Neutrophil microvesicles and their role in disease

Abstract

Microvesicles are formed through shedding from the plasma membrane, a process shared by almost all human cells. Microvesicles are highly abundant and have been detected in blood, urine, cerebrospinal fluid, and saliva. They contain a library of cargo derived from their parental cell during formation, including proteases, micro-RNAs and lipids and delivery of this parental cell-derived cargo to other cells can alter target cell function and drive disease. Cell specific molecules on the surface of microvesicles, obtained during microvesicle formation, allows their parental cell to be identified and populations of microvesicles to be investigated for roles in the pathogenesis of various diseases. For instance, recent work by our group has identified a role for neutrophil microvesicles in atherosclerosis. Microvesicle profiles could in future be associated with certain diseases and act as a biomarker to allow for earlier diagnosis. This short review will discuss some of the processes central to all microvesicles before focusing on neutrophil microvesicles, their potential role in cardiovascular disease and the mechanisms that may underpin this.

1. Introduction

Microvesicles (MVs) are membrane-derived extracellular vesicles formed by budding of the plasma membrane (Ratajczak and Janina, 2020; Yáñez-Mó et al., 2015) and are produced by a wide range of cell types including eosinophils (Akuthota et al., 2016), monocytes (Li et al., 2010), macrophages (Li et al., 2010), erythrocytes (Gkaliagkousi et al., 2019), platelets (Gkaliagkousi et al., 2019), and neutrophils (Gomez et al., 2020). MVs can contain an extensive and diverse repertoire of parental cell-derived cargo including anti-fungal proteins within neutrophil MVs (NMVs) (Shopova et al., 2020), tumour necrosis factor (TNF) in macrophage-derived MVs (Soni et al., 2016) and a wide range of microRNAs (miRNAs) in platelet (Qu et al., 2020), keratinocyte (Li et al., 2019), neutrophil (Gomez et al., 2020) and endothelial-derived MVs (Zhang et al., 2020).

MVs have important roles in cell-cell communication (Desrochers et al., 2016; Ratajczak and Ratajczak, 2021) and have been associated with diseases including diabetes (Bergen et al., 2020; Zhang et al., 2018), chronic obstructive pulmonary disease (COPD) (Soni et al., 2021), chronic kidney disease (CKD) (Fonseca et al., 2020), cancer (Das et al., 2019) and cardiovascular disease (CVD) (Gkaliagkousi et al., 2021; Huo et al., 2021; Plasín-Rodríguez et al., 2020; Sionis et al., 2018).

Due to this link with several diseases, MVs have become the subject of increasing research interest in recent years. One population of MVs to receive substantial attention are NMVs. Whilst generally considered short lived cells, neutrophil abundance in circulation and role in inflammatory diseases, including CVD (Gaul et al., 2017; Silvestre-Roig et al., 2020) has resulted in considerable interest in the role their MVs may also play in disease, particularly CVD (Chiva-Blanch et al., 2019; Sionis et al., 2018; Suades et al., 2019).

2. Neutrophil microvesicle stimuli

NMVs are produced constitutively, but also in response to a broad range of stimuli *in vitro* including fMLP (Hong et al., 2012), TNF-α (Johnson et al., 2013; Rhys et al., 2018), PMA and LPS (Pluskota et al., 2008). NMVs are also elevated in response to a high-fat diet in humans and mice (Gomez et al., 2020). It is worth noting that the environment at the time of NMV biogenesis likely dictates the capabilities of the NMVs, resulting in NMVs with disparate functions. For example, NMVs from resting neutrophils may exert more anti-inflammatory effects, reducing cytokine and reactive oxygen species (ROS) production in neutrophils, whereas apoptotic neutrophils or neutrophils exposed to opsonised particles produce more pro-coagulant and pro-inflammatory NMVs, respectively (Kolonics et al., 2021). Therefore, NMVs may possess different functions depending on their environment.

3. Neutrophil microvesicle biogenesis

MVs differ from another class of extracellular vesicle (EV), exosomes, broadly in terms of size (exosomes range from 30-150nm) and biogenesis (Yáñez-Mó et al., 2015). Exosomes are generated inside the cell, budding from early endosomes (Doyle and Wang, 2019) whilst MVs are generated from outward budding from the cell plasma membrane (Ratajczak and Janina, 2020; Yáñez-Mó et al., 2015).

