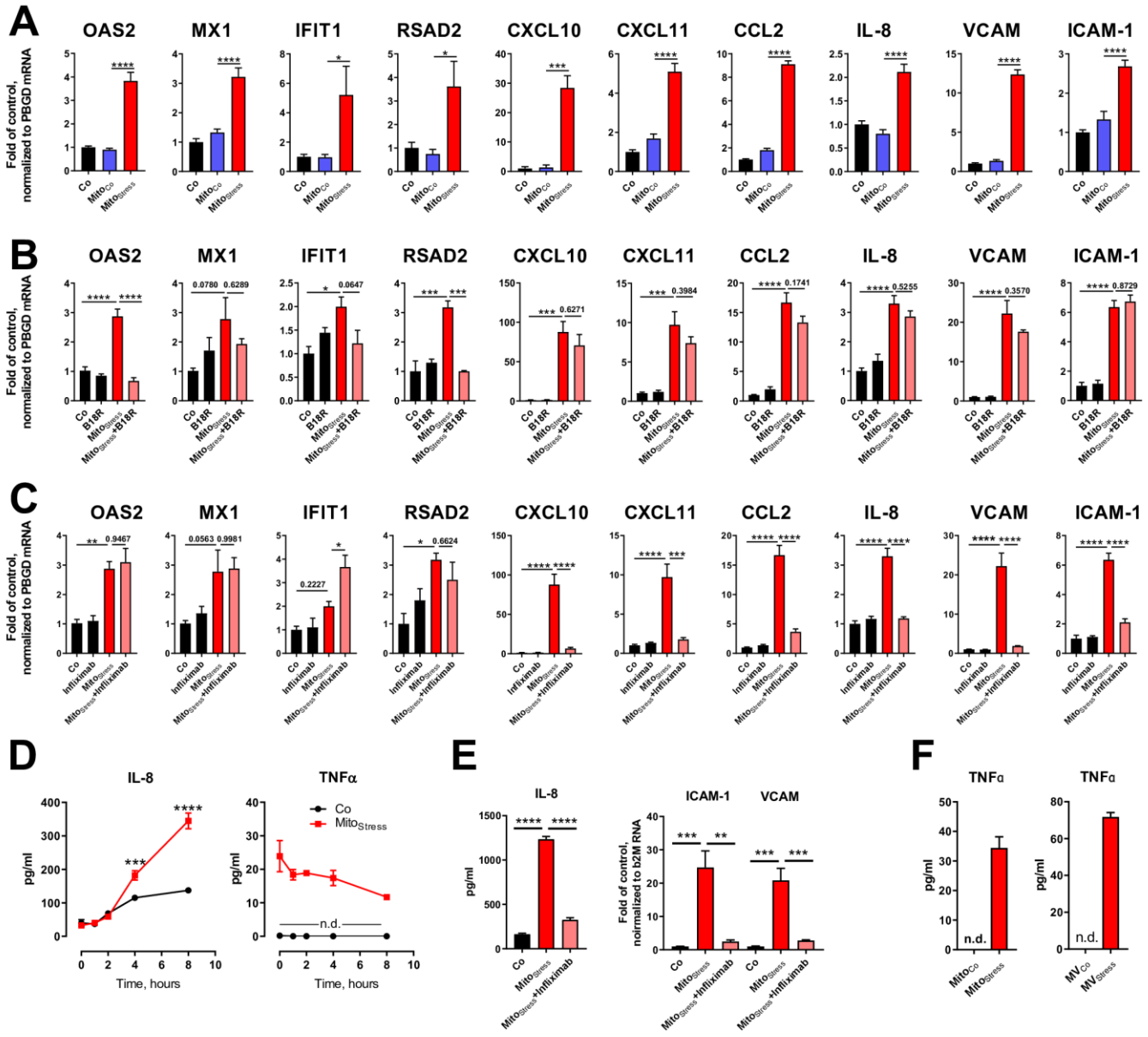
Online Figure III. Characterization of MVs, MV-production and ρ0 THP-1 monocytic cells. (A) Histogram of Calcein-labeled MV_{Stress} either subjected to sonication (blue) or not (red). MVs characterized by flow cytometry. **(B)** Characterization of mitochondrial activity in THP-1 and ρ0 THP-1 stimulated with LPS for 2h was performed by monitoring oxygen consumption rates (OCR) under normal and mitochondrial stress conditions using Seahorse XF24 Analyzer. **(C-E)** Flow cytometric quantification of LPS-induced MV release by **(C)** ρ0 or wt THP-1 monocytic cells; **(D)** THP-1 monocytic cells in presence or absence of pyruvate; **(E)** THP-1 monocytic cells in presence or absence of MitoTEMPO. MVs were isolated from culture supernatants 16h after stimulation. **(F)** and **(G)**, Flow cytometry-based characterization of mitoSOX-positivity of THP-1 monocytic cells stimulated with vehicle (blue), LPS (red) and LPS in presence of mitoTEMPO (black, panel F) or pyruvate (black, panel G). **(B to E)** Data shown as mean ±SEM, n=4. Unpaired t-test.

Online Figure IV



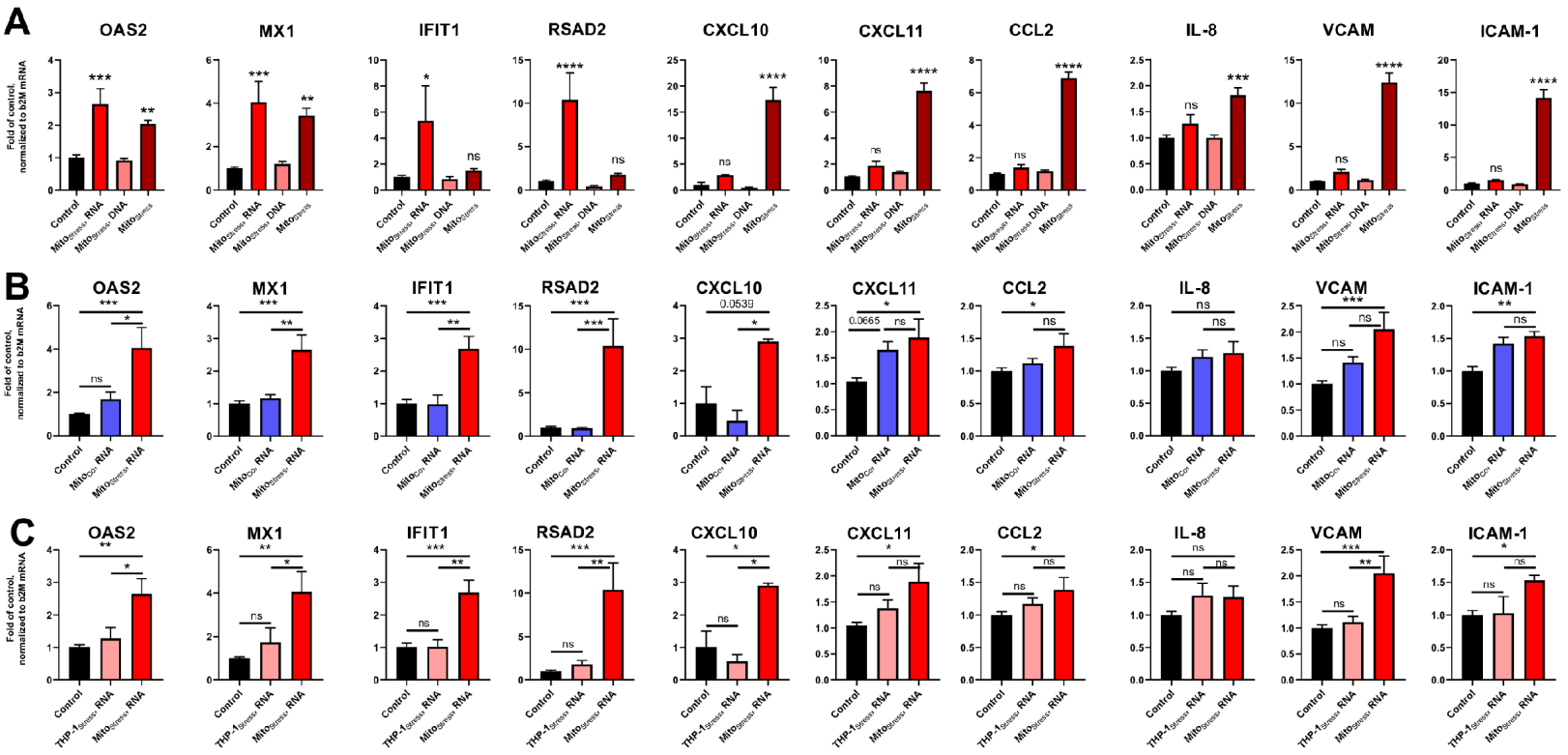
Online Figure IV. Characterization of MVs and mitochondria. (A-D) MV_{Stress} were pre-incubated with magnetic beads-coupled with anti-TOM22 antibodies and passed through paramagnetic columns to obtain TOM22-depleted vesicles (TOM22FT). MV_{Stress} were passed through paramagnetic columns without pre-incubation with antibodies to obtain sham-depleted MVs (ShamFT). **(A)** Histogram of flow-cytometric analysis of unbound MVs (flow through, FT) stained with anti-TOM22. ShamFT (blue), TOM22FT (red), unstained ShamFT (black). **(B)** Levels of mitochondrial 16S and cytoplasmic 18S rRNA in TOM22-depleted MVs (TOM22FT) as compared to sham-depleted MVs (ShamFT). **(C)** Ratio of mitochondrial 16S rRNA to cytosolic 18S rRNA in THP-1 cells compared to TOM22-depleted MVs (TOM22FT). **(D)** Histogram of flow-cytometric analysis of ShamFT (red), TOM22FT (blue), unlabeled MV_{Stress} (grey) for the presence of mitotracker and cytotracker. MVs were isolated from conditioned media of mitotracker- and cytotracker-labeled THP-1 cells stimulated with LPS. **(E)** Histogram of Calcein-labeled $Mito_{Stress}$ either subjected to sonication (blue) or not (red). Mitochondria characterized by flow cytometry. **(B and C)** Data shown as mean ±SEM, n=4. Unpaired t-test; **P < 0.01, *P < 0.05

