Neutrophil-derived extracellular vesicles induce renal endothelial inflammation in critical illness

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Abstract

Background

Circulating neutrophil-derived extracellular vesicles (NEVs) are elevated in critically-ill patients during systemic inflammatory states where they may work as a hidden reservoir of inflammation. However, there is a paucity of data examining the role of NEVs in the pathophysiology of acute kidney injury, specifically glomerular endothelial inflammation. We hypothesised that circulating NEVs cause significant glomerular endothelial inflammation and are thus fundamental to the pathophysiology of acute kidney injury in critically-ill patients.

Methods

To assess the role of NEVs in the development of systemic microvascular and renal glomerular inflammation, NEVs were isolated from lipopolysaccharide-treated healthy donor blood or critically-ill COVID-19 patients with acute respiratory distress syndrome. NEVs were incubated for 4 hours in our in vitro models comprising human umbilical vein endothelial cells or renal glomerular endothelial cells, co-cultured with peripheral blood mononuclear cells.

Results

NEVs were internalised by monocytes leading to their activation via the p38 pathway (NEVs vs untreated, p38 median fluorescence intensity (MFI), 694 vs 459, p=0.006), causing the release of tumour necrosis factor (NEVs vs untreated, TNF pg ml^{-1}, 676 vs 27, p=0.003), which subsequently increased cell adhesion molecule expression on human umbilical vein endothelial cells (NEVs vs untreated, E-Selectin MFI, 4120 vs 1438, p=0.008) or human renal glomerular endothelial cells (NEVs vs untreated, E-Selectin MFI, 2960 vs 931, p=0.003). These NEVs contained substantial amounts of matrix-metalloproteinase-8 and 9, inhibitors of which effectively ablated NEV-induced activation of the endothelial cells.

Conclusions

We have demonstrated a comprehensive pathway detailing how circulating NEVs cause significant renal endothelial inflammation in a monocyte-dependent fashion. Our data suggest a role for circulatory NEVs in the pathophysiology of acute kidney injury in critically-ill patients, including patients with COVID-19.

Introduction

Acute kidney injury (AKI) is defined as an abrupt decrease in kidney function[1] and commonly occurs in critically-ill patients with sepsis or acute respiratory distress syndrome (ARDS) as a part of a systemic inflammatory response.[2] The mortality of patients with AKI in an intensive care unit is high, with stepwise increases in mortality with increasing AKI severity[3]. This was particularly apparent during the COVID-19 pandemic where COVID-19 patients with AKI had significantly higher mortality than those without AKI.[4]

Attempts to elucidate the mechanism of AKI following systemic inflammation have largely focused on the tubular epithelium/endothelium where damage-associated molecular patterns/pathogen-associated molecular patterns, filtered via glomeruli, impair tubular epithelium and cause peritubular endothelial injury, resulting in interstitial oedema and leukocyte migration with subsequent tubular epithelial damage.[5] However, it is now increasingly appreciated that the glomerulus is a crucial site of injury during the development of AKI following systemic inflammation. Postmortem renal biopsies in septic patients demonstrated numerous infiltrating inflammatory cells such as monocytes within the glomerular space.[6] Indeed, collapsing glomerulopathy and endothelial injury or thrombotic microangiopathy were the most common findings besides acute tubular injury in COVID-19 patients.[7] Glomerular endothelial cell activation occurs in sepsis which can lead to increased vascular permeability and leakage, causing interstitial oedema[5] and this is demonstrated in lipopolysaccharide (LPS)-treated mice where the average fenestrae diameter of glomerular endothelial cells increased by 3-fold.[8] Whilst systemic inflammation plays a significant role in glomerular inflammation and injury in critically-unwell patients,[9] experimental treatments that target circulating mediators in AKI have not translated into clinical practice.[10] Thus, there is an urgent, unmet need to understand the mechanisms underlying the pathophysiology of AKI, which could identify new therapeutic targets and lead to novel therapeutic strategies.

Extracellular vesicles (EVs) (previously termed microvesicles) are membrane-circumscribed vesicles smaller than 1000nm released from injured and inflamed cells.[11] These vesicles transport a diverse array of molecular cargo and offer an alternative route for inter-cellular communication across both short and long distances more efficiently than soluble mediators.[12–14] A defining trait of EVs is their ability to transport a variety of molecules

to target cells within a protective membrane, safeguarding those mediators from degradation or dilution in bodily fluids or the bloodstream. Consequently, EVs could potentially function as a concealed reservoir of inflammation[15] and have been found to play profound roles in the pathophysiology of various inflammatory conditions.[11, 16] There has been particular interest in neutrophil-derived EVs (NEVs), as subcellar blueprints of neutrophil activation, and there are many reports of increased circulating NEVs in response to systemic inflammation.[17, 18] Consistent with this, we have previously found that NEVs are elevated in the blood of critically-ill patients with burns and sepsis,[19] which correlate with organ dysfunction and mortality. However, the mechanisms by which NEVs contribute to the pathophysiology of end-organ dysfunction in critically-ill patients, in particular AKI, remain unknown.

We hypothesised that circulating NEVs cause significant glomerular endothelial injury and are thus fundamental to the pathophysiology of AKI in critically-ill patients with inflammatory syndromes such as sepsis or ARDS. We aimed to investigate this and understand the mechanistic role of NEVs in the pathophysiology of AKI using in vitro coculture models of systemic microvascular and renal glomerular capillary inflammation.

Methods

Study approval

The 'Multiorgan Failure in COVID-19' study was approved by the North East Tyne and Wear South Research Ethics Committee (20/NE/0153). Patients who lacked capacity due to critical illness had either professional or personal consultee assent, and retrospective consent was obtained from survivors. Venesection of blood from healthy volunteers was performed following informed written consent.

EV incubation with *our in vitro* human models of systemic and glomerular microvascular inflammation

Human umbilical vein endothelial cells (HUVEC, Lonza) and primary human renal glomerular endothelial cells (HRGEC, Innoprot) were seeded overnight to achieve confluence. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation with Histopaque®-1077 and Histopaque®-1119, washed and added to HUVECs to create our in vitro systemic microvascular inflammation model, or to HRGECs to create our in vitro renal glomerular capillary inflammation model. After 2 hours, EVs (isolated from either stimulated blood of a healthy volunteer or a COVID-19 ARDS patient) were added to the HUVEC monoculture, HUVEC-PBMC coculture or HRGEC-PBMC coculture (PBMCs were isolated from the blood of another individual healthy volunteer), and incubated for 4 hours. Samples were never pooled. In some experiments, LPS (100ng ml^{-1}) was added instead of EVs and in others, cells were pretreated with 20 μM doxycycline (as a broad-spectrum matrix metalloproteinase (MMP) inhibitor), 20 μM MMP-8 inhibitor, 20 μM MMP-9 inhibitor, 10 mM Batimastat (tumour necrosis factor (TNF) converting enzyme inhibitor) or anti-TNF antibody. Doxycycline and anti-TNF antibody are water-soluble, whereas specific MMP inhibitors and Batimastat were solubilised in dimethyl sulfoxide (DMSO). Time-matched controls, including DMSO and post wash-supernatant, were also performed to verify that any biological effects were directly attributable to NEVs +/- inhibitors and not to any potential confounders (e.g. DMSO or any soluble mediators present within our samples). Following incubation, the cell-free supernatant underwent TNF analysis by enzyme-linked immunosorbent assay and the cells were studied by flow cytometry. For evaluating the activation of monocyte intracellular signalling pathways, cells were collected 1 hour after adding EVs.

