Evaluation of neutrophil microvesicle function in a novel tri-culture model of pulmonary vascular inflammation

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Introduction

Microvesicles (MVs) are medium size (0.1-1µm) cell membrane-derived extracellular vesicles carrying functional bioactive cargo and surface markers from their parental cells[1].

MVs are present in blood under healthy states, but their numbers are significantly increased in various inflammatory conditions. During sepsis or systemic inflammatory response syndrome, elevated levels of circulating neutrophil-derived MVs (N-MVs) are associated with clinical severity, suggesting potential as biomarkers and mediators of organ injury[2,3].

We previously found a dramatic increase of circulating MVs uptake within the vasculature by lung-marginated monocytes in a mouse model of endotoxaemia[4]. As a major site for accumulation of inflammatory cells during sepsis, uptake of N-MVs within pulmonary vasculature could influence inflammatory processes contributing to indirect acute lung injury. However, the interactions of N-MVs with leukocytes were studied in isolated systems, without considering their influence on endothelial cells and the course of inflammation.

Aim

To identify target cell-specific interactions and functions of N-MVs within the pulmonary vascular microenvironment, by developing an *in vitro* neutrophil-monocyte-endothelial cell 'tri-culture' model of inflammation that simulates the physiological environment.

Methods

Isolation of human leukocyte subpopulations

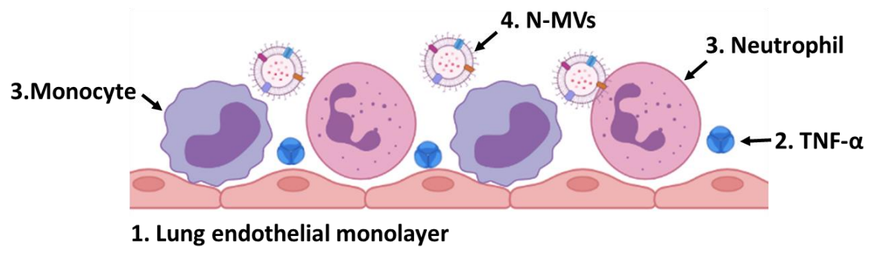
Neutrophils and peripheral blood mononuclear cells (PBMCs) were isolated from healthy volunteer blood by density gradient centrifugation, and monocytes from PBMCs by negative immunomagnetic bead selection (Miltenyi).

N-MVs production, labelling and uptake

N-MVs were generated by fMLP stimulation of isolated neutrophils. For uptake experiments, N-MVs were labelled with CFSE fluorescent dye, and were quantified by flow cytometry. To validate the uptake, the CFSE-MVs were pre-incubated with an anti-CD18 blocking antibody before their addition to the leukocytes.

'Tri-culture' model of pulmonary vascular inflammation

Confluent human lung microvascular endothelial cells (HLMECs) were pre-treated with low-dose tumour necrosis factor alpha (TNFα). After renewal of media, neutrophils and monocytes were added to HLMECs, allowed to adhere and then incubated with N-MVs for 3hrs.



Results

1. N-MVs are taken up by both neutrophils and monocytes, with integrin β2 (CD18) required for uptake by neutrophils, but not monocytes.

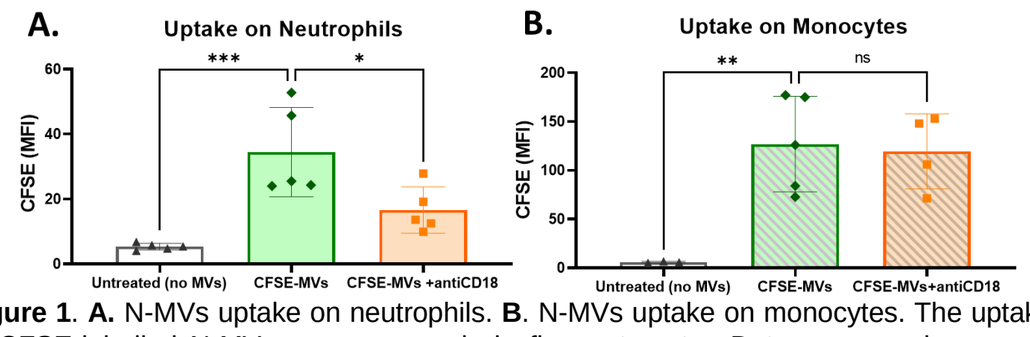


Figure 1. **A.** N-MVs uptake on neutrophils. **B**. N-MVs uptake on monocytes. The uptake of CFSE-labelled N-MVs was assessed via flow cytometry. Data expressed as mean fluorescent intensity (MFI). Mean±SD, (n=5), Ordinary one-way ANOVA,*p<0.05, **p<0.01, ***p<0.001.