Online Figure V



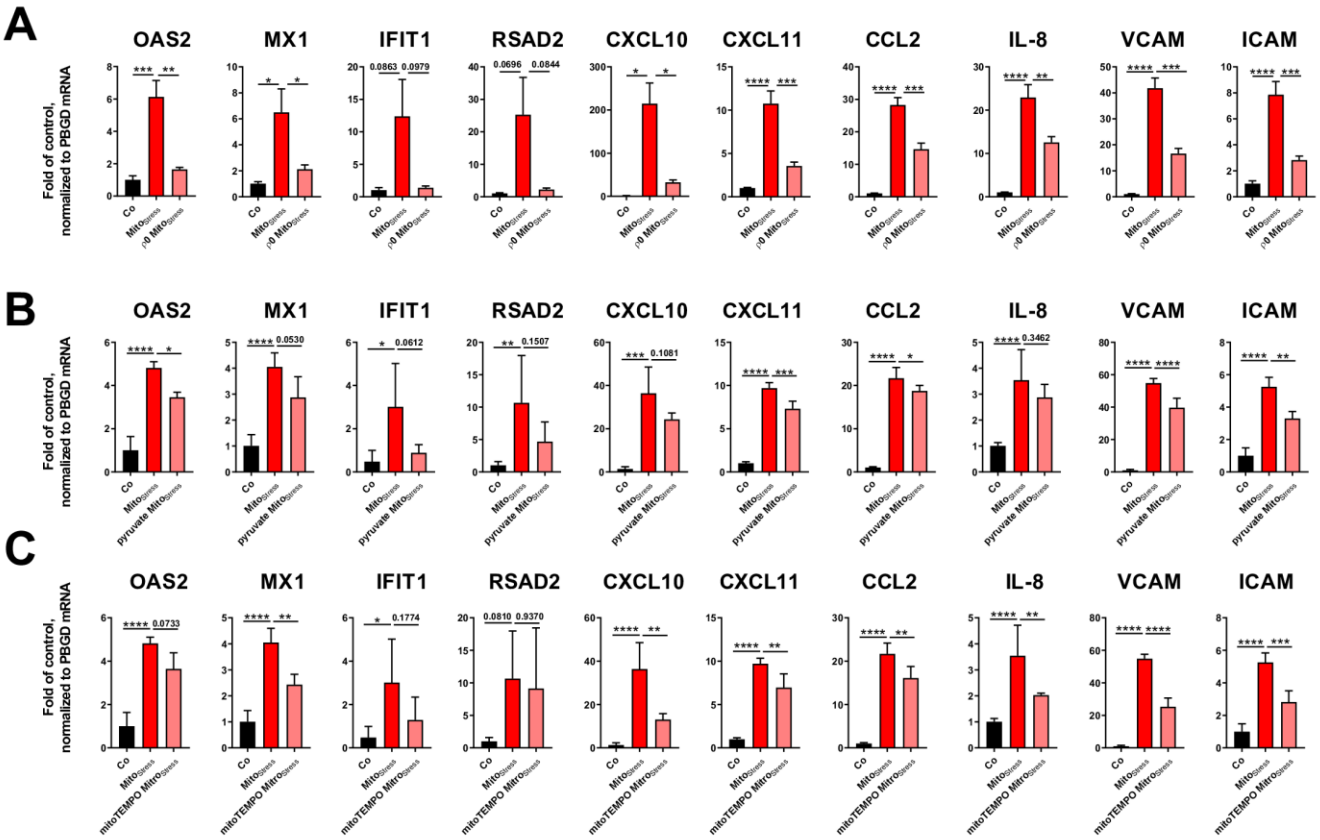
Online Figure V. Analysis of mRNA expression in HUVECs stimulated with isolated mitochondria and mitochondria-associated TNFα. Mitochondria were isolated from THP-1 monocytic cells after 16h stimulation with vehicle (MV_{Co}) or LPS (MV_{Stress}). **(A-C)** Fold induction of mRNA expression in HUVECs stimulated for 6h with $Mito_{Co}$ and $Mito_{Stress}$ as compared to untreated HUVECs was quantified by RT-qPCR. Stimulation with **(A)** Co- or $Mito_{Stress}$; **(B)** $Mito_{Stress}$ in presence or absence of B18R (5µg/ml); **(C)** $Mito_{Stress}$ in presence or absence of Infliximab (100µg/ml). **(D)** Levels of IL-8 (left) and TNFα (right) in supernatants of HUVECs stimulated with $Mito_{Stress}$ for indicated time-points quantified by ELISA. (E) Secretion of IL-8 protein (8h) and expression of ICAM and VCAM mRNA (6h) in stimulated HUVECs. Stimulation with $Mito_{Stress}$ pre-incubated with Infliximab (100µg/ml) or vehicle for 30min. $Mito_{Stress}$ were washed by centrifugation (18,000g, 15min) before addition to HUVECs in order to remove unbound antibody. **(F)** Levels of TNFα in $Mito_{Co}$ and $Mito_{Stress}$ (mitochondria isolated from 2*10^6 THP-1 monocytic cells) or MV_{Co} and MV_{Stress} (MVs isolated from 2ml conditioned media of 2*10^6 THP-1 monocytic cells) quantified by ELISA. Co = unstimulated; Data shown as mean ±SEM, n=4; One-way ANOVA test; ****P< 0.0001, ***P < 0.001, **P < 0.01, *P < 0.05