EV production, isolation and analysis

EVs were produced by LPS stimulation of healthy volunteer whole blood and isolated by centrifugation as previously described.[20] EVs were also isolated from blood obtained from patients with COVID-19-ARDS. EV subtypes were further isolated using an immunomagnetic bead separation method as previously described,[20] using MACS® MicroBeads at a ratio of 2µl beads to 1×10^6 EVs (Supplementary Figure 1): anti-CD66b conjugated beads were used for NEVs; and anti-CD61 conjugated beads for platelet-EVs, which are used as a control. Specifically, in COVID-19 patients, where the yield of EVs from patients was lower compared to LPS stimulated healthy volunteer blood, non-neutrophil EVs (i.e. those EVs that did not contain CD66b) were used as a control instead of platelet EVs.

Consistent with the 'Minimal Information for Studies of Extracellular Vesicles' guidelines,[21] EVs were identified using electron microscopy (Supplementary Figure 1) and flow cytometry using our previously described protocols that define EVs as: 1) less than 1µm, 2) double positive for specific precursor cell markers, and 3) sensitive to 0.1% Triton X-100 detergent (Supplementary Figure 2).[12, 19, 20, 22, 23] For EV subtype analysis, EV suspension was incubated with fluorescence-conjugated antibodies e.g., CD11b and CD66b to identify NEVs.[24]

Fluorescence labelling of EVs from isolated neutrophils and EV uptake experiment

Neutrophils were isolated from healthy volunteer blood by density gradient centrifugation and stimulated with 1µM N-formylmethionine-leucyl-phenylalanine (fMLP). Generated EVs were washed and incubated with 2µM CellTrace Far Red (CTFR) before being incubated with a HUVEC-PBMC coculture model for 4 hours.

Cell analysis by flow cytometry

After experiments using our in vitro human co-culture model of intravascular inflammation, endothelial cells and PBMCs were harvested, washed and stained with antibodies for cell identification and the quantification of surface and intracellular targets, using flow cytometry. Supplementary Figure 3 demonstrates the gating strategy used to identify cells.

Western blotting

MMP-8 and MMP-9 proteins in EVs were evaluated by Western blotting. In brief, EV pellets were treated with EV lysate buffer (containing 1% TritonX-100) and 50μg protein/sample was used. Samples were probed with rabbit anti-MMP-8 (1:1000) or rabbit anti-MMP-9 (1:1000) primary antibodies and blots were visualised using Super Signal West Femto Substrate. All raw Western Blot results are provided as supplementary data.

Statistics

All statistical analysis was performed using GraphPad Prism. Given the potential inaccuracies of normality testing with $n < 10$, non-parametric testing was used throughout. All experimental data was produced in a paired fashion, with each experimental condition being paired with the appropriate control. Therefore, non-parametric, paired analyses with corrections for multiple comparisons were performed, namely Friedman test with Dunn's correction for multiple comparisons for all data apart from data within Supplementary Figures 4a, 4b and 5a, which were performed using a two-tailed Wilcoxon matched pairs test. Each data point in each experimental condition was derived from a separate experiment performed on a separate day using samples from different volunteers/ patients e.g., within each experiment, separate blood donors for PBMC and EV isolation were used.

Results

NEVs induce endothelial activation in a monocyte-dependent manner

NEVs were isolated from LPS-treated healthy volunteer blood (to assimilate circulating NEVs from critically-ill patients with gram-negative sepsis) by immune-affinity beads as previously described.[20] In keeping with our previous data enumerating plasma concentrations of NEVs in critically-ill patients,[19] $1.0x10^4$ NEVs/µl were incubated with our in vitro systemic microvascular inflammation model consisting of HUVEC monoculture or HUVEC-PBMC coculture for 4 hours. We utilised a 'coculture' model of endothelial cells and PBMCs due to the well-reported close interaction of monocytes with glomerular endothelial cells in vivo.[24] NEVs significantly increased HUVEC expression of cellular adhesion molecules (CAM) E-selectin and vascular cell adhesion molecule-1 but only in the presence of PBMCs (Figures 1a and b), demonstrating that NEV-mediated endothelial upregulation of CAMs is a monocyte-dependent process. In contrast, platelet-EVs did not show any bioactivity on these cells either in mono- or co-culture (Figures 1a and b), and neither did the other controls, including post-wash supernatant from NEVs (to exclude the possibility of contamination from soluble mediators) or CD66b immune-affinity beads (Figure 1b and Supplementary Figures 4a and b, respectively).

NEVs activate monocytes, causing the release of TNF and endothelial activation

We then explored the mechanisms by which endothelial cells become activated in a monocyte-dependent manner. Following 4 hours of incubation with our in vitro co-culture, NEVs induced a significant upregulation of activation markers on monocytes, namely intercellular adhesion molecule-1 and tissue factor (Figure 1c), and a significant release of TNF into the supernatant (Figure 1d). To evaluate the role of TNF in NEV-induced cell activation, the HUVEC-PBMC coculture was incubated with NEVs in the presence of an anti-TNF antibody. This significantly reduced the upregulation of all CAMs on HUVECs (Figure 1b) while monocyte activation was largely preserved (Figure 1c), strongly suggesting that NEV-induced activation of HUVEC CAMs was dependent on TNF released from activated monocytes.

Next, we assessed how NEVs interacted with cells since it has been shown that proinflammatory EVs are taken up by target cells causing significant cellular inflammation.[25] Following 4 hours of incubation with our in vitro

co-culture, we found that CTFR-labelled NEVs from fMLP-stimulated isolated neutrophils were endocytosed by monocytes (Figure 1e); interestingly, HUVECs also took up NEVs despite not being activated in monoculture (Figure 1a and e).

NEVs activate the monocyte-endothelial cell co-culture in an MMP-8 and MMP-9-dependent fashion

We interrogated NEVs via western blotting for candidate molecules for monocyte activation and found significant amounts of MMP-8 and MMP-9 proteins (Figure 2a). These proteins were not detected in the post-wash supernatant and therefore the observed effects of EV samples cannot be ascribed to a contaminant of soluble MMPs. To evaluate the effect of these MMPs in NEV-induced cell activation, our HUVEC-PBMC coculture model was incubated with NEVs for 4 hours in the presence of various MMP inhibitors. First, doxycycline, an antibiotic and a pan-MMP inhibitor,[26] ablated the NEV-induced activation of HUVECs (Figures 2b), having decreased CAM upregulation on monocytes (Figure 2c) and caused a large reduction in TNF release (Supplementary Figure 5a). In addition, MMP-8 or MMP-9 specific inhibitors were used to reveal the bioactivity of each MMP contained within NEVs. Similar to doxycycline, MMP-8 inhibitor and MMP-9 inhibitor attenuated NEV-induced activation of endothelial cells and monocytes (Figures 2b and c) confirming that NEV bioactivity is mediated, at least in part, by MMP-8 and MMP-9. Importantly, DMSO, the carrier for MMP-8 and MMP-9 inhibitors, did not have any effect on NEV activity (Figures 2b and c).

To assess the possibility that MMP inhibitors reduced EV uptake (rather than directly inhibiting MMPs within NEVs), NEVs were labelled with CTFR and incubated with HUVEC-PBMC coculture for 4 hours in the presence of MMP inhibitors. NEV uptake by monocytes (Supplementary Figure 5b) and HUVECs (Supplementary Figure 5c) was similar in all conditions confirming that MMP inhibitors did not decrease NEV uptake by target cells. This uptake was abrogated at 4^{o}C suggesting that NEV uptake is an active process.

To exclude the possibility that MMP inhibitors have a non-specific inhibitory effect of TNF-converting enzyme, thereby reducing the release of soluble TNF from monocytes[27] (rather than a direct effect on MMPs within NEVs), HUVEC-PBMC coculture was treated with LPS for 4 hours in the presence of MMP inhibitors or Batimastat, and membrane TNF on monocytes was measured. As expected, Batimastat significantly

increased cell-surface membrane TNF expression, preventing soluble TNF release, whereas doxycycline, MMP-8 inhibitor and MMP-9 inhibitor did not affect cell surface TNF expression (Supplementary Figure 5d).