2. Evaluation of the tri-culture model: monocyte-dependent activation of neutrophils during LPS stimulation.

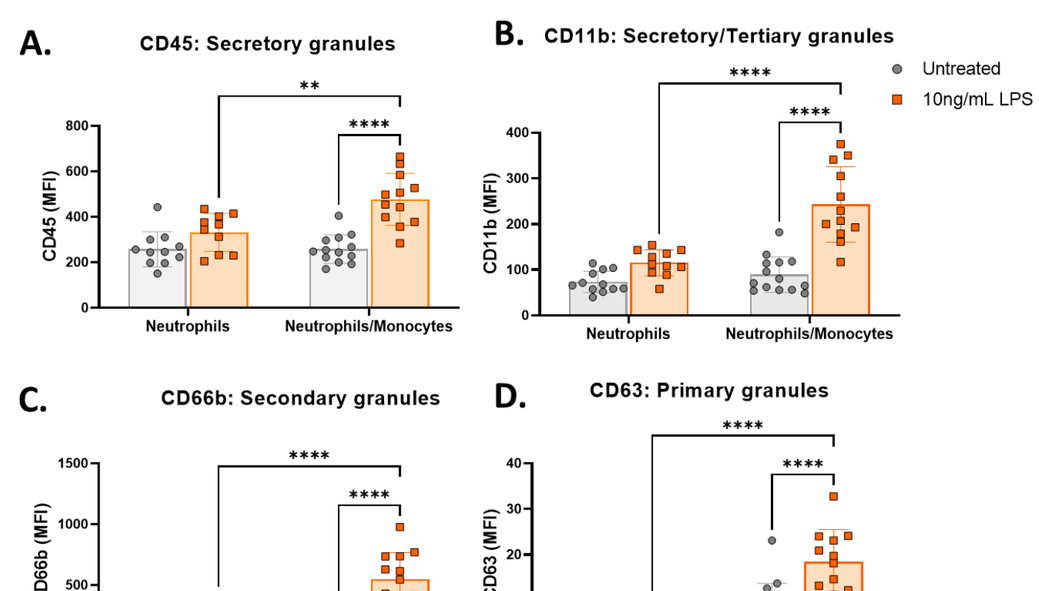


Figure 2. Evaluation of granule-derived neutrophil activation markers (**A-D**) via flow cytometry after the addition of monocytes at the LPS-treated cultures (3hours). Data expressed as mean fluorescent intensity (MFI) and analysed as mean ± SD via two-way ANOVA, (n=12), *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. LPS: lipopolysaccharide.

Results

3. N-MVs enhance neutrophil activation in the presence of monocytes.

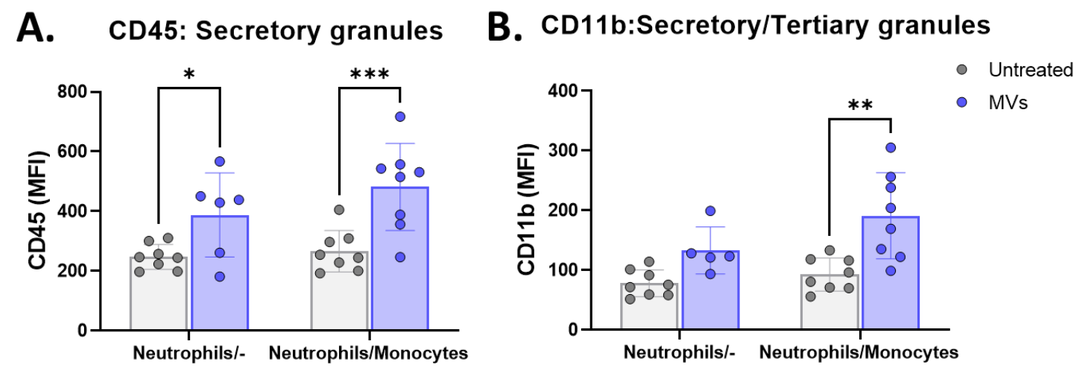


Figure 3. Addition of N-MVs (3hours) resulted in significant upregulation of surface activation markers on neutrophils: CD45 (**A**); CD11b (**B**); CD66b (**C**); and CD63 (**D**) as evaluated via flow cytometry. Mean ±SD, (n=5-8), Two-way ANOVA, p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