Online Figure VI



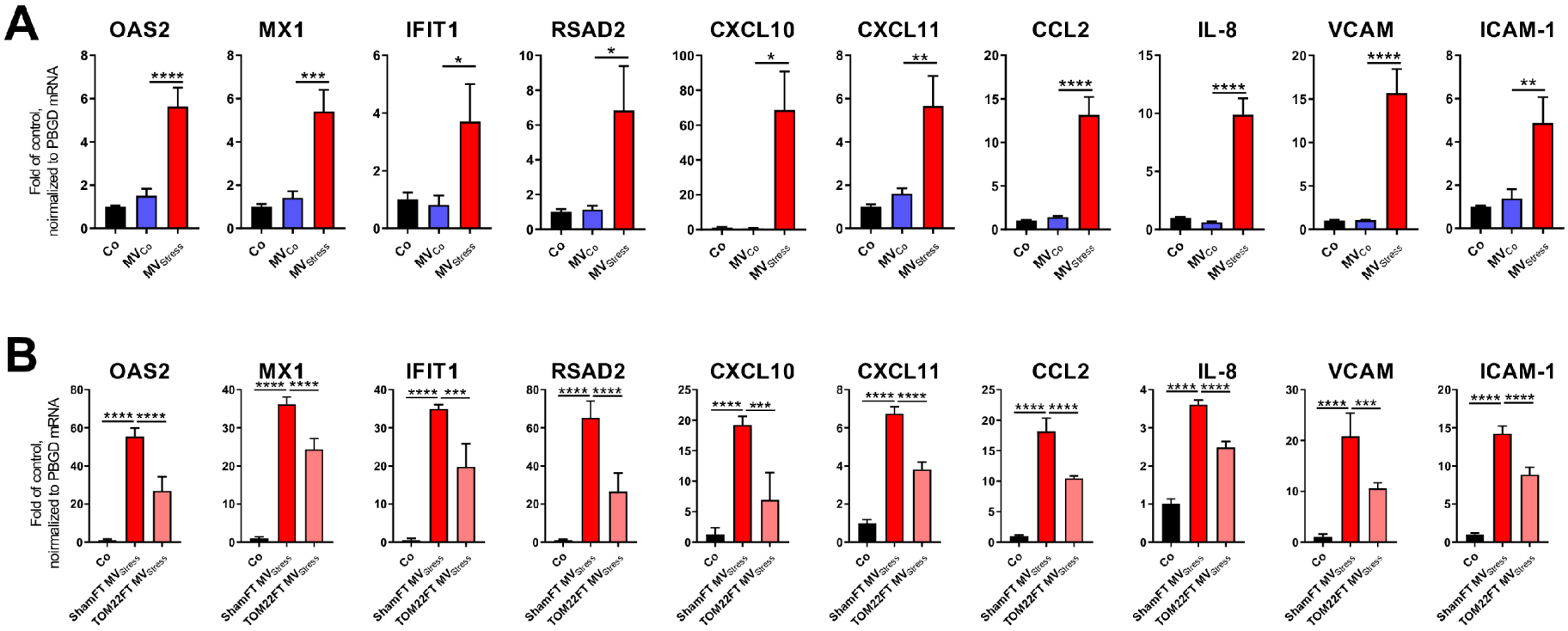
Online Figure VI. Characterization of MVs and mitochondria. (A-D) MV_{Stress} were pre-incubated with magnetic beads-coupled with anti-TOM22 antibodies and passed through paramagnetic columns to obtain TOM22-depleted vesicles (TOM22FT). MV_{Stress} were passed through paramagnetic columns without pre-incubation with antibodies to obtain sham-depleted MVs (ShamFT). **(A)** Histogram of flow-cytometric analysis of unbound MVs (flow through, FT) stained with anti-TOM22. ShamFT (blue), TOM22FT (red), unstained ShamFT (black). **(B)** Levels of mitochondrial 16S and cytoplasmic 18S rRNA in TOM22-depleted MVs (TOM22FT) as compared to sham-depleted MVs (ShamFT). **(C)** Ratio of mitochondrial 16S rRNA to cytosolic 18S rRNA in THP-1 cells compared to TOM22-depleted MVs (TOM22FT). **(D)** Histogram of flow-cytometric analysis of ShamFT (red), TOM22FT (blue), unlabeled MV_{Stress} (grey) for the presence of mitotracker and cytotracker. MVs were isolated from conditioned media of mitotracker- and cytotracker-labeled THP-1 cells stimulated with LPS. **(E)** Histogram of Calcein-labeled $Mito_{Stress}$ either subjected to sonication (blue) or not (red). Mitochondria characterized by flow cytometry. **(B and C)** Data shown as mean ±SEM, n=4. Unpaired t-test; **P < 0.01, *P < 0.05

Online Figure VII



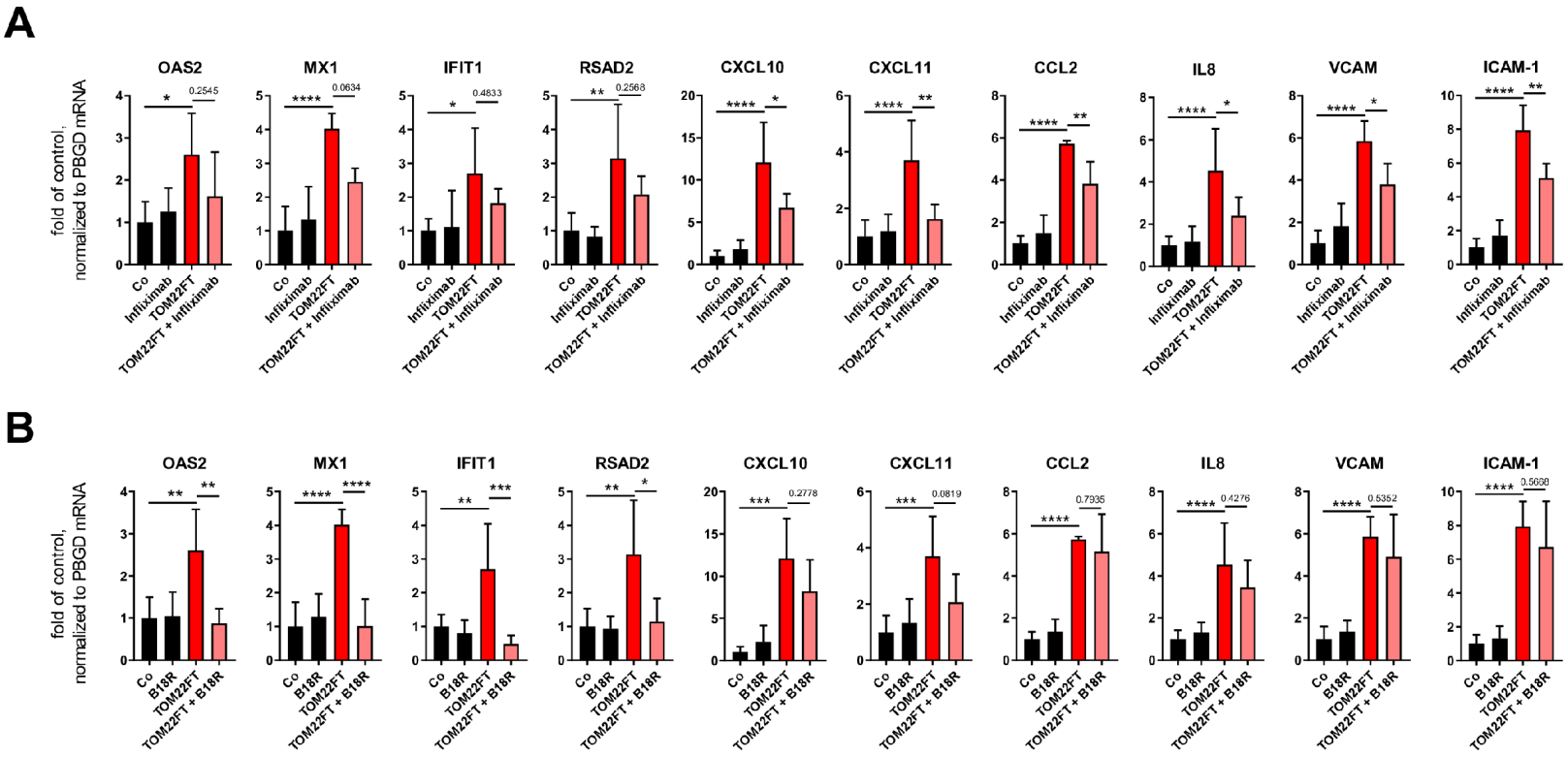
Online Figure VII. mRNA expression in HUVECs stimulated with isolated mitochondria. (A-C) Levels of selected mRNAs in endothelial cells (HUVEC) quantified by RT-qPCR. Total RNA was isolated 6h after stimulation. **(A)** Stimulation with $Mito_{Stress}$ isolated from THP-1 or ρ0 THP-1 monocytic cells ($Mito_{Stress}$ and ρ0 $Mito_{Stress}$, respectively). **(B and C)** Stimulation with $Mito_{Stress}$ isolated from THP-1 monocytic cells activated with LPS in presence or absence of pyruvate (1mM) **(B)** or mitoTEMPO (50μM) **(C)**. Co = unstimulated; Data shown as mean ±SEM, n=4; One-way ANOVA test; ****P< 0.0001, ***P < 0.001, **P < 0.01, *P < 0.05