Whilst specific research into NMV biogenesis is limited, the process of MV biogenesis is ubiquitous and unlikely to differ substantially between cells. Transient increases in intracellular calcium concentration, alterations in plasma membrane lipid composition and associated changes in membrane curvature, alongside changes in cytoskeletal organisation occur following stimulation and are central to MV biogenesis (Morel et al., 2011; Verderio et al., 2018). Cytosolic Ca^{2+} levels correlate with MV biogenesis in malignant breast cancer cells (Taylor et al., 2020) and treatment with calcium ionophores, resulting in increased Ca^{2+} concentration, leads to MV production (Roseblade et al., 2015). Structural rearrangements of the plasma membrane are calcium dependent. Ca^{2+} inhibits lipid transporting enzymes, flippases (Yu et al., 2018b), but is required by calpain which is linked to changes in cytoskeletal structure (figure 1). Decreases in flippase activity correlate with increased cytoplasmic Ca^{2+} levels, increased PS exposure and MV production. These correlations were abolished following pre-treatment of ECs with a calcium channel blocker (Nagata et al., 2016; Yu et al., 2018b) and similarly, inhibition of calpain reduced MV production (Giannella et al., 2021; Nolan et al., 2008; Välimäki et al., 2016).

Translocation of specific lipids between leaflets of the plasma membrane alters the curvature of the membrane. Flippases retain phosphatidylserine (PS), a negatively charged phospholipid normally asymmetrically distributed on the inner leaflet of the plasma membrane, within the cell whereas it is abundant on the surface of MVs (Kou et al., 2019; Wang et al., 2018; Yu et al., 2018a). When PS surface expression is disrupted, for instance in mice deficient in transmembrane protein 16 (TMEM16), a scramblase involved in PS exposure, MV biogenesis is impaired (Fujii et al., 2015). The conical structure of ceramide is also believed to induce membrane curvature and facilitate MV biogenesis. Endothelial cell (EC) MVs and circulating MVs (cMVs) present in the bloodstream are rich in ceramide and their

release dependent on the action of a-SMase, an enzyme responsible for catalysing the conversion of the membrane lipid sphingomyelin (SM) to ceramide (Serban et al., 2016).

Due to the mechanism through which MVs are generated they retain and express on their surface the same molecules as their parent cells making it possible to identify their cellular origin by analysing the expression marker proteins expressed on their surface. Neutrophil markers CD66b, CD11b, L-selectin and CD18 are all found expressed on the surface of NMVs (Gomez et al., 2020; Hong et al., 2012; Nolan et al., 2008). This allows for specific populations of MVs to be quantified and to validate the purity of preparations of MVs produced *in vitro* from isolated cells. This ability has made research into NMVs, and MVs more broadly possible.

