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## Neutrophil Derived Microvesicles: Emerging Role of a Key Mediator to the Immune Response

**Bobby L. Johnson III, Josh W. Kuethe, and Charles C. Caldwell\***

Division of Research, Department of Surgery, University of Cincinnati, Cincinnati, OH, USA

### Abstract

In response to infection and trauma, exquisite control of the innate inflammatory response is necessary to promote an anti-microbial response and minimize tissue injury. Over the course of the host response, activated leukocytes are essential for the initial response and can later become unresponsive or undergo apoptosis. Leukocytes, along the continuum of activation to apoptosis, have been shown to generate microvesicles. These vesicles can range in size from 0.1 to 1.0  $\mu\text{m}$  and can retain proteins, RNA and DNA of their parent cells. Importantly, neutrophil-derived microvesicles (NDMV) are robustly increased under inflammatory conditions. The aim of this review is to summarize the research to date upon NDMVs. This will include describing under which disease states NDMVs are increased, mechanisms underlying formation, and the impact of these vesicles upon cellular targets. Altogether, increased awareness of NDMVs during the host innate response may allow for diagnostic tools as well as potential novel therapies during infection and trauma.

### Keywords

Apoptosis; inflammatory; immune response; microvesicles; neutrophil derived microvesicles; sepsis

## INTRODUCTION

Microvesicles (MVs) are small intact vesicles ranging in size from 0.1–1.0  $\mu\text{m}$ . These extracellular vesicles have been observed to bleb off the surface of a number of cell types including erythrocytes, endothelial cells, lymphocytes, platelets, and myeloid cells [1]. Microvesicles have been implicated in a variety of disease processes including atherosclerosis [2], thrombosis [3], rheumatoid arthritis [4], cancer [5], inflammation [5, 6], transfusion-related acute lung injury [7], and sepsis [6]. Chargaff and West first described MPs in 1946 as a precipitable factor in platelet free plasma [8]. Later, in 1967 Wolf described lipid-rich MVs separated by ultracentrifugation from fresh plasma as “platelet dust” [9].

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\*Address correspondence to this author at the MSB SRU G479, Cincinnati, OH 45267-0558, USA; Tel: (513)-558-8674; Fax: (859)-558-8677; charles.caldwell@uc.edu.

### CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

Cells have been shown to generate MV after activation and during events leading to apoptosis [1]. These MV can contain membrane, cytoplasmic and nuclear components of the parent cell [1] and typically retain surface markers of their parent cells [10], but are distinct in composition and size from other subcellular structures, such as apoptotic bodies or exosomes [1]. Current literature suggests that MV generation is the end point of an activation cascade and / or the beginning of apoptosis requiring increases in intracellular calcium concentration, rearrangement of the cytoskeleton, and the budding of these vesicles from the plasma membrane [11], and as such functions as a terminal step of cell signaling.

Microvesicles express extracellular facing proteins that are known to be involved in a variety of important cellular processes and thus can act as bioactive effectors [12]. In fact, MVs can exert biological effects to host cells by stimulating receptors of the target cell, or by transferal of vesicle content, such as protein, lipids, mRNA, or microRNA, to the target cell [13]. Additionally, circulating microvesicles can act together with soluble mediators to enhance biological processes, such as coagulation [14]. Microvesicle compositions, even from the same cell type, can vary depending on the stimuli or disease state. Examples include: 1) endothelial cells, stimulated by plasminogen activator inhibitor-1 or TNF- $\alpha$ , generate microvesicles with distinct protein compositions [15]. 2) Platelet derived microvesicles derived just prior to cell apoptosis rather than cell activation, can differentially modulate cellular and physiological functions [16]. 3) Cancer cell microvesicles can transfer oncogenic content to bystander cells [17]. In this way, microvesicles act as distinct packets of information that transfer information between parent cells to target cells depending on the cellular environment or stimulus.