Online Figure VIII



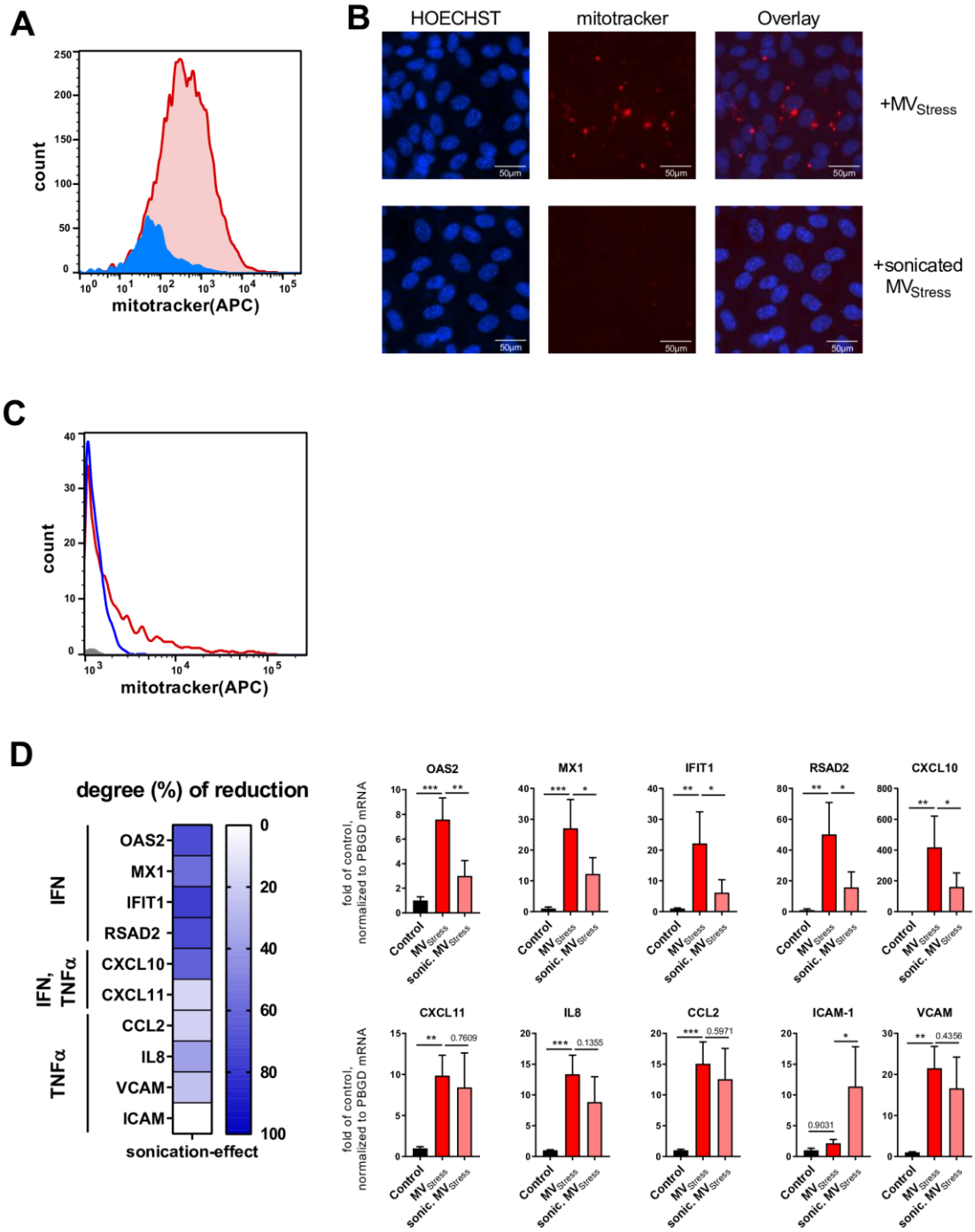
Online Figure VIII. mRNA expression in HUVECs stimulated with MVs. (A)(B) Levels of selected mRNAs in endothelial cells (HUVEC) quantified by RT-qPCR. Total RNA was isolated 6h after stimulation. HUVECs stimulated with **(A)** MV_{Co} or MV_{Stress}; **(B)** MV_{Stress} depleted for TOM22 positive vesicles (TOM22FT) or sham-depleted (ShamFT). MV_{Stress} were pre-incubated with magnetic beads-coupled with anti-TOM22 antibodies and passed through paramagnetic columns to obtain TOM22-depleted vesicles (TOM22FT). MV_{Stress} were passed through paramagnetic columns without pre-incubation with antibodies to obtain sham-depleted MVs (ShamFT). MVs were characterized by flow cytometry as depicted in online figure IV. Co = unstimulated; Data shown as mean ±SEM, n=4; One-way ANOVA test; ****P< 0.0001, ***P < 0.001, **P < 0.01, *P < 0.05

Online Figure IX



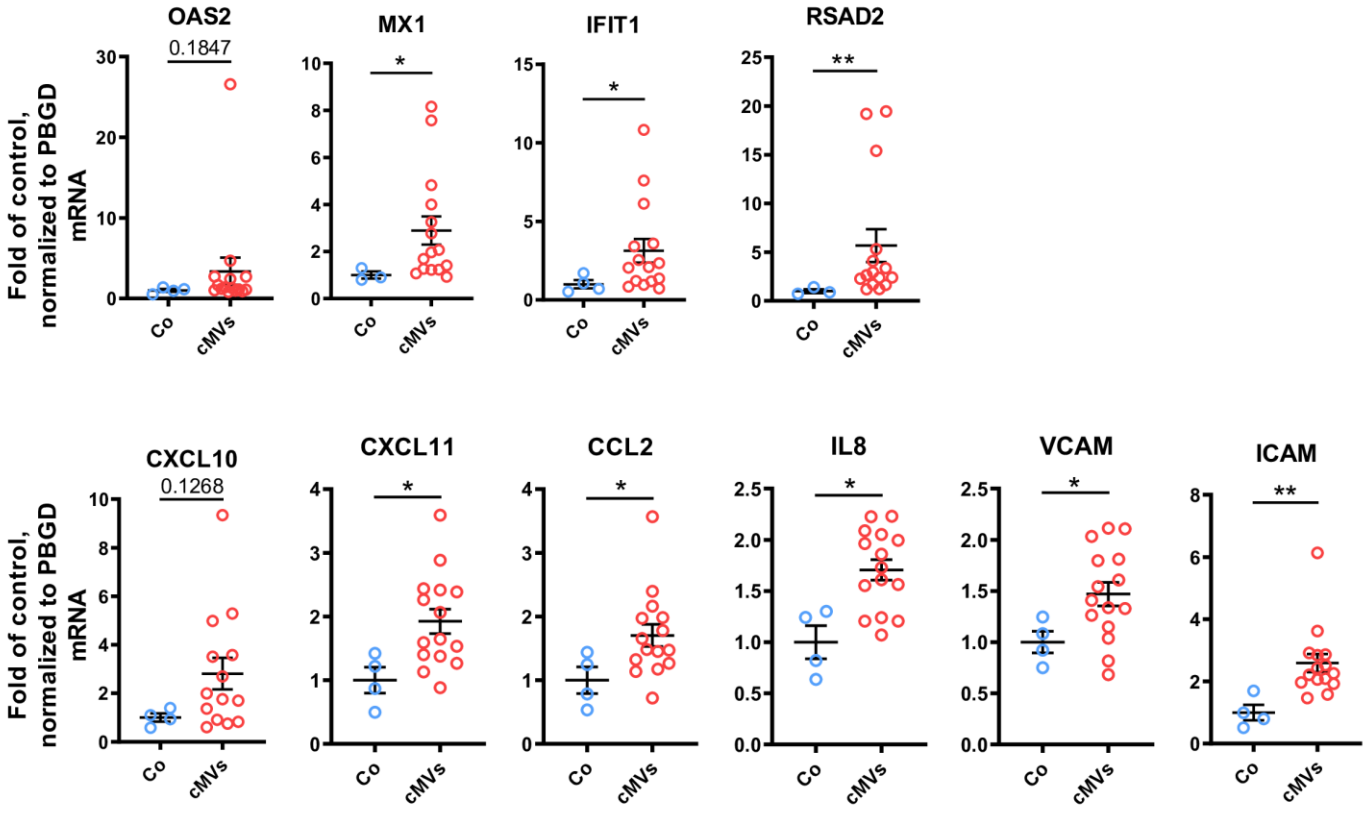
Online Figure IX. mRNA expression in HUVECs stimulated with MVs depleted of TOM22+ vesicles. MV_{Stress} were pre-incubated with magnetic beads-coupled with anti-TOM22 antibodies and passed through paramagnetic columns to obtain TOM22-depleted vesicles (TOM22FT). Levels of selected mRNAs in endothelial cells (HUVEC) quantified by RT-qPCR. Total RNA was isolated 6h after stimulation. Stimulation with TOM22FT: in presence or absence of B18R (5µg/ml) **(A)**; in presence or absence of Infliximab (100µg/ml) **(B)**. **(C)** Flow cytometry-based characterization of HUVECS for mitotracker-positivity after incubation with mitotracker-positive MV_{Stress} depleted for TOM22 positive vesicles (blue) or sham-depleted (red) at 37°C. HUVECs were incubated with sham-depleted MV_{Stress} at 4°C to define the threshold of active MV-uptake. **(A and B)** Data shown as mean ±SEM, n=4; One-way ANOVA test; ****P< 0.0001, ***P < 0.001, **P < 0.01, *P < 0.05