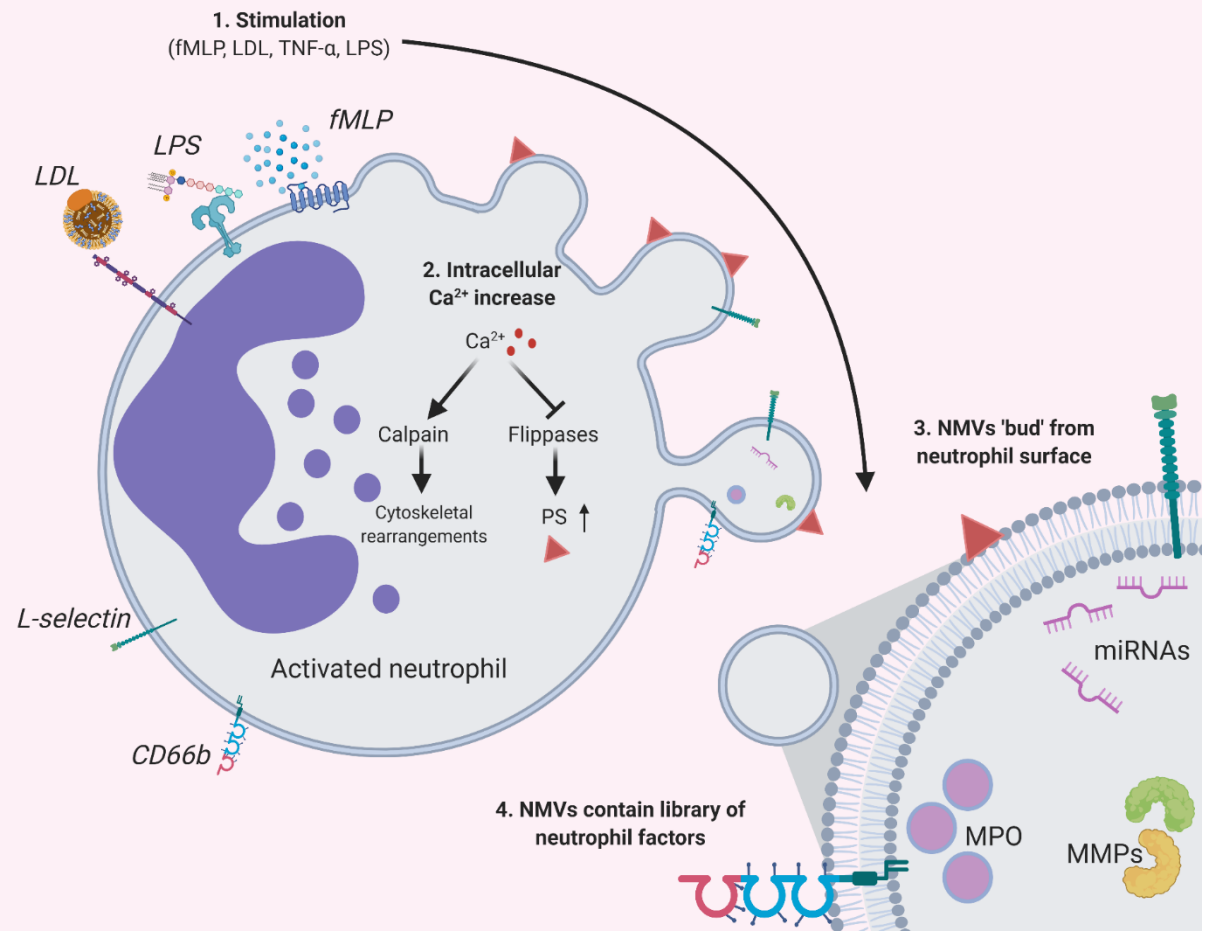

Figure 1 - Neutrophil microvesicle stimulation and formation. *Neutrophil microvesicles (NMVs) can be produced in response to a variety of stimuli including low-density lipoprotein (LDL), lipopolysaccharide (LPS) and N-formylmethionyl-leucyl-phenylalanine (fMLP) which trigger intracellular calcium ion increases and associated changes in the activity of enzymes governing cytoskeletal rearrangements and lipid translocation. Lipid accumulation at the cell membrane enhances membrane curvature and facilitates specific cargo loading into forming NMVs which are released via budding of the membrane.* ***Created with Biorender.com.***

4. Neutrophil microvesicle recruitment and internalisation

Neutrophils play an important role in cardiovascular inflammation. The interaction between neutrophils and ECs of the vasculature has been known about for many years and the cell surface receptors that underpin this interaction are well characterised. NMVs, because of their biogenesis, express these same receptors allowing their recruitment to and internalisation by ECs, providing a potential mechanism through which they may also modulate vascular inflammation.

Interestingly, NMVs are not recruited uniformly across the vasculature, instead accumulating at sites prone to atherosclerosis for example, greater numbers are observed at the atheroprone inner curvature of the aorta compared to atheroprotected outer curvature region in $ApoE^{-/-}$ mice (Gomez et al., 2020) an interaction governed by the binding of NMV expressed CD18 to EC expressed ICAM-1 (Gomez et al., 2020; Hong et al., 2012). Oscillatory shear stress (OSS), a feature of these atheroprone regions, induces EC inflammation and expression of the cell surface receptor ICAM-1 compared to ECs cultured statically or under high shear stress (HSS). OSS was confirmed to increase NMV recruitment to ECs *in vitro* (Gomez et al., 2020). In addition, following internalisation NMVs increased expression of EC surface receptors ICAM-1 and VCAM-1, alongside the chemokine CCL2 via RelA further increasing their recruitment.

In addition to ECs, NMVs also interact with platelets through αMβ2 binding to GPIbα, and PSGL-1 binding P-selectin (Pluskota et al., 2008; Rossaint et al., 2016). Platelets appear to induce NMV production through a GPIbα- and P-selectin-dependent mechanism and modulate NMV contents. For example, neutrophils co-cultured with platelets produced NMVs with higher arachidonic acid contents than neutrophils cultured alone (Rossaint et al., 2016). Fluorescently labelled platelets and NMVs co-localised and co-culture with NMVs increased platelet thromboxane (TxA_2) production indicating NMVs are internalised by platelets and can influence their function. TxA_2 production reduced when NMVs were pre-treated with blocking Mac-1 antibody implicating a Mac-1-dependent mechanism in NMV internalisation (Rossaint et al., 2016).