4. N-MVs induce the release of neutrophil extracellular traps (NETs) independently of monocytes.

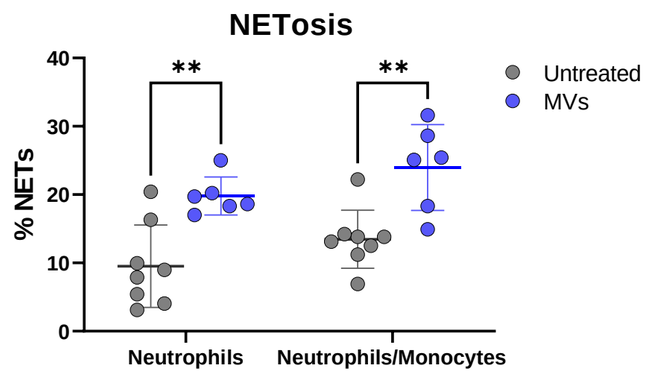


Figure 4. NETs were assessed via flow cytometry as the percentage of CD66b+ neutrophils (%) that co-express myeloperoxidase (MPO) and Sytox-Green. Mean ± SD, (n=6-7), Two-way ANOVA,**p<0.01.

5. N-MVs induce upregulation of monocyte tissue factor (TF) expression and soluble TNF- α release.

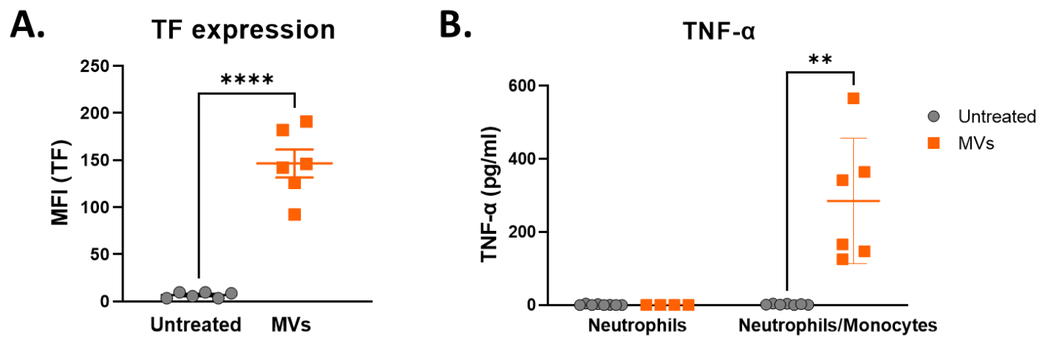


Figure 5. **A.** The N-MVs effect on monocytes was evaluated by surface expression of tissue factor via flow cytometric analysis and **B.** production of soluble TNF in tri-culture supernatant as measured with ELISA. Mean ± SD, (n=6-7), Unpaired two-tailed t test, **p<0.01,****p<0.0001.

Discussion

We successfully developed a physiologically-relevant tri-culture model to study inflammatory crosstalk between N-MVs with myeloid leukocytes within pulmonary vasculature during sepsis.

We demonstrated that N-MVs are taken up by monocytes and neutrophils and that they can produce significant activation of neutrophils, indirectly via monocyte-dependent mechanisms or directly in the case of NETs formation.

In addition, we showed that N-MVs induce significant activation of monocytes, and we propose local N-MV-induced TNF-α release as a potential mechanism of neutrophil-dependent lung injury.

Conclusion

Using this novel tri-culture model of pulmonary vascular inflammation, our results suggest a central role for N-MVs as mediators of acute vascular inflammation, leading ultimately to pulmonary endothelial dysfunction and development of indirect acute lung injury.

References

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Acknowledgments

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Chelsea and Westminster Hospital NHS Foundation Trust