Online Figure X



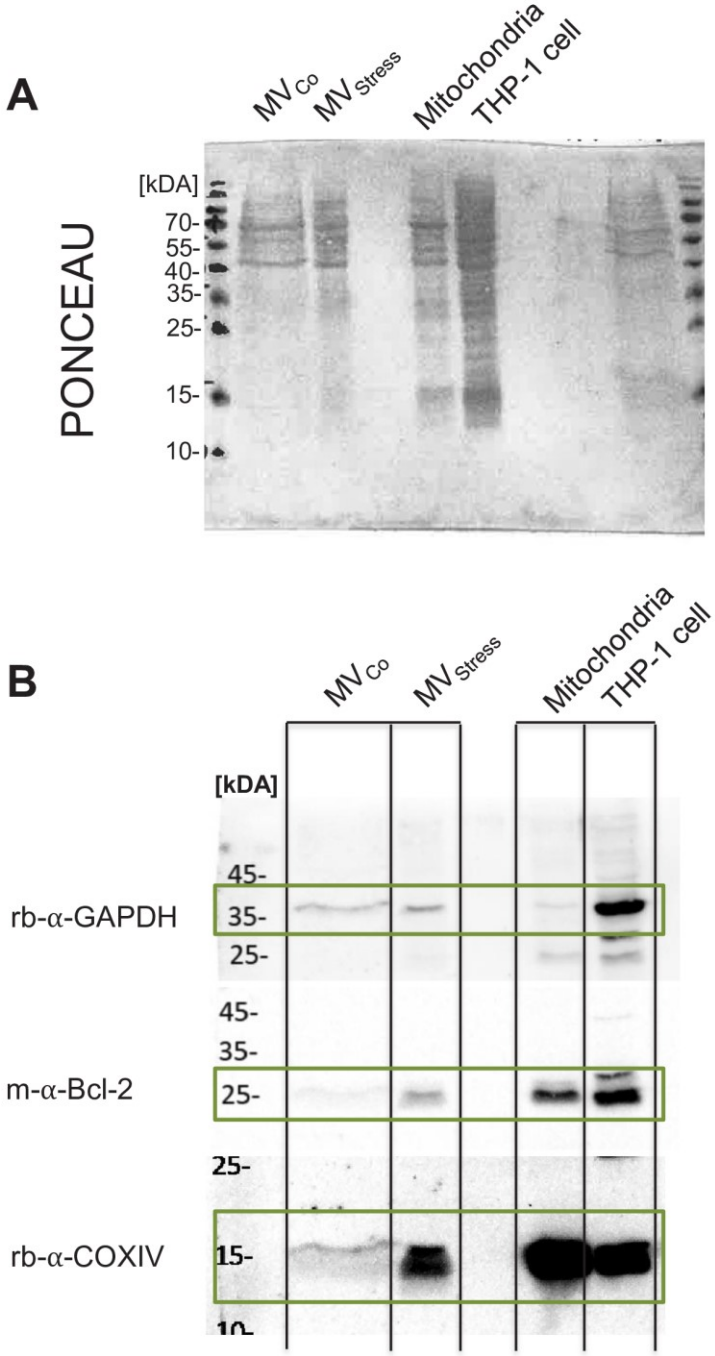
Online Figure X. Effect of disintegration of MV_{Stress} on their uptake and their capacity to induce mRNA expression in HUVECs. (A-C) MVs were isolated from conditioned media of mitotracker-labeled THP-1 cells stimulated with LPS. **(A)** Histogram of mitotracker-positive MV_{Stress} either subjected to sonication (blue) or not (red). MVs characterized by flow cytometry. **(B)** HUVECs were incubated with mitotracker-positive MV_{Stress}, intact or disintegrated by sonication, for 60min at 37°C. Afterwards excess of MVs was removed by washing. Nuclei were stained by Hoechst (1µg/ml in PBS) for 3min. **(C)** Flow cytometry-based characterization of HUVECS for mitotracker-positivity after incubation with mitotracker-positive MV_{Stress} either subjected to sonication (blue) or not (red) at 37°C. HUVECs were incubated with MV_{Stress} at 4°C to define the threshold of active MV-uptake. **(D)** HUVECs were stimulated with intact MV_{Stress} or sonicated MV_{Stress} for 6h. Levels of selected mRNAs in endothelial cells (HUVEC) were quantified by RT-qPCR. Data shown as mean ±SEM, n=4; One-way ANOVA test; ****P< 0.0001, ***P < 0.001, **P < 0.01, *P < 0.05

Online Figure XI



Online Figure XI. mRNA expression in HUVECs stimulated with MVs isolated from plasma from human low-grade endotoxinemia study. Circulating MVs (cMV) were isolated from plasma obtained from individuals 2h after LPS-injection (2ng/kg). HUVECs were stimulated with cMVs for 6h. Levels of selected mRNAs in endothelial cells (HUVEC) were quantified by RT-qPCR. Mann-Whitney, ****P< 0.0001, ***P < 0.001, **P < 0.01, *P < 0.05

Online Figure XII



Online Figure XII. Western Blot analysis of protein in lysates of MVs, isolated mitochondria and THP-1 monocytic cells. (A) Ponceau staining of Western Blot. **(B)** Western Blot analysis of COXIV, Bcl-2 and GAPDH protein in lysates of equal numbers of MV_{Co} and MV_{Stress}, mitochondria and THP-1 monocytic cells. Green rectangles indicate where parts of the image were cropped for main figure 1E.

4. Discussion

4.1 Extracellular mitochondria as a novel subset of extracellular vesicles

Interestingly, it has been reported that, platelets and mesenchymal stem cells can release mitochondria and microvesicle-encapsulated mitochondria [112][114], microvesicle-associated mitochondrial DNA is released in mouse models of hepatic inflammation [96] and activated human monocytes and mouse bone marrow–derived stromal cells can release microvesicles containing mitochondrial proteins [87][113]. Consistent with their bacterial origin, mitochondria present a potent source of damage-associated molecular patterns (DAMPs) [93] and may thus contribute to the pro-inflammatory activity of microvesicles. Mitochondrial DAMPs include *N*-formyl-methionyl dipeptides (fMLP), cardiolipin, ROS (reactive oxygen species), ATP and mitochondrial DNA [93].

In this thesis, I could show that activation of THP-1 monocytic cells by LPS-stimulation results in the release of microvesicle-encapsulated mitochondria and free mitochondria. This was demonstrated by, i) enrichment of mitochondrial proteins (Manuscript Figure 1C and 1E), ii) enrichment of mitochondrial ribosomal RNA (Manuscript Figure 1D), iii) detection of free and encapsulated mitochondria by electron-microscopy (Manuscript Figure 1F) and, iv) detection of TOM22+ vesicles by flow cytometry (Manuscript Figure 1G). TOM22 (translocase of the outer membrane, subunit 22) is an integral protein of the mitochondrial outer membrane.

Of note, the shedding of mitochondria-derived vesicles from mitochondria themselves has been proposed as a new form of mitochondrial quality control [116]. However, the size of these mitochondria-derived vesicles ranges between 70-150nm [116]. In this thesis, vesicles were obtained after 18,000g centrifugation from conditioned media of THP-1 monocytic cells. Nanoparticle-tracking analysis (NTA) of these vesicles revealed their average size to be 200nm (Manuscript Online Figure ID) and these vesicles could be labeled positively with Annexin-V (Manuscript Online Figure IB). On the other hand, vesicles obtained after 100,000g centrifugation had an average size of 100nm (Manuscript Online Figure ID) and were enriched in the protein ALIX (ALG-2-interacting protein X) (Manuscript Online Figure IC), an exosomal marker [18]. Importantly, free mitochondria and microvesicle-encapsulated mitochondria, detected by electron-microscopy (Manuscript Figure 1F), were ranging from 200-500nm in size - similarly to mitochondria released by activated platelets [112]. Taken together, these data show that upon proinflammatory challenge, monocytic cells not only release microvesicles enriched in mitochondrial content but that they release mitochondria constituting a novel subset of extracellular vesicles that share certain properties (size, Annexin-V positive labeling)

with microvesicles. However, these data do not exclude that mitochondria-derived vesicles are also released by activated monocytic cells as part of the extracellular vesicle fraction obtained after 100,000g centrifugation.

Importantly, only microvesicles released by LPS-activated (MV_{Stress}), but not vehicle-treated (MV_{Co}), THP-1 monocytic cells were able to induce endothelial cell (HUVEC) activation in serum-free conditions (Manuscript Figure 1A and 1B). To control for LPS-carryover by microvesicles, all endothelial cell stimulations were performed in absence of serum (Manuscript Figure 1B). As described in the introduction, endothelial cells will only recognize LPS when it is bound by CD14 provided by serum [82]. Depletion of TOM22+ vesicles (free mitochondria) from MV_{Stress} (Manuscript Online Figure IVA and IVB), significantly reduced the capacity of MV_{Stress} to activate endothelial cells (Manuscript Figure 3A). This observation showed a significant contribution of free mitochondria to the proinflammatory potential of MV_{Stress}, in line with the literature suggesting mitochondria as a potent source of DAMPs [93]. However, also microvesicles released from vehicle-treated THP-1 cells contained mitochondrial proteins (Manuscript Figure 1C and 1E), TOM22+ vesicles (Manuscript Figure 1G) and mitochondrial ribosomal RNA (Manuscript Figure 1D), although to a lower degree. Interestingly, when comparing the proinflammatory potential of mitochondria isolated from LPS ($Mito_{Stress}$) and vehicle ($Mito_{Co}$) stimulated monocytic cells, only $Mito_{Stress}$ were able to induce endothelial cell activation at equivalent concentration (Manuscript Figure 3B). These data indicated that the proinflammatory potential of released mitochondria depends on cellular or mitochondrial activation state and not simply on the presence of mitochondrial contents.