NEVs induce activation of monocyte intracellular p38 MAPK pathway

To explore the intracellular signalling pathway involved in monocyte activation induced by NEVs, we investigated the potential role of mitogen-activated protein kinases (MAPK). We found that NEVs significantly activated the intracellular p38 MAPK pathway of monocytes and this activation was markedly attenuated by an MMP-8 inhibitor (Figure 2d), although there was no significant activation of other MAPK pathways (Supplementary Figures 6a and b).

NEV activation of HRGECs is largely mediated by MMPs

We then assessed whether we could apply the above data to primary HRGECs to increase the translatability of our findings specifically to the glomerulus. As with HUVECs, NEVs induced significant HRGEC inflammation (Figure 3a), monocyte CAM expression and release of soluble TNF (Supplementary Figure 7a b). Anti-TNF antibody abolished endothelial inflammation (Figure 3a), confirming that NEV-induced HRGEC activation was also dependent on TNF released from inflamed monocytes. To evaluate the role of MMPs in NEV-induced cell activation, an HRGEC-PBMC coculture was incubated with NEVs in the presence of MMP inhibitors. Similar to the HUVEC-PBMC coculture, all inhibitors attenuated the upregulation of CAMs on HRGECs (Figure 3b) and the activation of monocytes (Figure 3c), reconfirming that NEV activity in our endothelial co-culture models is mediated to a substantial extent by MMPs.

NEVs from COVID-19-ARDS patients induced upregulation of CAMs on HRGECs

NEVs isolated from the plasma of individual critically-ill COVID-19 patients were incubated with our HRGEC-PBMC coculture for 4 hours and non-neutrophil EVs (CD66b-ve) isolated from the same patient acted as a control. A smaller number of COVID-19-EVs ($6.0x10^2$/µl) were used than LPS-NEVs ($1.0x10^4$/µl) due to the constraints of EV yield from clinical samples. A significant amount of MMP-8 protein was contained within these NEVs but not within non-neutrophil EVs (CD66b-ve) (Figure 4a). Similar to NEVs isolated from LPS-stimulated healthy blood, NEVs from COVID-19-ARDS patients induced significant upregulation of

CAMs on HRGECs (Figure 4b), activation of monocytes (Figure 4c) and the release of soluble TNF (Figure 4d), demonstrating that plasma NEVs induce glomerular endothelial cell activation in COVID-19 patients.

Discussion

In this study, we report several novel findings regarding the biological and functional activity of circulating NEVs in critically-ill patients and their potential role in the pathophysiology of AKI. Using our in vitro systemic microvascular inflammation model we provide a comprehensive pathway detailing how circulating NEVs cause significant microvascular inflammation in a monocyte-dependent fashion (Figure 5). NEVs are internalised by monocytes, which are then activated via the p38 pathway and subsequently enter a pro-inflammatory state, releasing TNF and causing significant endothelial inflammation. Importantly, this inflammation is mediated by cargo associated with NEVs, in particular MMP-8 and MMP-9, and this proinflammatory activity is attenuated by MMP inhibitors. Moreover, using our in vitro renal glomerular capillary inflammation model and NEVs isolated from COVID-19-ARDS patients, we confirmed that these results and elucidated mechanisms are also applicable to microvascular inflammation within the kidney and for critically-ill COVID-19 patients. Taken together, our results highlight a potentially significant role of NEVs in AKI development in critically-ill patients including COVID-19-ARDS, which may be a novel therapeutic target.

More than half of critically-ill patients in intensive care develop AKI, leading to increased mortality and morbidity; sepsis is the most common cause of AKI in intensive care patients.[3] Several mechanisms have been postulated to explain the pathophysiology of AKI which is likely due to a combination of a dysregulated immune response, systemic inflammation, hemodynamic changes, microcirculatory dysfunction and metabolic reprogramming.[5, 9, 28] Another factor to consider, specifically for critically-ill patients with respiratory failure and/or ARDS, is the effect of interorgan cross-talk between the lungs and the kidneys since a significant proportion of these patients subsequently develop AKI. This was particularly true during the COVID-19 pandemic where AKI was a serious complication in critically-ill COVID-19-ARDS patients[7] with up to 55% of these patients requiring renal replacement therapy,[29, 30] leading to higher mortality compared to patients without AKI.[4] The mechanism of lung-kidney interaction has not been fully elucidated but has been hypothesised to include the direct effect of hypoxia and hypercapnia, impaired renal haemodynamics by mechanical ventilation, and perhaps most pertinently, the release of inflammatory mediators from the lungs caused by proinflammatory pathologies such as ARDS.[31] However, circulating soluble cytokines are

reportedly lower in COVID-19-ARDS patients than in non-COVID-19-ARDS patients,[32] despite the increased incidence of AKI in COVID-19-ARDS, suggesting that other circulating mediators are involved in the amplification of systemic inflammation and subsequent organ failure.

The importance of EVs as alternative mediators of inflammation has become increasingly apparent and numerous investigations have identified increased levels of circulating EVs, specifically originating from granulocytes or neutrophils in patients with systemic inflammation.[17, 33] Previously, we have demonstrated that NEVs are raised in critically-ill patients with burns and severe sepsis, and these NEV numbers correlate with outcomes including mortality.[19] EVs act as efficient mediators of intercellular communication during the development of systemic inflammation due to their characteristic lipid bilayer membrane enclosing a variety of molecular cargo, which ensures EVs and their cargo exist stably within the circulation. To the best of our knowledge, there has only been one study investigating EVs in AKI patients, which found higher levels of circulating endothelial and platelet EVs in children who developed kidney injury after cardiac surgery.[34] Although this study illustrated an association between circulating EVs and worsening renal function, it remained unknown how these EVs may be involved in the pathophysiology of kidney injury.

In the present study we focused on the mechanistic role of NEVs in the pathophysiology of AKI and, in particular, glomerular endothelial cell inflammation as the glomerulus is the first renal cellular barrier in direct contact with circulating plasma mediators.[35] Interestingly, the glomerular capillary has specific characteristics very similar to pulmonary capillary vessels where leukocytes including monocytes adhere and crawl on the glomerular endothelium during systemic inflammation.[24] We have previously reported in mice that circulating EVs were taken up by marginated intravascular monocytes in the pulmonary circulation and this occurred several-fold times more under systemic inflammatory conditions compared to resting conditions.[22] These circulating EVs induced pulmonary microvasculature endothelial cellular inflammation as well as increased pulmonary oedema and receptor for advanced glycation endproducts in ex-vivo isolated murine lungs, but only in the presence of monocytes.[20] These findings suggest that monocytes play a crucial role in the pathophysiology of EV-mediated endothelial injury and inflammation in pulmonary microvasculature. Monocytes are not normally present within healthy kidneys but are recruited to the renal vasculature during

inflammation, in a similar fashion to the pulmonary vasculature as described above.[36, 37] Our data demonstrate that circulating NEVs in isolation do not activate renal glomerular endothelial cells alone but instead, monocytes internalise these EVs, become inflamed and release TNF which subsequently activates the glomerular endothelium. Our results highlight further the role of marginated monocytes as a communication hub, processing inflammatory signals from circulatory NEVs, and transposing this inflammation to activate and injure glomerular endothelial cells.