5. Neutrophil microvesicle cargo and the link to cardiovascular disease

Leukocyte MVs (LMVs) have long been identified within atherosclerotic plaques (Canault et al., 2007) and cMVs are decreased in patients treated with statins (Suades et al., 2013). Higher proportions of leukocyte cMVs have been reported in patients with familial hypercholesteremia (FH), a condition which increases the risk of CVD, compared with healthy controls (Suades et al., 2014). FH patients with confirmed atherosclerotic plaques exhibit higher total Annexin V positive cMV levels and higher levels of cMVs of neutrophil, granulocyte, platelet, and EC origin compared with patients without atherosclerotic plaques (Chiva-Blanch et al., 2019). This cluster of cMVs improved predictions of plaque presence. There is also evidence to suggest cMVs are also linked to severity of CVD symptoms. FH patients who later developed a cardiovascular event (CVE) demonstrated significantly higher cMV levels of activated platelet, pan-leukocyte, and neutrophil origin (Suades et al., 2019) and levels of lymphocyte, activated platelet and neutrophil cMVs were associated with mortality at follow-up (Suades et al., 2019**).** Additionally, cMVs of granulocyte and neutrophil origin were elevated in STEMI patients with cardiogenic shock (CS) compared with non-CS STEMI patients (Sionis et al., 2018) and

platelet and EC MVs were elevated in severe hypertensive patients compared with controls (Preston et al., 2003). Diabetes mellitus (DM) is a pro-inflammatory, pro-thrombotic disease and a risk factor for CVD. Platelet and erythrocyte MVs were found to be elevated in type II DM (Gkaliagkousi et al., 2019) and platelet, EC and monocyte MVs were elevated in type I DM (Bergen et al., 2020; Nomura et al., 2004). Together, these studies provide good evidence of a link between cMVs and CVD (Table 1).

Disease	MV origin	References
Atherosclerosis	neutrophil/ granulocyte, platelet, and EC	(Chiva-Blanch et al., 2019) (Gomez et al., 2020)
Familial hypercholesteremia	Leukocyte	(Suades et al., 2014)
Cardiogenic shock	Granulocyte/neutrophil	(Suades et al., 2014)
Hypertension	Platelet/EC	(Preston et al., 2003)
Diabetes Mellitus	Platelet/erythrocyte/ EC/monocyte	(Gkaliagkousi et al., 2019; Nomura et al., 2009; Nomura et al., 2004)
Peripartum cardiomyopathy	Monocyte	(Walenta et al., 2012)
Coronary artery disease	Platelet	(Gkaliagkousi et al., 2021)

Table 1 - Link between microvesicles and cardiovascular disease*. Summarises the link between elevated levels of MVs from multiple cell origins and cardiovascular disease.*

There is also evidence to suggest NMVs exert effects that contribute to CVD. The role of neutrophils in general in CVD is becoming increasingly well understood. Recently, the release of neutrophil extracellular traps (NETs) consisting of chromatin associated with cytosolic and granule proteins, in a process known as NETosis, has been implicated in EC dysfunction, thrombosis and atherosclerosis (Döring et al., 2020; Folco et al., 2018; Franck et al., 2018). NETs increase EC tissue factor expression and activity leading to enhanced clotting of plasma (Folco et al., 2018). NMVs contain many of the same granule proteins as NETs and recent studies have indicated cytoskeleton disassembly and NMV shedding occurs in neutrophils just prior to NETosis (Thiam et al., 2020). However, any link between NMVs and NETosis in terms of function is remains unclear.

A recent publication from our group describes a specific mechanistic link between NMVs and atherosclerosis (Gomez et al., 2020). NMVs isolated from mice fed a high-fat Western diet were enriched in micro-RNA (miR) 155 (miR-155) and miR-223 compared to mice fed chow. NMVs were preferentially recruited to and internalisation by ECs in atheroprone regions of the aorta of $ApoE^{-/-}$ mice. Delivery of miR-155 drove expression of pro-inflammatory genes for adhesion molecules ICAM-1 and VCAM-1, and the cytokine CCL2 through NF-κB signalling and enhanced monocyte recruitment

(figure 2) (Gomez et al., 2020). Alongside miR-155, NMVs were also enriched in miR-223. Interestingly, miR-155 has been identified as a biomarker of ischemic stroke, in this instance associated with MVs of EC origin (Zhang et al., 2020) and both miR-155 and miR-223 have been associated with coronary heart disease (CAD) (Singh et al., 2020) and the occurrence of CVE in peripheral artery disease (PAD) patients (Barbalata et al., 2020).