The release of mitochondria from platelets upon activation [112] and mitochondrial DAMPs upon traumatic injury [94] into the circulation has been previously reported. In this thesis, I describe increased levels of circulating TOM22+ vesicles 2h after low-dose (2ng/kg) endotoxin injection in healthy volunteers (Manuscript Figure 4G). It is important to note that in control experiments, it was possible to show enrichment of mitochondrial ribosomal RNA in microvesicles isolated from plasma 2h after LPS-injection as compared to microvesicles isolated from plasma before LPS-injection. Considering that TOM22 is a subunit of the mitochondrial outer membrane protein TOM (translocase of the outer membrane), it is not possible to determine the cellular origin of free mitochondria in the circulation by this method. Additionally, rapid clearance of microvesicles has been shown in the circulation [117][118], hence the actual degree of TOM22+ vesicle release after LPS-challenge might be underestimated as only freely circulating vesicles can be quantified. These *in vivo* data support the *in vitro* observations that free mitochondria are released from LPS-activated cells and suggest a role of free mitochondria in inflammation *in vivo*.

4.2 TNF α as mitochondria-associated cytokine

Previously, a role for mitochondria in the assembly of the inflammasome and processing of pro-IL-1β to IL-1β has been reported [119]. On the one hand, association of the inflammasome adaptor protein ASC (Apoptosis-associated speck-like protein containing a CARD) and NLRP3 (NACHT, LRR and PYD domains-containing protein 3) with mitochondria has been shown [119]. On the other hand, release of ASC specks from activated cells, subsequent transfer to macrophages and inflammasome activation has been reported [120]. Additionally, Ferrari et al [121] showed that extracellular ATP can trigger the release of IL-1β from LPS-primed macrophages. Thus, it was possible that released mitochondria, derived from activated monocytic cells, activate the inflammasome in target cells by transfer of inflammasome components or by production of extracellular ATP as a danger-signal. However, when LPS-primed THP-1 macrophages were stimulated with MV_{Stress}, no induction of IL-1β release by MV_{Stress} was observed (Manuscript Online Figure IIA and IIB). These data indicated that the proinflammatory components of MV_{Stress} were neither inflammasome components nor ATP - potentially associated with or produced by free or microvesicle-encapsulated mitochondria. Moreover, release of MV-associated IL-1β from LPS-activated monocytic cells and its ability to induce endothelial cell activation has been previously reported [115]. However, similar to the report by Wang et al, the proinflammatory effect of MV_{Stress} was only partially blocked in presence of the interleukin 1 receptor antagonist Anakinra (Manuscript Online Figure IIC to IIE), which indicated that a major proportion of the inflammatory activity of MV_{Stress} can be attributed to other components.

When analyzing gene expression patterns of endothelial cells stimulated with monocytic microvesicles and isolated mitochondria, I observed that MV_{Stress} and $Mito_{Stress}$ were both inducing TNF-response genes (Manuscript Figure 4). This was validated by using an antibody (Infliximab) targeting TNFα, which was able to block the ability of $Mito_{Stress}$ to induce TNF-response genes (Manuscript Figure 4C). It was possible that TNFα was released by endothelial cells upon incubation with $Mito_{Stress}$ and then signal to endothelial cells in an autocrine manner. However, while $Mito_{Stress}$ induced release of IL-8 over time, TNFα was detected in the supernatant of endothelial cells immediately upon addition of $Mito_{Stress}$ (Manuscript Online Figure VD). Additionally, when $Mito_{Stress}$ had been preincubated with Infliximab and subsequently washed, Infliximab-incubated $Mito_{Stress}$ were unable to induce endothelial cell activation as determined by IL-8 protein release and ICAM-1 and VCAM mRNA expression (Manuscript Online Figure VE). Moreover, it was only possible to detect TNFα in pellets of $Mito_{Stress}$ and MV_{Stress} but not $Mito_{Co}$ or MV_{Co} (Manuscript Online Figure VF). On a side note, TNFα was detected at a concentration of about 30pg/ml, which is close to the ELISA detection limit, yet still sufficient to induce robust TNF-responses. Altogether, these data

suggest that TNFα is transferred in association with $Mito_{Stress}$/MV_{Stress}, but the nature of this association is not known. TNFα exists in a transmembrane form (tmTNFα), which itself can be recognized by TNF-receptors [122], and a soluble form (sTNFα), which is shed by metalloproteinase-mediated cleavage of tmTNFα [122]. Interestingly, tmTNFα has been documented to induce even stronger responses in target cells than sTNFα [123]. The anti-TNF antibody employed in this thesis, Infliximab, recognizes both the soluble and membrane bound form of TNFα, it is thus not possible to judge which form of TNFα is associated with $Mito_{Stress}$ and MV_{Stress}. On the other hand, the possible localization of TNF and TNF-receptors to mitochondria intracellularly has been reported [124][125][126], indicating that TNFα could be associated with mitochondria as part of a protein complex.

4.3 Mitochondrial RNA as DAMP

As mentioned above, mitochondria are considered to be a source of potential damage-associated molecular patterns (DAMPs), including mitochondrial nucleic acids [93][109]. Primarily mitochondrial DNA (mtDNA) has been recognized for its ability to activate proinflammatory responses via recognition through nucleic acid sensors (cGAS, TLR9) resulting in the induction of type I interferon signaling [109]. The proinflammatory capacity of mtDNA may depend on modifications such as oxidation or hypomethylation of CpG [109]. Release of mtDNA with the potential to induce proinflammatory responses has been reported in trauma [94] and in association with hepatocyte microvesicles [96]. On the other hand, mitochondria can also be a source of potentially immunogenic double-stranded RNA, as the mitochondrial genome is circular and transcribed in both directions [127]. Moreover, mitochondrial RNA has been shown to have a higher inflammatory potential than other types of RNA present in mammalian cells, which was attributed to a lower degree of nucleoside modifications (e.g. hypomethylation of CpG) [128]. There are several open questions regarding the role of mitochondrial nucleic acids as DAMPs: i) how are mitochondrial nucleic acids released from cells, ii) are these always derived from damaged or dying cells, iii) in what form are they released (free, in association with proteins, inside vesicles), iv) how can they be recognized by endosomally located receptors (TLRs), and v) what defines their proinflammatory potential [109].

In this thesis, I could show that $Mito_{Stress}$, but not $Mito_{Co}$, induce type I IFN signaling in endothelial cells (Manuscript Figure 4A and 4B), which was blocked in presence of the IFN-decoy receptor B18R (Manuscript Figure 4C). Surprisingly, the potential of $Mito_{Stress}$ to induce type I IFN responses was associated with mitochondrial RNA but not DNA (Manuscript Online Figure VIA) and total cellular RNA isolated from LPS-activated THP-1 cells was not able to induce such responses (Manuscript Online Figure VIC). I also observed a higher degree of

oxidation of mitochondrial ribosomal RNA isolated from $Mito_{Stress}$ than $Mito_{Co}$ (Manuscript Online Figure VID), which might explain why RNA from $Mito_{Stress}$ but not $Mito_{Co}$ was able to induce type I IFN signaling in endothelial cells (Manuscript Online Figure VIB).

These data, however, do not exclude hypomethylated mitochondrial DNA as a general inflammatory stimulus. Importantly, the highest concentrations of isolated mitochondrial RNA and DNA used for stimulation were 5µg/ml, at which a reproducible induction of type I IFN signaling in endothelial cells by mitochondrial RNA but not DNA was observed. In a previous study it was shown that 10µg/ml of mitochondrial DNA, in combination with 10nM N-formyl-Met-Leu-Phe (fMLP), were able to activate TLR9 [94], which also induces interferon signaling pathways. On the other hand, mitochondrial RNA has been shown to be inflammatory (induction of TNF and IL-8) at similar concentrations (5µg/ml) in complex with lipofectin [128], which was added in order to facilitate uptake and recognition by endosomal toll-like receptors. These data support the observation (Manuscript Online Figure VI) that mitochondrial RNA has the highest biological activity in this context. Furthermore, oxidation of mitochondrial DNA has been shown to enhance its proinflammatory potential [95]. While this suggests that the higher degree of oxidation observed in RNA isolated from $Mito_{Stress}$ may be responsible for the differences in inflammatory potential, these experiments do not proof causality. One way to address this experimentally, would be to further oxidize isolated RNA and test if this enhances its potential to induce type I IFN signaling. On the other hand, additional mitochondrial components such as N-formyl-Met-Leu-Phe (fMLP) may enhance the inflammatory potential of mitochondrial RNA similarly to what was observed for mitochondrial DNA [94], but this was not addressed in this thesis.