In general, microvesicles can mediate the immune response under pathophysiological conditions. Of interest, neutrophil derived microvesicles (NDMVs) are currently one of the least studied microvesicle populations, despite the neutrophil being an integral part of the immune response. Similar to the cell from which they originate, NDMVs are involved in a number of distinct autocrine and paracrine immunological processes. The remainder of this review will focus only upon NDMVs, specifically, increased numbers during disease, generation during inflammatory conditions, impact on target cells, and potential future directions.

## NUMBERS OF NDMV ARE INCREASED IN DISEASE STATES

Neutrophil derived microvesicles are present in very small amounts in normal physiologic conditions [18]. However, NDMVs are reported to be elevated in a variety of inflammatory disorders [18a, 18c, 19] (Table 1). The increase of NDMV numbers during pathophysiological processes could be used, advantageously, as diagnostic indicator of inflammation. For example, an early report that investigated patients with various kidney disorders, found NDMV populations were significantly increased in patients with acute vasculitis, chronic vasculitis, IgA nephropathy, and tubulointerstitial nephritis, as well as patients who were undergoing hemodialysis [19a]. Another study demonstrated that elevated circulating NDMVs alone can be an independent predictor of atherosclerosis burden [19b]. NDMVs from patients with anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis have elevated levels compared to normal counterparts and have a pro-

inflammatory effect on endothelial cells, thus contributing to the disease process [20]. Additionally, in patients with systemic lupus erythematosus (SLE) and anti-phospholipid syndrome, both autoimmune conditions that affect the vasculature, NDMVs are also increased and associated with increased plasmin generation [19c]. Because of these associations, quantification of NDMVs could be used as a prognostic indicator of vascular diseases.

During infectious conditions, systemic NDMV numbers are observed to be elevated. Plasma from patients with community acquired pneumonia showed a statistically significant increase in circulating NDMVs, compared to healthy individuals [18c]. During meningococcal sepsis, the level of circulating NDMVs is significantly increased compared to baseline levels, while other microvesicle populations are not significantly changed [18a]. Neutrophils from trauma patients more robustly generated microvesicles than did neutrophils from healthy individuals, leading to elevated NDMVs in these patients [21]. The question of whether the robust increase in NDMVs continually or preferentially during the early or latter stages of disease pathophysiology remains to be answered.

In the circulation, platelet derived microvesicles are the largest microvesicle population observed [19c]. In contrast, at the sites of infection or inflammation, NDMVs have been shown to predominate [6, 18c, 22]. One study demonstrated that NDMVs were over 14-fold more concentrated in a blister vs. human serum, despite the serum containing 10-fold more neutrophils than the blister [18c]. Consistent with this, NDMVs were significantly increased in BAL samples from patients with pneumonia and increased in abdominal washings from patients with abdominal sepsis. NDMVs also predominated as the primary MV population at infectious foci [6]. Acute Respiratory Distress Syndrome (ARDS) is a condition usually seen in patients with sepsis or severe trauma characterized by lung inflammation, which leads to impaired gas exchange and worsening inflammation. It was observed that neutrophil derived microvesicles were the most numerous MV populations in bronchial washings in patients with ARDS [22]. Interestingly in this same study, ARDS patients who had a higher NDMV level had a survival benefit [22]. We speculate that in lung inflammation, NDMVs have an anti-inflammatory effect upon leukocytes and this suppression of the host immune response would limit impaired gas exchange, leading to improved outcomes.

Altogether, the presence of NDMVs in these conditions could be used as a novel and rapid marker of inflammation or infection. While this is an interesting prospect, the aforementioned studies quantifying NDMV levels in patients utilized multiple methods of enumerating NDMVs. Some of these studies measured NDMVs directly from patient samples, while others isolated and stimulated neutrophils to generate NDMVs, which were subsequently quantified. Until future studies develop a standardized method of collection and analysis [10, 23], interpretation of these data in a day-today clinical setting will be problematic.