We also found that EV-mediated activation of the monocyte and glomerular endothelium was mediated by cargo contained within NEVs, in particular MMP-8 and MMP-9, via the intracellular p38 pathway. Consistent with this, it has been previously reported that p38 MAPK has an important role in the pathogenesis of glomerular inflammation.[38] MMPs are a member of the superfamily of metzincin proteases, a family of multidomain zinc-dependent endopeptidases, which degrade extracellular matrices such as collagens, proteoglycans gelatins and elastins, and several non-extracellular matrix substrates including chemokines, cytokines and cell-surface receptors.[39] Among the family of MMPs, MMP-8 and MMP-9 are known to be released from neutrophils and whilst it has been previously shown that NEVs contain MMP-9,[40] our data also highlight that neutrophils additionally release MMP-8 within EVs. The bioactivity of either MMP-8 or 9 with EVs had not been explored, but here we demonstrate for the first time that MMPs within NEVs, harvested from either LPS-stimulated human blood or blood of COVID-19-ARDS patients, were functionally active, inducing significant monocyte and subsequently glomerular endothelial inflammation. These MMPs have been previously associated with critically-ill patients. For example, MMP-8 is known to have a critical role in sepsis, is associated with increased mortality and organ failure and genetic ablation or pharmacologic inhibition of MMP-8 activity confers a survival advantage in a murine model of sepsis.[41] Furthermore, plasma MMP-9 levels are associated with COVID-19 mortality and plasma MMP-9 is raised in COVID-19 patients with AKI.[42] However, previous studies have not demonstrated how MMP-8/9 induces AKI. Our study strongly supports that circulating MMP-8/9 originates from activated neutrophils, and is transported to glomerular endothelial cells via NEVs, causing significant inflammation and subsequent kidney injury during sepsis or COVID-19-ARDS.

There are some limitations to this study. First, we evaluated the bioactivity of NEVs and their role in glomerular endothelial inflammation using an *in vitro* cell culture assay. There is growing interest in 'kidney-on-chip' organoid models which can be used to mirror certain aspects of the glomerular endothelial capillary bed such as the filtration barrier.[43] However, they have mainly been used for studying drug and molecule transport, reabsorption and toxicity rather than modelling diseases, nor have they vital immune cells incorporated into such organoids.[44] Instead, we carefully developed in vitro co-culture models, based on our previous experience with pulmonary microvascular inflammation models,[22, 45] which allowed us to mechanistically assess the molecular pathways involved in NEV-dependent glomerular inflammation. Second, our study has examined the role of EVs rather than exosomes (or non-vesicular extracellular particles), the role of which was outside the scope of this study. Third, we did not evaluate the effects of NEVs on other functional parameters of endothelial cell injury, such as changes in endothelial permeability or vascular resistance, which may further our understanding of the role of NEVs in the pathophysiology of AKI through different pathways. Fourthly, whilst it is clear that MMP-8/9 plays a vital functional role in NEV-mediated glomerular endothelial cell inflammation, it would be germane for future studies to investigate the role of other EV-associated mediators such as cathepsin G or neutrophil elastase. Finally, due to the limited volume of patient-derived samples, we were unable to perform inhibitor experiments on COVID-19 ARDS NEVs with appropriate time-matched controls. Nevertheless, the biological activity observed was highly similar to that of NEVs derived from LPS-stimulated blood. Therefore, we would expect that the effects of COVID-19 NEVs are also mediated via an MMP-dependent pathway, although further experiments would be required to confirm this.

In summary, our results demonstrate that circulating NEVs cause significant renal endothelial inflammation in a monocyte-dependent fashion, which is mediated by cargo contained within NEVs, in particular MMP-8 and MMP-9. These data detail new insights into the pathophysiology of glomerular inflammation during AKI and illustrate that circulating NEVs may be a potential therapeutic target in critically-ill patients with systemic inflammation and AKI.

Authors contributions

Research study design: JS, YI, KW, KO, FT, MT, SS

Conduct of experimentation: YI, JS, TY, EB, DT, AG, XC, PS

Data acquisition: JS, YI, TY

Data analysis: JS, YI, TY, EB, AG, SB, MT, SS

Supply of materials: PP, UW, RS, AR, PS, AG, KO, FT, MT, SS

Writing of the manuscript: JS, YI, TY, MT, SS

All authors were involved in the drafting of the article or revising it critically for important intellectual content, gave their final approval of the version to be published and agreed to be accountable for all aspects of the work thereby ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Declaration of interests

FWKT has received research project grants from AstraZeneca Limited, Boehringer Ingelheim, MedImmune, OncoOne, Rigel Pharmaceuticals, and Thornton and Ross Ltd and has consultancy agreements with OncoOne and Rigel Pharmaceuticals. ACG reports that outside of this work he has received consulting fees from AstraZeneca, Beckman Coulter, and VVB Bio and speaker fees from Fresenius Kabi. The remaining authors declare no other conflicts of interest.

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References

1. Section 2: AKI Definition. *Kidney Int Suppl (2011)* 2012; **2**: 19–36
2. Ronco C, Bellomo R, Kellum JA. Acute kidney injury. *The Lancet* 2019; **394**: 1949–64
3. Hoste EAJ, Bagshaw SM, Bellomo R, et al. Epidemiology of acute kidney injury in critically ill patients: the multinational AKI-EPI study. *Intensive Care Med* 2015; **41**: 1411–23
4. Gasparini M, Khan S, Patel JM, et al. Renal impairment and its impact on clinical outcomes in patients who are critically ill with COVID-19: a multicentre observational study. *Anaesthesia* 2021; **76**: 320–6
5. Peerapornratana S, Manrique-Caballero CL, Gómez H, Kellum JA. Acute kidney injury from sepsis: current concepts, epidemiology, pathophysiology, prevention and treatment. *Kidney Int* 2019; **96**: 1083–99
6. Lerolle N, Nochy D, Guérot E, et al. Histopathology of septic shock induced acute kidney injury: apoptosis and leukocytic infiltration. *Intensive Care Med* 2010; **36**: 471–8
7. Akilesh S, Nast CC, Yamashita M, et al. Multicenter Clinicopathologic Correlation of Kidney Biopsies Performed in COVID-19 Patients Presenting With Acute Kidney Injury or Proteinuria. *American Journal of Kidney Diseases* 2021; **77**: 82-93.e1
8. Xu C, Chang A, Hack BK, Eadon MT, Alper SL, Cunningham PN. TNF-mediated damage to glomerular endothelium is an important determinant of acute kidney injury in sepsis. *Kidney Int* 2014; **85**: 72–81
9. He F-F, Wang Y-M, Chen Y-Y, Huang W, Li Z-Q, Zhang C. Sepsis-induced AKI: From pathogenesis to therapeutic approaches. *Front Pharmacol* 2022; **13**
10. Pickkers P, Murray PT, Ostermann M. New drugs for acute kidney injury. *Intensive Care Med* 2022; **48**: 1796–8
11. Soni S, Tirlapur N, O'Dea KP, Takata M, Wilson MR. Microvesicles as new therapeutic targets for the treatment of the acute respiratory distress syndrome (ARDS). *Expert Opin Ther Targets* 2019; **23**: 931–41
12. Soni S, Wilson MR, O'Dea KP, et al. Alveolar macrophage-derived microvesicles mediate acute lung injury. *Thorax* 2016; **71**: 1020–9
13. Soni S, O'Dea KP, Tan YY, et al. ATP redirects cytokine trafficking and promotes novel membrane TNF signaling *via* microvesicles. *The FASEB Journal* 2019; **33**: 6442–55
14. Buzas EI. The roles of extracellular vesicles in the immune system. *Nat Rev Immunol* 2023; **23**: 236–50
15. Maile R. Extracellular vesicles: secret agents of inflammation and the importance of their identification. *J Leukoc Biol* 2023; **114**: 384–6
16. Lanyu Z, Feilong H. Emerging role of extracellular vesicles in lung injury and inflammation. *Biomedicine & Pharmacotherapy* 2019; **113**: 108748