4.4 Linking mitochondrial activity to the proinflammatory capacity of microvesicles

Metabolic status, in particular whether a cell is relying on glycolysis or oxidative phosphorylation, is critical in defining the type of cellular immune response [89]. For instance, glycolysis is associated with short-term immune responses, whereas oxidative phosphorylation is favored in long-term immune responses [89]. LPS-activation of leukocytes promotes the switching of cellular metabolism from oxidative phosphorylation to glycolysis [89]. Additionally, LPS-stimulation is known to induce oxidative stress in cells [129].

Here, I describe that the proinflammatory potential of $Mito_{Stress}$ and MV_{Stress} was dramatically reduced when mitochondria (Manuscript Figure 3C) and microvesicles (Manuscript Figure 2B) were derived from LPS-activated but non-respiring cells (Manuscript Online Figure IIIB). Moreover, when MV-shedding cells had been stimulated with LPS in

presence of a mitochondrial ROS scavenger or pyruvate-supplementation, the proinflammatory capacity of $Mito_{Stress}$ (Manuscript Figure 4D) and MV_{Stress} (Manuscript Figure 2C and 2D) was also significantly reduced. I could show that both the mitochondrial ROS scavenger and pyruvate-supplementation reduced mitochondrial ROS production of LPS-activated THP-1 cells (Manuscript Online Figure IIIF and IIIG). In this context, pyruvate may act directly as an anti-oxidant [130] and/or by preserving the mitochondrial membrane potential and reducing mitochondrial stress [131]. Together, these data show that mitochondrial-activity determines the proinflammatory potential of microvesicles released by monocytic cells, possibly via oxidative modifications or ROS-dependent signaling. Specifically in the context of mitochondrial transfer between cells these data may explain the seemingly contradictory inflammatory [96][112] or cytoprotective [113][114] effects observed in these earlier studies. In this thesis the metabolic activity of extracellular mitochondria was not assessed and how the transfer might influence the cellular metabolism of target endothelial cells – for instance, by enhancing oxidative phosphorylation – was not studied, but would be an interesting topic for future investigations.

On the other hand, these data demonstrate that microvesicles have the ability to transmit information on cellular metabolic stress between cells. It has been shown that different subpopulations of cells in the blood rely differently on oxidative phosphorylation or glycolysis [132]. While monocytes are more or less energetically balanced between oxidative phosphorylation and glycolysis, neutrophils entirely rely entirely on glycolysis whereas platelets and lymphocytes are mostly dependent on oxidative phosphorylation for ATP-production [132]. Importantly, these cell types, including neutrophils, still react by significant oxidative stress (oxidative burst) upon activation [132]. LPS-activation of monocytic cells, which provokes oxidative burst and mitochondrial stress, drastically enhanced the release of free and microvesicle-encapsulated mitochondrial. Therefore, it is possible that detection of extracellular mitochondria under non-traumatic conditions (i.e. release by viable cells) could be a biomarker for cellular metabolic stress culminating in oxidative burst. However, the experiments performed on the thesis did not support a direct role of mitochondrial ROS production in the release of mitochondria from cells, as reduction of mitochondrial ROS by scavenging (mitoTEMPO treatment) or pyruvate supplementation did not affect the release of mitochondria and microvesicles (Manuscript Online Figure IIID-IIIG). Therefore, it is likely that the responsible signaling pathway lies upstream of mitochondrial ROS production and the release of mitochondria is not triggered by mitochondrial ROS itself. At this point, the use of extracellular mitochondria as biomarkers for cellular stress additionally faces the challenge that free mitochondria are unlikely to inherit plasma membrane proteins from their parental cells, making it necessary to find other markers to determine their cellular origin. On the other hand, microvesicle-encapsulated mitochondria are currently difficult to detect in the circulation and it

would be necessary to either i) use a combination of membrane-permeable mitochondria-specific dyes and antibody-based detection of plasma membrane proteins associated with the microvesicles, or ii) develop a suitable permeabilization and fixation protocol of microvesicles in order to detect plasma membrane proteins on the outside and mitochondrial proteins on the inside of the vesicle.

4.5 Linking mitochondrial content to the proinflammatory activity of microvesicles

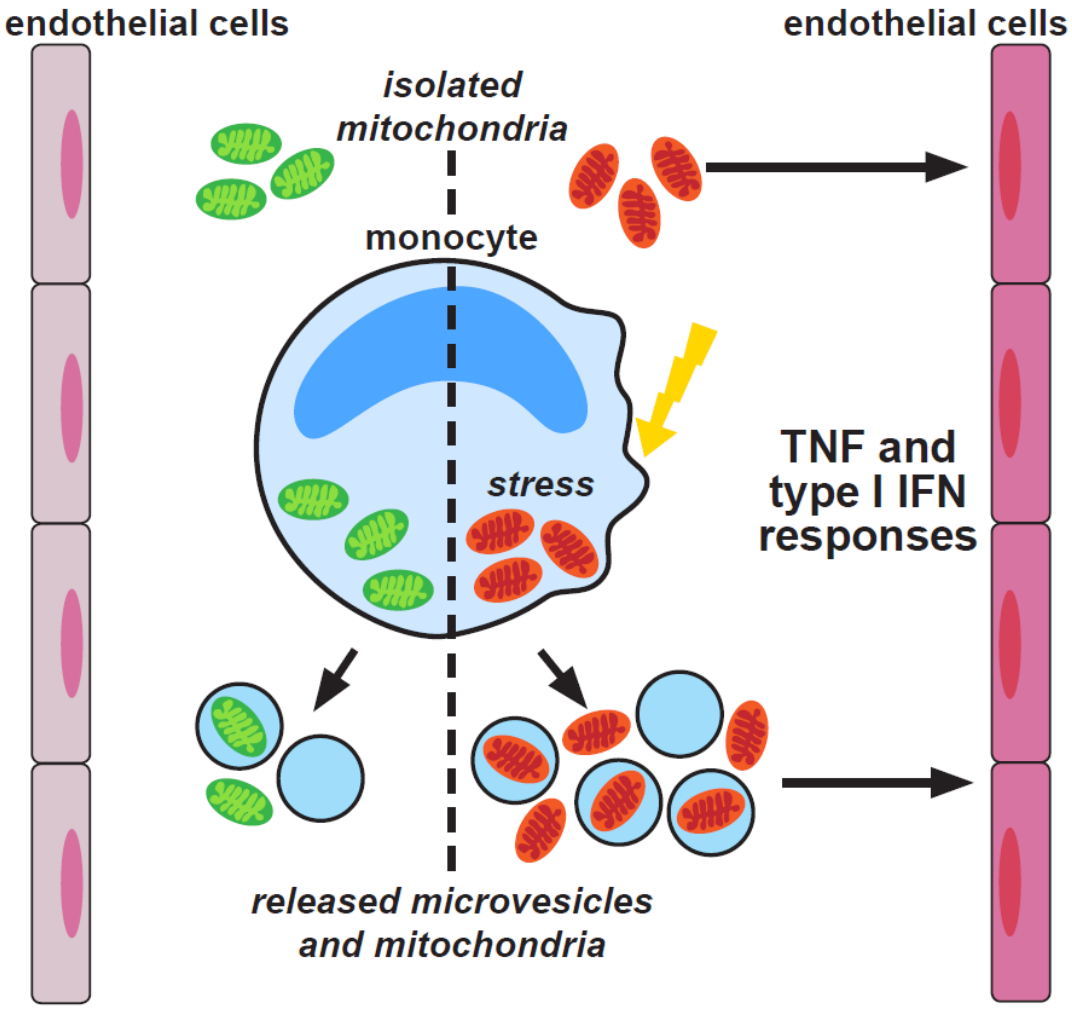
Similarly to stressed mitochondria, MV_{Stress}, but not MV_{Co}, were able to induce type I interferon and TNF-signaling in endothelial cells (Manuscript Figure 4E). The ability of MV_{Stress} to induce both of these signaling pathways was significantly reduced upon depletion of free mitochondria (TOM22+ vesicles) (Manuscript Figure 4F). The remaining TOM22-depleted MV_{Stress} still contained microvesicles enriched in mitochondrial content (Manuscript Online Figure IVC and IVD). Importantly, the capacity of TOM22-depleted MV_{Stress} to induce TNF and type I IFN responses was significantly reduced in presence of Infliximab and B18R, respectively (Manuscript Online Figure IXA and IXB). These data show that the proinflammatory capacity of MV_{Stress} can be linked to the presence of free mitochondria and microvesicles enriched in mitochondrial content.