### **Microvesicle Stimulation and Mechanisms of formation**

Similar to other global microvesicle populations, neutrophil derived microvesicles can be elicited from both apoptotic and activated cells by a large number of stimuli. Microparticle composition appears to be different based on stimuli used for generation. This is illustrated

by a recent study by Dalli *et al.*, demonstrating different NDMV protein composition depending on neutrophil state, and discrete impact endothelial cell gene expression profiles, depending on stimulus used for NDMV generation [18c]. For organizational purposes, we will separate these compounds into 3 broad categories: bacterial byproducts, host factors/cytokines, and exogenous compounds. In general, bacteria have been observed to cause neutrophils to spawn NDMVs [18b, 24]. Strikingly, these microvesicles are subsequently observed to curtail bacterial growth [18b]. Bacterial byproducts such as endotoxin [25] and fMLP [26] are potent inducers of NDMV generation. In terms of host mediators, TNF- $\alpha$  [27], complement [26e, 28], IL-8 [26a], and Platelet activating factor [20] have all been demonstrated to increase NDMVs generation. Other exogenous compounds such as the protein kinase C activator, PMA [20, 26a, 26b], ionomycin [26a, 26b], and nitric oxide synthase inhibitor, L-NAME [29], have also been found to increase NDMV production. A complete list of compounds, and references to date, dealing with the generation neutrophil derived microvesicles can be found in Table 2.

Currently the methodologies utilized to investigate mechanisms underlying NDMV generation are quite varied. For example, a number of papers “prime” neutrophils prior to the incubation with the molecule of interest, while others do not. In addition, there is great variability on incubation lengths for the stimulus of interest. Furthermore, NDMV quantification is varied in a number of ways, such by protein amount or absolute or indirect counting by flow cytometry. There is no consensus on which cell surface marker(s) should be utilized to identify NDMVs. Indeed, neutrophil specific antibody titration curves should be presented to show specificity of the antibody for NDMV. Annexin positivity (to detect extracellular expression of phosphatidylserine) or negativity as it relates to NDMV enumeration is also something in which there is no consensus in the field. It is quite possible that MV expression of phosphatidylserine will vary depending on the timing of MV derivation; ie., during the early or latter stages of the disease state [30]. However, without standardized methodology to characterize and enumerate NDMVs, it will be difficult to temporally compare differences of NDMV generation with different stimuli.

A number of papers have evaluated intracellular molecular mechanisms of NDMV generation. Multiple studies have looked at NDMVs being generated through cell activation specifically investigating compounds that increase intracellular calcium concentration [18b, 25b, 26a, 26b]. Increased intracellular calcium is known to increase calpain activity [31]. The enzyme calpain is normally responsible for the degradation of talin, an essential structural component of the neutrophil cytoskeleton and the shedding of membrane-derived vesicles [32]. Because of the relationship of calcium, calpain, and microvesicle formation, most studies have evaluated microvesicle formation in the presence of reagents that increase intracellular calcium, namely, fMLP, PMA (a protein kinase C activator), and L-NAME. Consistent with this, calpain inhibition decreased NDMV generation after neutrophil activation [25a, 29]. In terms of NDMV generation during cellular during the events leading to apoptosis, TNF- $\alpha$  alone has been shown to be sufficient to generate NDMVs, through an NF- $\kappa$ B and/or Caspase 8 dependent pathway [27]. Caspase 8 activates Caspase 3, and caspase 3 can activate Calpain *via* cleavage of the calpain inhibitor calpastatin [33]. Altogether, the majority of these studies investigating NDMV generation utilize *in vitro*

systems. One challenge going forward is to verify that these *in vitro* elucidated mechanisms using *in vivo* models.

### NDMV Impact on Target Cells

The number of NDMVs can be increased in a number of inflammatory disease states, and their presence appears to be able to influence the immunological process (Fig. 1). Although there have been a number of *in vitro* studies describing how NDMVs can mediate target cell function, there is a paucity of reports describing how manipulating NDMV numbers *in vivo* could impact the pathophysiology being studied. It remains to be determined whether NDMVs can augment or depress the inflammatory response. Currently, the literature suggests that this response is dependent upon the state of neutrophil/how the NDMV is generated, as well as dependent upon the cellular target. The effects of NDMVs on cells can be seen on Table 3, and are discussed below.