17. Nieuwland R, Berckmans RJ, McGregor S, et al. Cellular origin and procoagulant properties of microparticles in meningococcal sepsis. *Blood* 2000; **95**: 930–5
18. Daniel L, Fakhouri F, Joly D, et al. Increase of circulating neutrophil and platelet microparticles during acute vasculitis and hemodialysis. *Kidney Int* 2006; **69**: 1416–23
19. O'Dea KP, Porter JR, Tirlapur N, et al. Circulating Microvesicles Are Elevated Acutely following Major Burns Injury and Associated with Clinical Severity. *PLoS One* 2016; **11**: e0167801
20. Tan YY, O'Dea KP, Tsiridou DM, et al. Circulating Myeloid Cell–derived Extracellular Vesicles as Mediators of Indirect Acute Lung Injury. *Am J Respir Cell Mol Biol* 2023; **68**: 140–9
21. Welsh JA, Goberdhan DCI, O'Driscoll L, et al. Minimal information for studies of extracellular vesicles (MISEV2023): From basic to advanced approaches. *J Extracell Vesicles* 2024; **13**
22. O'Dea KP, Tan YY, Shah S, et al. Monocytes mediate homing of circulating microvesicles to the pulmonary vasculature during low-grade systemic inflammation. *J Extracell Vesicles* 2020; **9**
23. Soni S, Garner JL, O'Dea KP, et al. Intra-alveolar neutrophil-derived microvesicles are associated with disease severity in COPD. *American Journal of Physiology-Lung Cellular and Molecular Physiology* 2021; **320**: L73–83
24. Kitching AR, Hickey MJ. Immune cell behaviour and dynamics in the kidney — insights from in vivo imaging. *Nat Rev Nephrol* 2022; **18**: 22–37
25. Soni S, O'Dea KP, Abe E, et al. Microvesicle-Mediated Communication Within the Alveolar Space: Mechanisms of Uptake by Epithelial Cells and Alveolar Macrophages. *Front Immunol* 2022; **13**
26. Cabral-Pacheco GA, Garza-Veloz I, Castruita-De la Rosa C, et al. The Roles of Matrix Metalloproteinases and Their Inhibitors in Human Diseases. *Int J Mol Sci* 2020; **21**: 9739
27. Lee E-J, Moon P-G, Baek M-C, Kim H-S. Comparison of the Effects of Matrix Metalloproteinase Inhibitors on TNF-α Release from Activated Microglia and TNF-α Converting Enzyme Activity. *Biomol Ther (Seoul)* 2014; **22**: 414–9
28. Fani F, Regolisti G, Delsante M, et al. Recent advances in the pathogenetic mechanisms of sepsis-associated acute kidney injury. *J Nephrol* 2018; **31**: 351–9
29. Mohamed MMB, Lukitsch I, Torres-Ortiz AE, et al. Acute Kidney Injury Associated with Coronavirus Disease 2019 in Urban New Orleans. *Kidney360* 2020; **1**: 614–22
30. Cummings MJ, Baldwin MR, Abrams D, et al. Epidemiology, clinical course, and outcomes of critically ill adults with COVID-19 in New York City: a prospective cohort study. *The Lancet* 2020; **395**: 1763–70
31. Alge J, Dolan K, Angelo J, Thadani S, Virk M, Akcan Arikan A. Two to Tango: Kidney-Lung Interaction in Acute Kidney Injury and Acute Respiratory Distress Syndrome. *Front Pediatr* 2021; **9**
32. Leisman DE, Ronner L, Pinotti R, et al. Cytokine elevation in severe and critical COVID-19: a rapid systematic review, meta-analysis, and comparison with other inflammatory syndromes. *Lancet Respir Med* 2020; **8**: 1233–44
33. Fujimi S, Ogura H, Tanaka H, et al. Activated Polymorphonuclear Leukocytes Enhance Production of Leukocyte Microparticles with Increased Adhesion Molecules in Patients with Sepsis. *The Journal of Trauma: Injury, Infection, and Critical Care* 2002; **52**: 443–8

34. Sullo N, Mariani S, JnTala M, et al. An Observational Cohort Feasibility Study to Identify Microvesicle and Micro-RNA Biomarkers of Acute Kidney Injury Following Pediatric Cardiac Surgery. *Pediatric Critical Care Medicine* 2018; **19**: 816–30

35. Sol M, Kamps JAAM, van den Born J, et al. Glomerular Endothelial Cells as Instigators of Glomerular Sclerotic Diseases. *Front Pharmacol* 2020; **11**

36. Nachiappa Ganesh R, Garcia G, Truong L. Monocytes and Macrophages in Kidney Disease and Homeostasis. *Int J Mol Sci* 2024; **25**: 3763

37. Cormican S, Negi N, Naicker SD, et al. Chronic Kidney Disease Is Characterized by Expansion of a Distinct Proinflammatory Intermediate Monocyte Subtype and by Increased Monocyte Adhesion to Endothelial Cells. *Journal of the American Society of Nephrology* 2023; **34**: 793–808

38. Sheryanna A, Bhangal G, McDaid J, et al. Inhibition of p38 Mitogen-Activated Protein Kinase Is Effective in the Treatment of Experimental Crescentic Glomerulonephritis and Suppresses Monocyte Chemoattractant Protein-1 but Not IL-1β or IL-6. *Journal of the American Society of Nephrology* 2007; **18**: 1167–79

39. de Almeida LGN, Thode H, Eslambolchi Y, et al. Matrix Metalloproteinases: From Molecular Mechanisms to Physiology, Pathophysiology, and Pharmacology. *Pharmacol Rev* 2022; **74**: 714–70

40. Butin-Israeli V, Houser MC, Feng M, et al. Deposition of microparticles by neutrophils onto inflamed epithelium: a new mechanism to disrupt epithelial intercellular adhesions and promote transepithelial migration. *The FASEB Journal* 2016; **30**: 4007–20

41. Solan PD, Dunsmore KE, Denenberg AG, Odoms K, Zingarelli B, Wong HR. A novel role for matrix metalloproteinase-8 in sepsis*. *Crit Care Med* 2012; **40**: 379–87

42. D`Avila-Mesquita C, Couto AES, Campos LCB, et al. MMP-2 and MMP-9 levels in plasma are altered and associated with mortality in COVID-19 patients. *Biomedicine & Pharmacotherapy* 2021; **142**: 112067

43. Petrosyan A, Cravedi P, Villani V, et al. A glomerulus-on-a-chip to recapitulate the human glomerular filtration barrier. *Nat Commun* 2019; **10**: 3656

44. Ingber DE. Human organs-on-chips for disease modelling, drug development and personalized medicine. *Nat Rev Genet* 2022; **23**: 467–91

45. Tsiridou DM, Sachouli E, Takata M, O'Dea KP. Neutrophil-Derived Microvesicles Enhance Pulmonary Vascular Inflammation via a Toll-like Receptor 4 Signaling-Dependent Mechanism. *The FASEB Journal* 2022; **36**

Legends to Figures

Fig 1. NEV stimulation of a HUVEC monoculture and HUVEC-PBMC co-culture, attenuated by anti-TNF therapy.

Neutrophil EVs (NEVs) and platelet EVs (PEVs) were isolated from LPS-treated healthy volunteer blood. EVs ($1.0x10^4$/µl) were incubated with a HUVEC monoculture or a HUVEC-peripheral blood mononuclear cell (PBMCs) co-culture for 4 hours and expression (mean fluorescence intensity (MFI)) of cell adhesions molecules (CAMs) on HUVECs and monocytes were assessed. Platelet EVs (PEVs) and post-wash supernatant (PWSN) acted as controls. (a) Neither NEVs nor PEVs increased E-selectin or vascular cell adhesion molecule 1 (VCAM) expression on HUVECs in monoculture. (b) When in co-culture, NEVs caused significant upregulation of E-selectin and VCAM expression on HUVECs, which was associated with (c) upregulation of intercellular cell adhesion molecule 1 (ICAM-1) and tissue factor (TF) on monocytes and (d) the release of soluble tumour necrosis factor (TNF) into the supernatant. Furthermore, anti-TNF antibodies ablated the NEV-induced CAM increase on (b) HUVECs but not (c) monocytes. (e) In separate experiments, the incubation of cell tracer far red (CTFR)-labelled-NEVs with a HUVEC-PBMC co-culture led to significant NEV uptake by both HUVECs and monocytes. Data were analysed by Friedman test with Dunn's correction for multiple comparisons. n = 4-6, * p <0.05, ** p <0.01, *** p <0.001.