In addition, NMVs have other important effects on EC function and integrity. NMVs contain myeloperoxidase (MPO) shown to disturb EC morphology and membrane integrity (Pitanga et al., 2014). Interestingly, MPO expressed on NMV surfaces had enhanced enzymatic activity compared to soluble protein, suggesting NMVs may enhance proteolytic activity (Slater et al., 2017). NMVs also inhibit wound healing response and proliferation (Slater et al., 2017), and increase EC senescence (El Habhab et al., 2020). Treatment of ECs with rat splenocyte MVs (SMVs) generated by LPS stimulation increased senescence-associated β-galactosidase (SA-β-Gal) activity and senescence markers, P16 and P21 (El Habhab et al., 2020). When rat NMVs were specifically immuno-depleted SA-β-Gal activity decreased by half (El Habhab et al., 2020). NMVs were found to activate the endothelium through CD18-specific interactions increasing ICAM-1, ROS, IL-6, and IL-8 production (Hong et al., 2012). NMVs produced in response to fMLP stimulation are also rich in matrix metalloproteinase 9 (MMP-9) and following adhesion to epithelial cell monolayers have been shown to disrupt intercellular adhesions through the MMP-9-dependent degradation of desmoglein 2 (Dsg-2) (Butin-Israeli et al., 2016). Degradation of Dsg-2 facilitates neutrophil transepithelial migration, an important process in many inflammatory diseases (Butin-Israeli et al., 2016).

Furthermore, apoptotic neutrophils and neutrophils treated with opsonised particles, but not resting neutrophils, produced NMVs which decreased coagulation time of citrated plasma (Kolonics et al., 2021) suggesting a pro-coagulant capacity under some situations. The mechanism for this is not clear; however there is evidence to suggest neutrophils may express tissue factor (Darbousset et al., 2012) which may become incorporated into NMVs. Together, these findings are suggestive of a role of NMVs, in the pathogenesis of CVD.