Interestingly, NDMVs can have an autocrine impact with respect to neutrophil chemotaxis. This is consistent with concept that microvesicle formation is intertwined with uropod formation of a polarized cell and the content of the MV [34]. It has been demonstrated that NDMVs can enhance chemotaxis due to expression of the surface adhesion molecules L-selectin and P-selectin glycoprotein 1 on the NDMV [29]. In contrast, it has been shown that NDMVs inhibit chemotaxis dependent upon annexin 1 expression [35]. It is likely that the explanation for these seemingly disparate results is due to the mechanism of NDMV generation. In the former study, NDMVs were generated by fMLP stimulation coupled with L-NAME incubation. In the latter case, NDMVs were generated without the L-NAME incubation. These studies implicate the importance of nitric oxide upon MV function during formation.

NDMVs can exert a pro-inflammatory response on platelets. It has also been observed that NDMVs can cause an increase in platelet expression of P-selectin *via* binding and activation of  $\alpha_{IIb}\beta_3$  integrin [25b]. NDMVs isolated from septic humans activated JNK1-specific signaling in endothelial cells resulting in the release of IL-6 and MCP-1 [26d]. Consistent with NDMV-mediated increases in inflammation, another study demonstrated that NDMVs caused endothelial cells to generate IL-6 and IL-8, as well as increased expression of adhesion molecules [26c]. Additionally, NDMVs were found to bind to endothelial cells *via* CD18, resulting in increased ICAM-1 expression as well as increased reactive oxygen species generation by endothelial cells [20]. NDMVs were also shown to increase endothelial cell tissue factor expression, which was associated with the generation of a factor Xa-dependent procoagulant response [26d]. While these examples are ways in which NDMVs would enhance the inflammatory response, NDMVs generated from neutrophils co-incubated with endothelial cells were shown to down-regulate STAT1, NF- $\kappa$ B, CCL8, and CXCL6 expression in endothelial cells, which would result in an anti-inflammatory processes or decreased pro-inflammatory actions [18c]. Interestingly, Lim *et al.*, recently demonstrated that inhibition of NDMV generation resulted in increase in increased vascular leakage, suggesting that NDMVs, in normal physiologic conditions, are in part, responsible for maintaining vascular integrity [36].

NDMV have been demonstrated to exert an overall anti-inflammatory or inflammation-resolving effect on cells of a myeloid lineage. In mature macrophages, NDMV-macrophage cell contact is sufficient for blocking macrophage phagocytosis [26e]. NDMVs also block the inflammatory response of macrophages to zymosan and LPS [26e]. Specifically NDMVs, block the zymosan activation of macrophages by inhibiting NF- $\kappa$ B phosphorylation and nuclear translocation [37]; this inhibition was found to be through NDMV activation of macrophage MerTK (Mer-receptor tyrosine kinase) and PI3K/Akt pathways [37]. Phosphatidylserine expression on NDMVs is thought to be the mechanism for MerTK activation on macrophages, as PS blockade inhibits MerTK activation [38]. Interestingly, PS expression on other cell-derived microvesicles did not result in activation of this pathway [38]. In addition, NDMVs from both apoptotic and zymogen stimulated neutrophils cause macrophages to generate distinct lipid pro-resolving mediators [39], which would also serve to contribute to a counter inflammatory response. Alterations in TGF- $\beta$ 1 production have been observed after incubation with NDMVs [26e, 40]. TGF- $\beta$ 1 can enhance regulatory T-Cell differentiation [41], as well as block macrophage and lymphocyte activation. More recently, NDMVs have been shown to decrease production of IFN- $\gamma$  and TNF- $\alpha$ , while increasing release of TGF- $\beta$ 1 in IL-2 activated natural killer cells [40b].

Similar to mature macrophages, in monocyte-derived dendritic cells, NDMVs caused a change in morphology consistent with reduced phagocytic activity and increased release of TGF- $\beta$ 1 [42]. In this same study, when NDMVs were co-incubated with monocyte-derived dendritic cells in the presence of LPS, activation marker expression decreased, inflammatory cytokine production decreased, and macrophages demonstrated a reduced capacity to induce T cell proliferation [42]. Finally, the presence of NDMVs was observed to decrease neutrophil-HUVEC adherence [35]. We speculate that these aforementioned actions would blunt proinflammatory actions of myeloid cells during the immune response.