Fig 2. Effect of MMP inhibitors on NEV-induced CAM upregulation on HUVECs and monocyte activation.

(a) Matrix metalloproteinase (MMP)-8 and MMP-9 proteins were contained within NEVs as shown in the representative Western Blot images. (b and c) To evaluate the functional role of MMP-8 and MMP-9 in NEV-induced cell activation, a HUVEC-PBMC coculture was incubated with NEVs ($1.0x10^4$/µl) isolated from LPS-treated healthy volunteer blood for 4 hours in the presence of MMP inhibitors doxycycline (pan-MMP inhibitor), a specific MMP-8 inhibitor and a specific MMP-9 inhibitor. NEVs with dimethyl sulfoxide

(DMSO), the carrier for MMP-8 and MMP-9 inhibitors, acted as a control. All inhibitors attenuated NEV-induced activation of (b) HUVECs and (c) monocytes, showing that NEV bioactivity is mediated by MMPs. (d) To explore the intracellular pathway of NEV-MMP-induced activation of monocytes, a HUEVC-PBMC coculture was incubated with NEVs with and without MMP-8 inhibitor for 1 hour. NEVs significantly activated the monocyte intracellular p38 mitogen-activated protein kinase (MAPK) pathway, and this activation was attenuated by MMP-8 inhibitor, showing that NEVs activate the monocyte intracellular p38 MAPK pathway, dependent on MMPs. Data were analysed by Friedman test with Dunn's correction for multiple comparisons. n = 5- 8, * p <0.05, ** p <0.01, *** p <0.001.

Fig 3. NEV activation of an HRGEC-PBMC co-culture with inhibition of CAM upregulation using anti-TNF therapy and MMP inhibitors.

To explore the effect of NEVs on the glomerular microvasculature, human primary glomerular endothelial cells (HRGECs) were incubated with NEVs and PEVs ($1.0x10^4$/μl) in an HRGEC-PBMC coculture for 4 hours. Again, where appropriate, platelet EVs (PEV), post-wash supernatant (PWSN) and NEV with dimethyl sulfoxide (DMSO) acted as controls. (a) NEVs, but not PEVs or PWSN, significantly induced upregulation of CAMs on HRGECs, which was attenuated by anti-TNF antibodies, suggesting that CAM activation was dependent on TNF released from activated monocytes. To evaluate the role of MMPs in NEV-induced cell activation, separate experiments utilised MMP inhibitors, which were added to the HRGEC-PBMC coculture alongside NEVs for 4 hours. (b) MMP inhibition effectively attenuated the upregulation of CAMs on HRGECs and (c) the activation of monocytes. Data were analysed by Friedman test with Dunn's correction for multiple comparisons. n = 5, * P<0.05, ** p < 0.01.

Fig 4. Effect of COVID-19-NEVs on CAM upregulation on HRGECs.

To evaluate the bioactivity of NEVs in COVID-19-ARDS patients, NEVs and non-NEVs (CD66b-ve) were isolated from the plasma of COVID-19-ARDS patients. (a) First, MMP-8 protein was contained within NEVs but not within non-NEVs (CD66b-ve) or the supernatant controls, as shown in the representative Western Blot image. Subsequently, NEVs (6.0x102/μl), alongside non-neutrophil EVs (CD66b-ve) and PWSN controls, were incubated with an HRGEC-PBMC coculture for 4 hours. (b) Incubation with NEVs led to significant

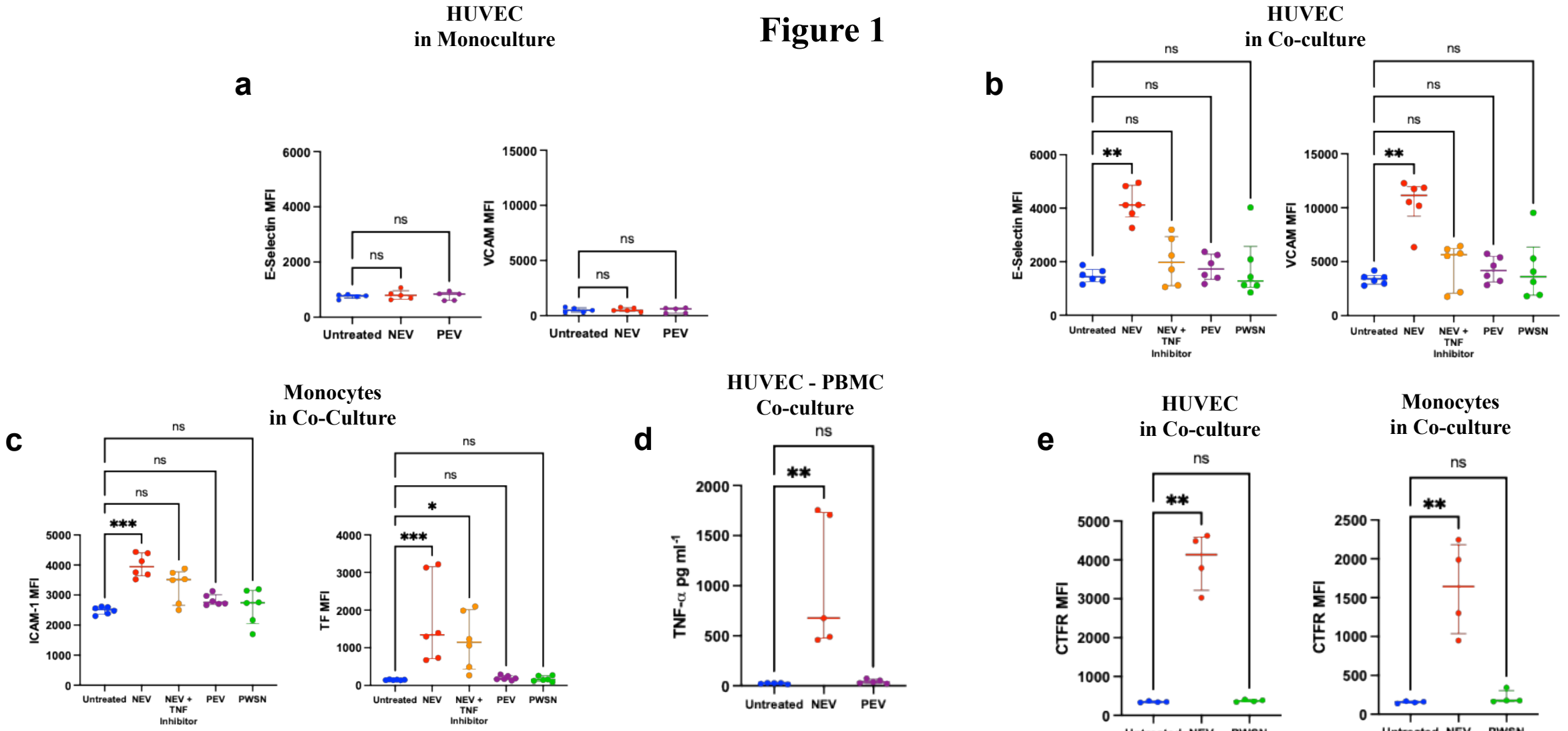
HRGEC activation, (c) the upregulation of CAMs on monocytes and (d) the release of TNF into the supernatant. Data were analysed by Friedman test with Dunn's correction for multiple comparisons. n = 5-6, * p <0.05, ** p < 0.01.