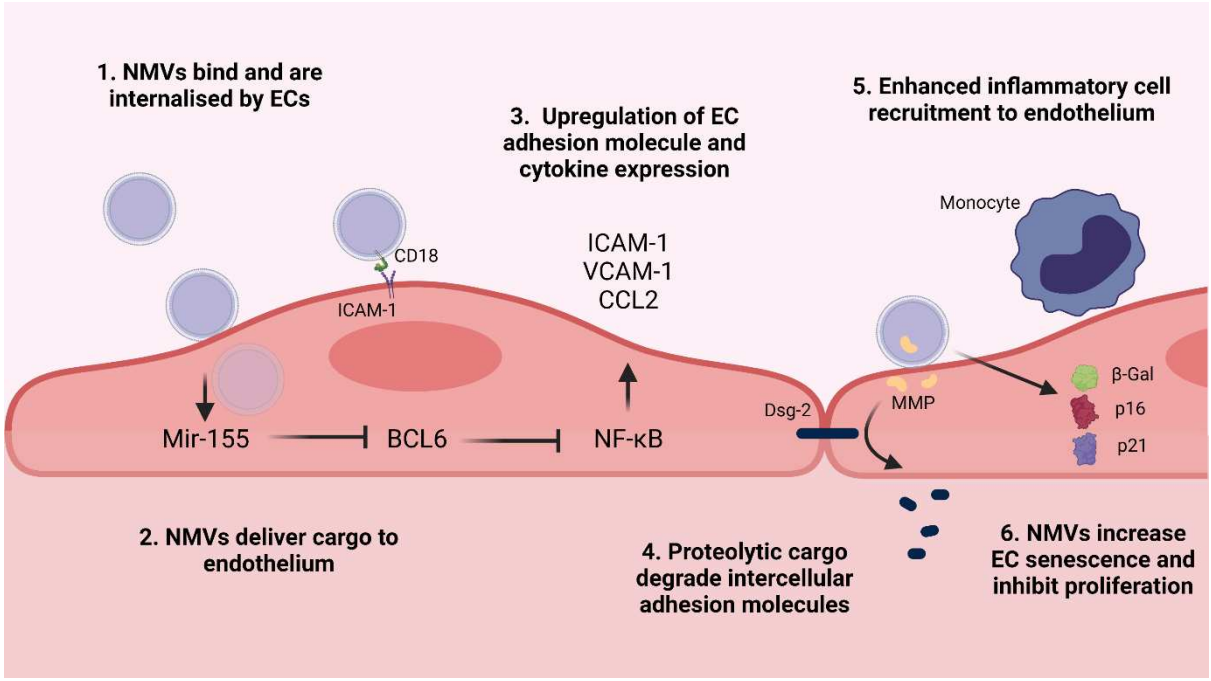

Figure 2 - NMVs drive endothelial cell dysfunction. *NMVs are recruited to endothelium via ICAM-1 and CD18 interactions where they are internalised and deliver cargo, including micro-RNA-155 (MiR-155). MiR-155 inhibits B-cell lymphoma 6 (BCL6) a transcriptional repressor of NF-κB. NF-κB drives inflammatory activation of the endothelium and increased intracellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1) and C-C motif chemokine ligand 2 (CCL2) expression leading to inflammatory cell recruitment to the endothelium. NMVs contain matrix metalloproteinases (MMP) capable of cleaving intercellular adhesion molecule, desmoglein-2 (Dsg-2). NMVs have also been reported to increase EC senescence markers β-Galactosidase (β-Gal), p16 and p21.* ***Created with Biorender.com.***

Whilst research into NMVs has largely uncovered a pro-inflammatory effect on ECs, NMVs have been shown to exert anti-inflammatory effects too. For instance, neutrophils adhered to human umbilical vein ECs (HUVECs) released NMVs that when incubated with fresh neutrophils reduced their adhesion to a HUVEC monolayer in a mechanism dependent on annexin 1 (Dalli et al., 2008).There is also evidence that NMVs impart anti-inflammatory effects in other cell types. For instance, NMVs increase release of IL-2, IL-12 and TGF-β whilst reducing TNF-α and IFN-γ release in natural killer cells, in a process dependent on NMV phosphatidylserine (Pliyev et al., 2014). NMVs also limited activation of macrophages in a mouse model of arthritis (Rhys et al., 2018) and induced the release of stored TGF-β (Eken et al., 2013), an important anti-inflammatory cytokine. NMVs also alter expression of IL-8 and PGE2 in chondrocytes, resulting in cartilage protection in rheumatoid arthritis (Headland et al., 2015).

6. Therapeutic implications

The association between NMVs, and MVs more broadly, with CVD makes them potentially valuable biomarkers of disease presence (Chiva-Blanch et al., 2019) and predictors of future CVE (Sionis et al., 2018; Suades et al., 2019). Relatively simple blood tests may in future be used to detect elevated levels of cMVs to predict disease and monitor treatment effectiveness. A more comprehensive understanding of how MVs deliver their cargo to specific cells may allow for more targeted delivery of drugs to fight disease. It is already well established that lipid nanoparticles are effective vectors for

the transport of some drugs. For example, the Pfizer-BioNTech BNT162b2 mRNA COVID-19 vaccine utilises this technology to deliver mRNA specifically to the cytoplasm of cells where it can be translated into the spike protein and was the first mRNA vaccine to be granted emergency use authorisation by the FDA (Polack et al., 2020). Incorporating drugs into lipid nanoparticles may protect nucleic acid based therapeutics from degradation and loading cell surface molecules onto lipid nanoparticles may allow for more targeted delivery of drugs to specific cell types.

Designing therapeutics to reduce levels of cMVs found to be elevated in many chronic diseases may provide an additional treatment option for patients. There is evidence to suggest statins reduce generation of leukocyte, platelet and EC MVs as well as markers of parental cell activation (Suades et al., 2013). Combination therapy consisting of statins and the angiotensin II receptor agonist Losartan reduces monocyte-derived MVs in hypertensive patients with type II diabetes mellitus (Nomura et al., 2004) and platelet anti-aggregant reduces EC MVs in patients with CKD (García-Menéndez et al., 2019). If a causal link between elevated cMVs and chronic disease is established, a reduction in cMV concentrations is likely to offer therapeutic benefits. It is encouraging that several widely available and generally safe drugs currently in use appear to achieve this reduction.

7. Conclusion

More research is required into MVs in disease. Their formation in response to stimuli and their ability to deliver specific cargo to target cells makes MVs an interesting subject area and a promising field for future therapeutics. A better understanding of MV populations in disease could allow for early detection of disease and a better understanding of the mechanisms that control the packaging of cargo into MVs and their delivery to target cells may facilitate more specific and effective delivery of drugs to treat these diseases.

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