Consistent with other models [43], one constant observation concerning NDMV, regardless of target cell, is that when the NDMV is annexin V positive, the impact upon the immune response is invariably immune suppressive. Ingestion of phosphatidylserine expressing neutrophils by macrophages leads to alterations in PGE-2 [44] and IL-10 [45] levels. Additionally, blockage of PS expression on NDMVs attenuated the anti-inflammatory response in NK cells [40b]. Changes in PGE-2 could serve to modulate the macrophage immune response decreasing inflammatory cytokine secretion [46]. IL-10 can suppress the antigen-presenting capacity of antigen presenting cells, regulate phagocytosis on macrophage subsets, and decrease inflammatory cytokine production by neutrophils [47]. Thus, we speculate that increased NDMV numbers generated from apoptotic cells may act as a brake on pro-inflammatory functions.

NDMV generation during sepsis may illustrate the way NDMV numbers would influence the pathophysiology of a disease. Sepsis is the leading cause of morbidity and mortality in surgical intensive care units [48]. This disease is characterized by an overwhelming acute inflammatory response and impaired immune responses [49], as well as endothelial dysfunction, and a systemic prothrombotic state. These all often culminate in end-organ hypo-perfusion or micro-vessel thrombosis, which can lead to multi-organ failure and eventually death. In a patient who is septic, NDMV generating mediators, as seen in Table 1,



whether bacteria, bacterial byproducts or host-derived mediators, are abundant. We speculate that these mediators result in NDMV production that is excessive. Elevated NDMVs then can cause increased coagulation [26d] and platelet adhesion [25b] leading to micro-thrombosis, increased inflammation of the vasculature [26c, 26d] and increase reactive oxygen species [20, 26c] leading to impaired vascular function. Taken together, we postulate that a sustained overabundance of NDMVs may be contributing to an anti-inflammatory immune status. This would be detrimental during sepsis, as an immunosuppressed phenotype has been shown to be associated with an inability to clear infections and to worsened outcomes [50].

## CONCLUSION

The field of NDMVs remains an area of biology where much remains to be elucidated. The impact of microvesicles on the host inflammatory response highlights the need for more research into this area. NDMVs as biomarkers for disease and targets for medical intervention holds much promise, however, a standardized methodology for NDMV qualification and quantification needs to be agreed upon. Additionally, more work is needed to elucidate the molecular mechanisms of NDMV generation and their subsequent impact on target cells. By understanding the effects NDMVs have on target cells, and by identifying molecular targets involved in NDMV generation, the clinician will potentially be able to modulate the immune system through control of NDMV production.