Moreover, in a human endotoxemia model, in which 15 healthy volunteers receive low-dose (2ng/kg) injections of LPS, I could document an increase of circulating TOM22+ vesicles 2h after injection (Manuscript Figure 4G). Interestingly, the kinetics of TNFα levels detected in plasma of these subjects overlapped with the changes in TOM22+ vesicle levels (Manuscript Figure 4H). Furthermore, significantly increased levels of soluble VCAM, a measure for endothelial cell activation, were detected 8h after injection (Manuscript Figure 4I). It was possible to show that microvesicles, isolated from plasma obtained 2h after LPS-injection, were able to induce robust activation of type I IFN and TNF responses in endothelial cells (Manuscript Online Figure XI). These *in vivo* data support the *in vitro* findings that microvesicles released by LPS-activated cells induce type I IFN and TNF responses.

However, as I have also discussed in the previous chapter, the detection of free mitochondria (TOM22-positive events) does not inform about the cellular origin of these mitochondria, and the specific detection of microvesicle-encapsulated mitochondria was not possible at this point. In order to better elucidate the mechanism and cellular origin, further studies may test the effects of different damage- or pathogen-associated molecular patterns (DAMPs/PAMPs) on the release of mitochondria and on their inflammatory capacity by *ex vivo* stimulation of whole-blood or isolated primary cells (e.g. primary monocytes or macrophages).

4.6 Uptake of microvesicles with mitochondrial content

Different mechanisms of extracellular vesicle signaling between cells have been described [12]. These include surface binding of the vesicles to target cells via adhesion molecules, as well as their uptake by macropinocytosis, phagocytosis, clathrin-, caveolin- or lipidraft-mediated endocytosis, and membrane fusion [12][133]. Wang et al [115] previously demonstrated that microvesicles released by LPS-activated monocytic cells bind to endothelial cells. In this thesis, I could show that mitotracker-positive MV_{Stress} are taken up by endothelial cells (Manuscript Online Figure XB and XC). Interestingly, disintegration of MV_{Stress} by sonication (Manuscript Online Figure XA) reduced both their uptake (Manuscript Online Figure XB and XC) and their ability to induce a type I IFN response, while not reducing their capacity to induce a TNF-response (Manuscript Online Figure XD). Considering that TNF α was identified as one of the active components of MV_{Stress}, this observation is consistent with the known recognition of TNF α by TNF-receptors expressed on the cell surface. On the other hand, the significantly reduced type I IFN response might be explained by the requirement for uptake of nucleic acids embedded in microvesicles and their intracellular sensing by pattern recognition receptors [134], similar to RNA-lipofectin complexes [128]. Additionally it should be considered that transport of RNA within free mitochondria and microvesicle-encapsulated mitochondria, may protect it from degradation by nucleases in the circulation.



Scheme 5. Graphical Abstract. (1) LPS-activated monocytic cells release mitochondria and mitochondria embedded in microvesicles. (2) Both, free mitochondria and microvesicle-embedded mitochondria contribute to the ability of microvesicles to activate endothelial cells (3) This proinflammatory capacity is determined by the mitochondrial activity of parental cells rather than the mere presence of mitochondrial content. (4) Free mitochondria and microvesicle-embedded mitochondria from stressed monocytic cells induce TNF and type I IFN responses in endothelial cells.

4.7 Summary and Outlook

In summary, I observed the release of mitochondria and mitochondria encapsulated in microvesicles by LPS-activated THP-1 monocytic cells and that these mitochondria constitute a novel subset of extracellular vesicles (Manuscript Figure 1). I showed that the proinflammatory capacity of mitochondria and microvesicle-encapsulated mitochondria is determined by the mitochondrial activity of their parental cells (Manuscript Figures 2 and 3). This finding is in sharp contrast to the current view that the mere presence of mitochondrial material confers proinflammatory potential. Furthermore, I demonstrated that mitochondria and microvesicle-encapsulated mitochondria released from stressed cells induce both TNF and type I IFN responses in endothelial cells (Manuscript Figure 4).

Unexpectedly, I found that the principal components responsible for this proinflammatory activity were mitochondria-associated TNF and mitochondrial RNA. It will require further experiments to elucidate the nature of TNF-mitochondria association, as well as the differences between mitochondrial RNA in stressed mitochondria and non-stressed mitochondria defining its interferonogenic capacity. In this thesis, an antibody-based immunoprecipitation targeting 8-hydryoxyguanine (8-oxoG) was employed to assess the degree of oxidative modification of RNA. While this experiment showed that RNA isolated from stressed mitochondria was more oxidized, it does not proof that 8-oxoG modifications are responsible for the increased interferonogenic capacity.

These findings provide novel insights into the mechanisms by which microvesicles and mitochondria can mediate inflammation and the basis for the development of new therapeutic approaches targeting mitochondria (e.g. mitochondrial ROS production or stress) and their release by parental cells or uptake by target cells, which will require detailed studies of the responsible mechanisms.

This work may be relevant to further understanding of the role of mitochondria in inflammatory diseases involving TNF and interferon signaling, mitochondrial stress or mitochondrial DAMPs, such as trauma [94], atherosclerosis [135], psoriasis [136], systemic lupus erythematosus [137], rheumatoid arthritis [138] and neurodegenerative diseases [139].

Further work should explore the release mechanisms and the pathophysiological relevance of free mitochondria and microvesicle-encapsulated mitochondria and their potential as a biomarker that could be easily obtained from the circulation, e.g. by studying mouse models with cell type-specific, fluorescently-tagged mitochondria and primary cells derived from different genetic backgrounds.

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Curriculum Vitae

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Conference Poster Presentations

2015 February	**Malondialdehyde (MDA) Microparticles (MVs) as modulators of inflammation and thrombosis** CCHD Bridging the gap Symposium, *Vienna, Austria*
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2016 May	**The generation of malondialdehyde (MDA) positive microvesicles (MVs)** EAS (European Atherosclerosis Society) Congress, *Innsbruck, Austria*
2016 June	**Mitochondrial activity is a major contributor to the pro-inflammatory capacity of microvesicles** Keystone Symposium – Exosomes/Microvesicles: Novel Mechanisms of Cell-Cell Communication, *Keystone, Colorado, USA*
2016 September	**Mitochondrial activity is a major contributor to the pro-inflammatory capacity of microvesicles** CCVM (Center for Cardiovascular Medicine) kickoff Symposium, *Vienna, Austria*
2017 April	**Mitochondrial activity is a major contributor to the pro-inflammatory capacity of microvesicles** CCHD Bridging the gap Symposium, *Vienna, Austria*
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2017 April	**Pharmacological screening approach to understand release mechanisms of monocytic microvesicles enriched in mitochondrial content** SFB54 InThro Retreat, *Vienna, Austria*
2017 June	**Mitochondrial activity is a major contributor to the pro-inflammatory capacity of microvesicles** 22^{e} colloque provincial Immuno-Inflammation, *Eastman, Québec, Canada*
2018 April	**Mitochondria of parental cells control the capacity of monocyte-derived microvesicles to activate endothelial cells** FCVB meeting, *Vienna, Austria*
2018 May	**Screening approach to target the release of monocytic microvesicles carrying malondialdehyde (MDA)-adducts** SFB54 InThro Retreat, *Vienna, Austria*
2019 June	**Characterizing the release and inflammatory activity of microvesicles carrying malondialdehyde (MDA)-adducts** SFB54 InThro Retreat, *Vienna, Austria*
2019 June	**The number, content and pro-inflammatory potential of microvesicles and extracellular mitochondria released by LPS-activated monocytic cells: effect of glucose availability** SFB54 InThro Retreat, *Vienna, Austria*

Conference Talks

2016 February	**The generation of malondialdehyde(MDA)-positive microvesicles** Joint PhD Symposium "Bridge Gaps Cross Roads", *Vienna, Austria*
2016 March	**The generation of malondialdehyde(MDA)-positive microvesicles (MVs)** SFB54 InThro Symposium, *Vienna, Austria*
2017 April	**Mitochondrial activity is a major contributor to the pro-inflammatory capacity of microvesicles** EAS (European Atherosclerosis Society) Congress, Prague, Czech Republic
2018 February	**Mitochondria of parental cells control the capacity of monocyte-derived microvesicles to activate endothelial cells.** GTH meeting, *Vienna, Austria*
2018 May	**Mitochondria contribute to content and pro-inflammatory capacity of monocytic microvesicles** SFB54 InThro Symposium, *Vienna, Austria*
2018 June	**Mitochondria contribute to content and pro-inflammatory capacity of monocytic microvesicles** KILM Summer Symposium, *Vienna, Austria*
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- Experience as private tutor (mathematics) and university tutor (biochemistry course, supervision of bachelor thesis/student)

List of publications

- **Puhm F,** Afonyushkin T, Resch U, Obermayer G, Rohde M, Penz T, Schuster M, Wagner G, Rendeiro AF, Melki I, Kaun C, Wojta J, Bock C, Jilma B, Mackman N, Boilard E, Binder CJ. Mitochondria Are a Subset of Extracellular Vesicles Released by Activated Monocytes and Induce Type I IFN and TNF Responses in Endothelial Cells. (2019) *Circ Res.*

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