Fig 5. Schematic overview of circulating NEV-induced HRGEC activation via an MMP-dependent pathway.

Circulating NEVs, which contain MMP-8 and MMP-9 proteins, are taken up by monocytes, which activate the p38 intracellular MAPK signalling pathway. Subsequently, this causes monocytes to display a proinflammatory phenotype, as demonstrated by the upregulation of CAMs leading to TNF release. This secreted TNF causes significant activation of glomerular endothelial cells.

Figures

Figure 1



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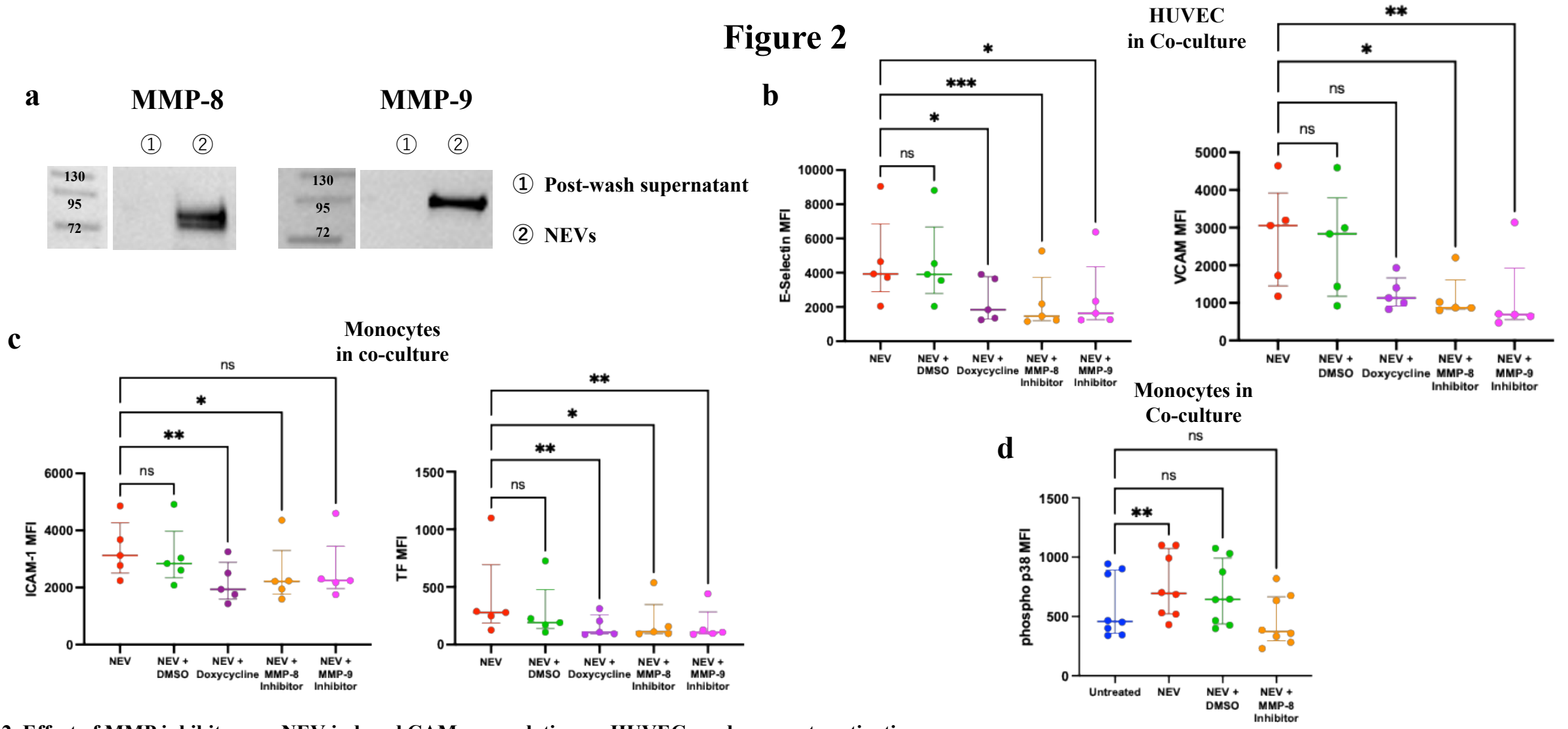

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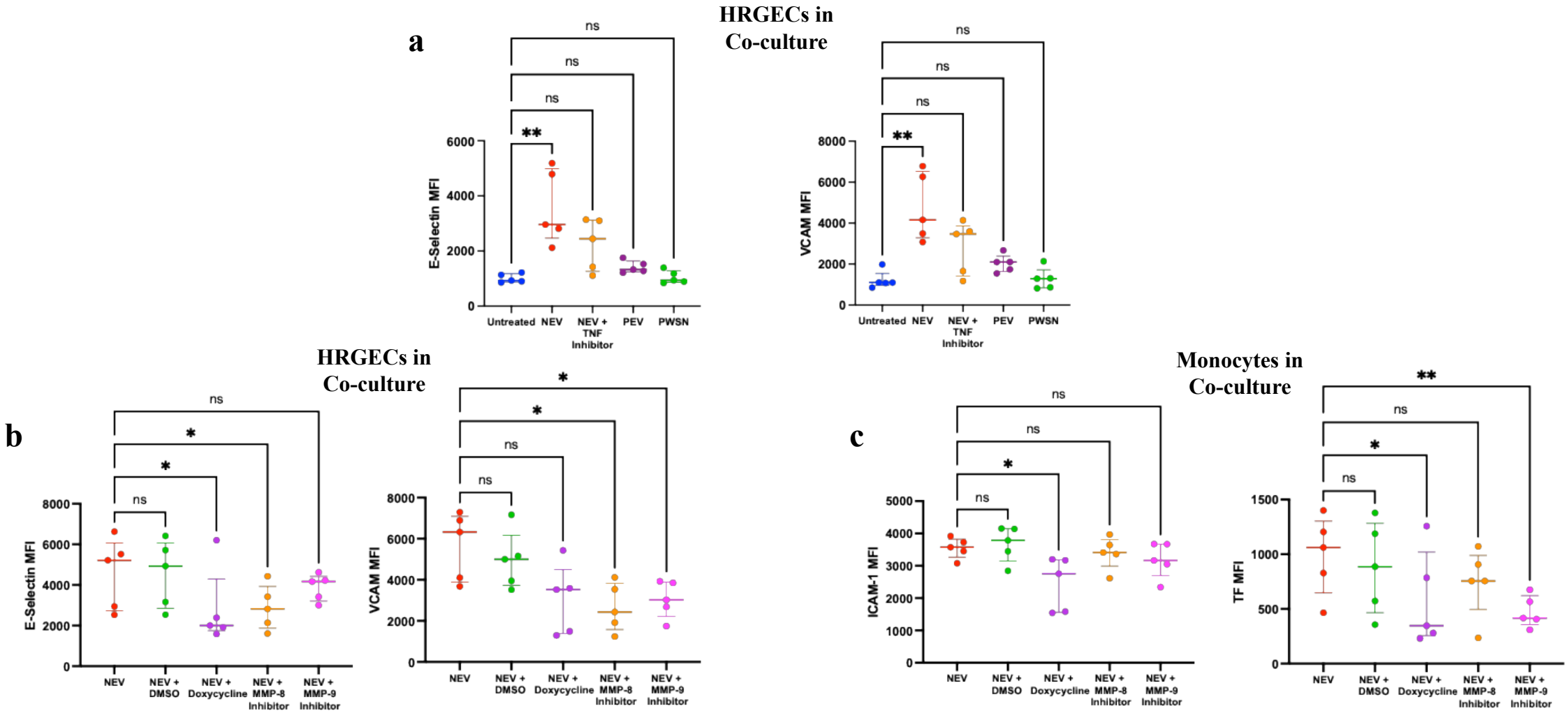