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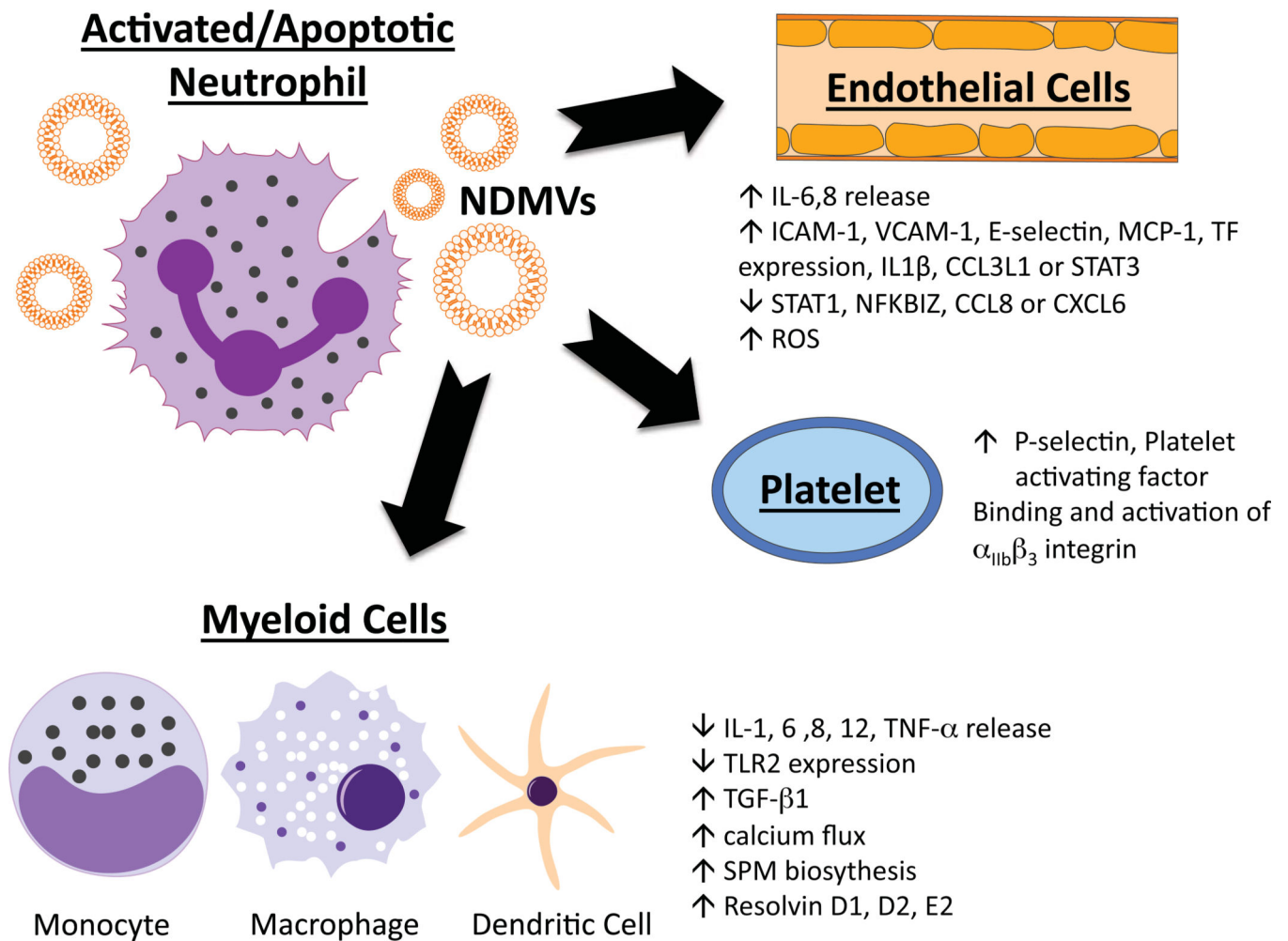
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**Fig. 1. Impact of NDMVs on various cells**

Abbreviations: SPM - Specialized pro-resolving mediator.

**Table 1**

Presence of NDMVs in human disease.

Human Disease	Source	References
Cardiovascular Disease/Atherosclerosis	Platelet-poor plasma (PRP)	[19b, 51]
Systemic Lupus Erythematosus, Anti-phospholipid Syndrome	PRP	[51]
ANCA-vasculitis	PolymorphPrep (Blood)	[20]
Pneumonia/ARDS	BAL / PRP / Blood	[6, 19a, 22]
Tubulo-interstitial nephritis, Vasculitis, IgA Nephropathy	Blood	[19a]
Sepsis	Blood / Peritoneal Lavage	[6, 18]
Localized Infection	Blister exudate	[18c]

Abbreviations: NDMV – Neutrophil Derived Microvesicle, ARDS – acute respiratory distress syndrome, BAL – Bronchoalveolar lavage (BAL).

Table 2

Stimuli for NDMV generation.

NDMV Induction Stimulus	Concentration	Incubation Time	Species: (H)human (M)ouse (R)ats	Quantification Method	Notes	References
<b>Bacterial Byproducts</b>						
- Bacteria	n/a	20 min	H, R	FACS, EM		[18b, 24]
- LPS	5 ug/mL	90 min	H	FACS		[25]
- fMLP	10 uM – 1 M	5 min-2 hr	H, M	FACS		[19a, 20-22, 26, 29, 35, 37, 42,52]
<b>Host Factors</b>						
- Spontaneous	n/a	15 min	H	FACS	<i>a</i> ,	[21-22, 52b, 52c, 53]
- TNF- $\alpha$	1-10 ng/mL	20 min	H, M	FACS	<i>b</i> ,	[19a, 20, 27, 54]
- Complement	6-9 ug/mL, 100 ng/mL	20-25 min	M	EM		[26e, 28]
- IL-8	100 ng/mL	2 hr	H	FACS		[26d]
- PAF	500 nM	15-30 min	H	FACS		[25b]
- Calcium	1 mM	15-30 min	H	FACS		[25b]
- ANCA	200 ug/mL	1 hr	H	FACS	<i>c</i> ,	[20]
<b>Exogenous Compounds</b>						
- PMA	10 nM – 5 M	20 min	H	FACS, EM		[18b, 25b, 26a, 26b]
- Ionomycin	0.5-5 mM	20 min	H	FACS		[26a, 26b]
- L-NAME	30 uM	1 hr	H	FACS		[29]

<sup>a</sup> Samples taken from patients with ARDS [22]<sup>b</sup> Neutrophils pre-incubated overnight for synchronization [54]<sup>c</sup> Used TNF- $\alpha$  to prime neutrophils [20]

Abbreviations: FACS - Fluorescent Activated Cell Sorting, EM - Electron Microscopy, LPS – Lipopolysaccharide, PAF – Platelet Activating Factor, L-NAME – NG-nitro-L-arginine methyl ester, ANCA – anti-neutrophil cytoplasmic antibody



**Table 3**

NDMV impact upon host cell.

Target Cell	NDMV Source	Cell Impact	Relevant Mechanisms	References
Endothelial cells	fMLP, IL-8, TNF- $\alpha$ , MPO	↑ IL-6,8 release ↑ ICAM-1, VCAM-1, E-selectin, MCP-1, TF expression, IL1 $\beta$ , CCL3L1 or STAT3 ↓ STAT1, NFKBIZ, CCL8 or CXCL6 ↑ ROS	Induction of JNK1 pathway NDMPs bind to Endothelial cells <i>via</i> CD18	[18c, 20, 26c, 26d]
Macrophage	fMLP, C5a	↓ IL-1, 6, 8, 10, 12, TNF- $\alpha$ release ↓ TLR2 expression ↑ TGF- $\beta$ 1 ↑ calcium flux ↑ SPM biosynthesis ↑ Resolvin D1, D2, E2	Induction of MerTK-dependent anti-inflammatory pathway Activates PI3K/Akt pathway	[18c, 26e, 37–38, 40a]
Immature macrophages	fMLP	↑ TGF- $\beta$ 1, ↓ CD40, CD80, CD83, CD86, and HLA-DP DQ DR expression ↓ IL-8, IL-10, IL-12, and TNF- $\alpha$ release		[40a, 42]
Neutrophils	fMLP	↓ cell/cell adhesion	Annexin-1 presence on MP implicated in decreased in adhesion	[35]
Platelets	PMA, LPS	↑ P-selectin, Platelet activating factor	Binding and activation of $\alpha_{IIb}\beta_3$ integrin	[25]
Monocyte	Neat - BAL/Serum	↑ phagocytotic activity ↑ or ↓ cell activation <sup>a</sup>		[27]
Bacteria	S. Aureus	↓ bacterial growth	Increased bacterial clumping	[18b]

<sup>a</sup>Increased activity if NDMP phagocytosed by THP-1 cell, decreased if NDMP interacts with cell, but is not phagocytosed

Abbreviations: MPO - Myeloperoxidase, ROS - Reactive Oxygen Species, SPM - Specialized pro-resolving mediator, THP1 - Human acute monocytic leukemia cell line, BAL - Bronchoalveolar Lavage