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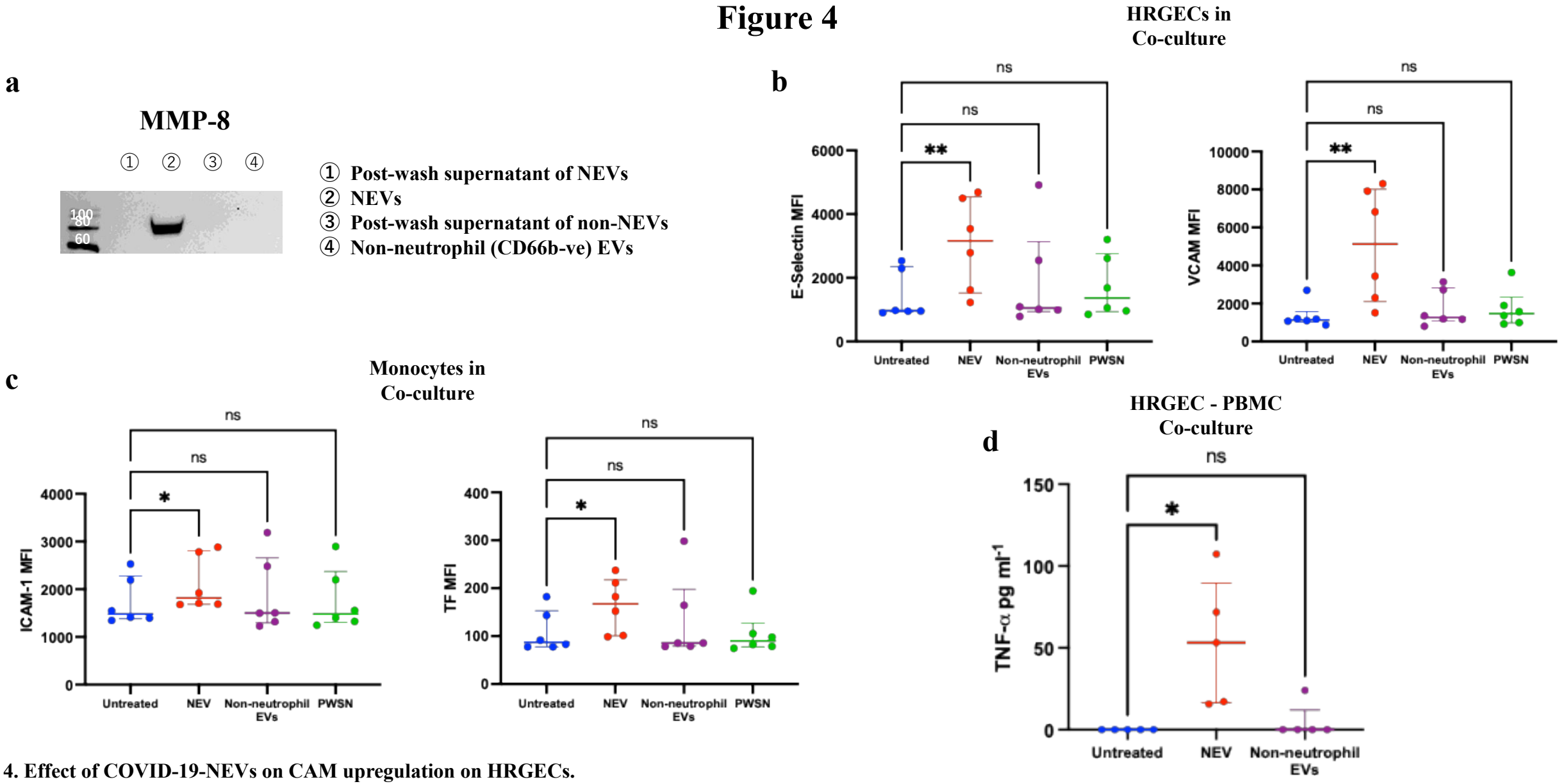

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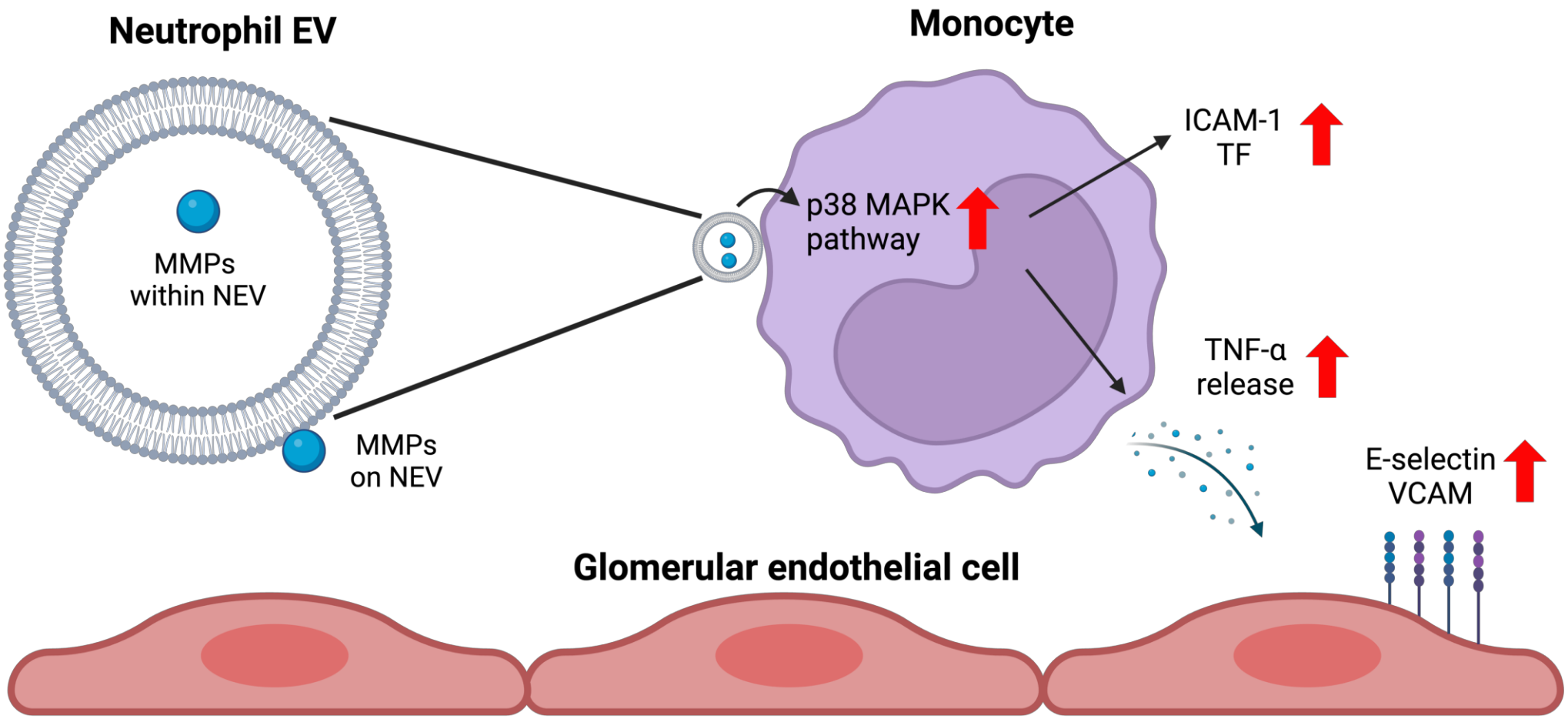

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