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Mechanisms underlying mouse TNF-α stimulated neutrophil derived microparticle generation

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ABSTRACT

Despite advances in understanding and treatment of sepsis, it remains a disease with high mortality. Neutrophil Derived Microparticles (NDMPs) are present during sepsis and can modulate the immune system. As TNF-α is a cytokine that predominates in the initial stages of sepsis, we evaluated whether and how TNF-α can induce NDMPs in mice. We observed that TNF-α treatment results in increased NDMP numbers. We also determined that the activation of either TNF receptor 1 (TNFr1) or TNF receptor 2 (TNFr2) resulted in increased NDMP numbers and that activation of both resulted in an additive increase. Inhibition of Caspase 8 diminishes NDMPs generated through TNFr1 activation and inhibition of NF-κB abrogates NDMPs generated through activation of both TNFr1 and TNFr2. We conclude that the early production of TNF-α during sepsis can increase NDMP numbers through activation of the Caspase 8 pathway or NF-κB.

1. Introduction

Sepsis is a serious medical condition characterized by a systemic inflammatory response due to a severe infection. Despite advances in understanding of sepsis, treatment remains supportive and the disease continues to account for a significant number of deaths in the critically ill population [1,2], with mortality rates ranging between 35 and 50% [1].

Tumor necrosis factor-α (TNF-α) predominates in the initial phases of sepsis and is one of the first cytokines released into the circulation during the inflammatory response to infectious stimuli [3]. It has diverse effects *in vivo* as a mediator of the immune response, in part, through activation of neutrophils [4]. Observed during sepsis are: impaired neutrophil recruitment to sites of infection, prolonged accumulation of neutrophils to remote sites, and dysregulation of neutrophil function [5]. TNF-α induced multi-organ damage has been shown to be primarily mediated by neutrophils [6–8], and this damage is ameliorated in neutrophil depleted pigs after TNF-α administration [9]. A better understanding of TNF-α activation of neutrophils is needed to more completely understand sepsis pathophysiology.

Microparticles (MPs) are small, intact vesicles ranging from 0.3–1.0 μm that bleb off the cell membranes, and are generated during cell activation and cell apoptosis, both of which occur during sepsis [10]. Host cytokines such as c5A [11], TNF-α [12,13], IL-8 [14], as well as bacteria [15] and bacterial byproducts, such as fMLP [14,16] and endotoxin [17] can increase MP formation. Neutrophils, cytokines, and bacterial products, are present in the septic milieu, and as such Neutrophil Derived Microparticles (NDMPs) may serve as potential mediators in the pathophysiology of this disease.

In vitro studies have demonstrated that NDMPs can have an inflammatory impact on endothelial cells, increasing both IL-6 and IL-8 production [18] and increasing expression of ICAM-1, VCAM-1, and E-selectin [14]. In addition, NDMPs been shown to increase in platelet expression of P-selectin [19]. NDMPs have also been shown to decrease macrophage IL-6, IL-8, IL-10, and TNF-α production[20,21], while increasing TGF-β1. Recently, we demonstrated that MPs accumulate at infected foci in critically ill patients during sepsis and are predominantly of neutrophil derivation [22]. In addition, we have shown that NDMPs are phagocytosed by monocytes, causing the amplified activation of the ingesting leukocytes and the deactivation of non-ingesting leukocytes, suggesting that they contribute to the disordered immune response during sepsis [22].

To date, the generation of NDMPs following TNF-α administration *in vitro* has been described in three papers [12,13,23], however these papers use TNF-α in conjunction with other agents. In this study, we hypothesized that mouse NDMPs could be generated by administration of TNF-α alone to unactivated neutrophils. To address the mechanisms, not addressed in previous literature, we investigated the role of TNF-α in NDMP generation. We specifically looked at the role of TNF Receptor 1 (TNFr1) and TNF Receptor 2 (TNFr2), as well as MP generation as it relates to pathways

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involved in apoptosis and cellular fate decisions, namely the caspase and NF-κB pathways. Altogether, increased understanding into the way NDMPs are generated and their effects on cellular function may lead to greater understanding of the inflammatory response and sepsis.

2. Materials and methods

2.1. Materials

Male C57BL/6 and TNFr1 KO (stock number: 003242) mice between 6 and 8 weeks of age were obtained from Jackson Labs (Bar Harbor, ME). All experiments involving animals were performed under protocols approved by the Institutional Animal Care and Use Committee of the University of Cincinnati (08-09-19-01). Mouse and human TNF-α Recombinant Protein (rmTNF-α and rhTNF-α, respectively) were purchased from Preprotech (Rocky Hill, NJ). NF-κB inhibitor (BAY 11-7085) and Caspase 8 inhibitor (Z-IETD-FMK) were purchased from R&D systems (Minneapolis, MN). The pharmacological agents were solubilized in DMSO (American Bioanalytical, Natick, MA). The amount of DMSO was consistent in all samples groups.

2.2. Neutrophil isolation

Mouse neutrophils from bone marrow were isolated from C57Bl/6 mice as previously described [6]. The femurs and tibias were flushed with Hanks' Balanced Salt Solution without calcium & Magnesium (HBSS) (Mediatech, Inc. Manassas, VA, USA). Bone marrow particulate was disaggregated and filtered in a 70 micron filter, then centrifuged at 400*xg* for 10 min at 23 °C. The cells in the pellet were resuspended in HBSS, placed on a 55% Percoll gradient, and centrifuged at 1000 *x g* for 30 min at 23 °C. The pellet was washed once more. The final neutrophil preparation was re-suspended in HBSS. The purity and viability of the neutrophils (90% purity >99% viability, respectively) was determined by flow cytometry. Neutrophil purity was assessed by flow cytometry after cell staining with fluorescently conjugated antibodies to Ly-6G, CD19, CD3 and F4/80 with >99% of the cells negative for F4/80 and CD3. The main cell contaminant in this preparation was due to B cells. Further purification was not conducted by centrifugation due to risk of non-specific neutrophil activation [7] or by magnetic beads due to microparticle-sized contaminants introduced by the beads themselves.

2.3. Microparticle generation and analysis

Isolated neutrophils from bone marrow preparation were plated and cultured for the indicated time at 37 °C with stated treatments. The cell/microparticle mixture was labeled for Ly-6G and Annexin V without manipulation as previously described [22,24]. Flow cytometry data acquisition and analysis were performed on a Beckman Coulter Epic flow cytometer using Kaluza software (Indianapolis, IN, USA). Neutrophil derived microparticle numbers were assessed using the neutrophil specific marker [25], Ly-6G (Clone: 1A8, BD Pharmingen, San Diego, CA, United States); and Annexin V (BD Pharmingen). Particles within the 0.3–1.0 μm range that were labeled with both Ly-6G and Annexin V were identified as NDMPs [26].

2.4. Statistical analysis

Statistical comparisons were performed using Student *t* Test (two groups), or one-way ANOVA with Tukey post hoc test, (more than two groups) using Prism 5.0 (GraphPad Software, La Jolla, CA, United States). The mean and standard error of the mean were calculated in experiments containing multiple data points. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Mouse TNF-α treatment results in increased neutrophil-derived microparticle generation

Using flow cytometry, forward- and side-channels were set to analyze particles using 0.5-, 0.9-, and 3.0-μm latex beads for calibration as previously described [27] (Fig. 1A). The forward and side

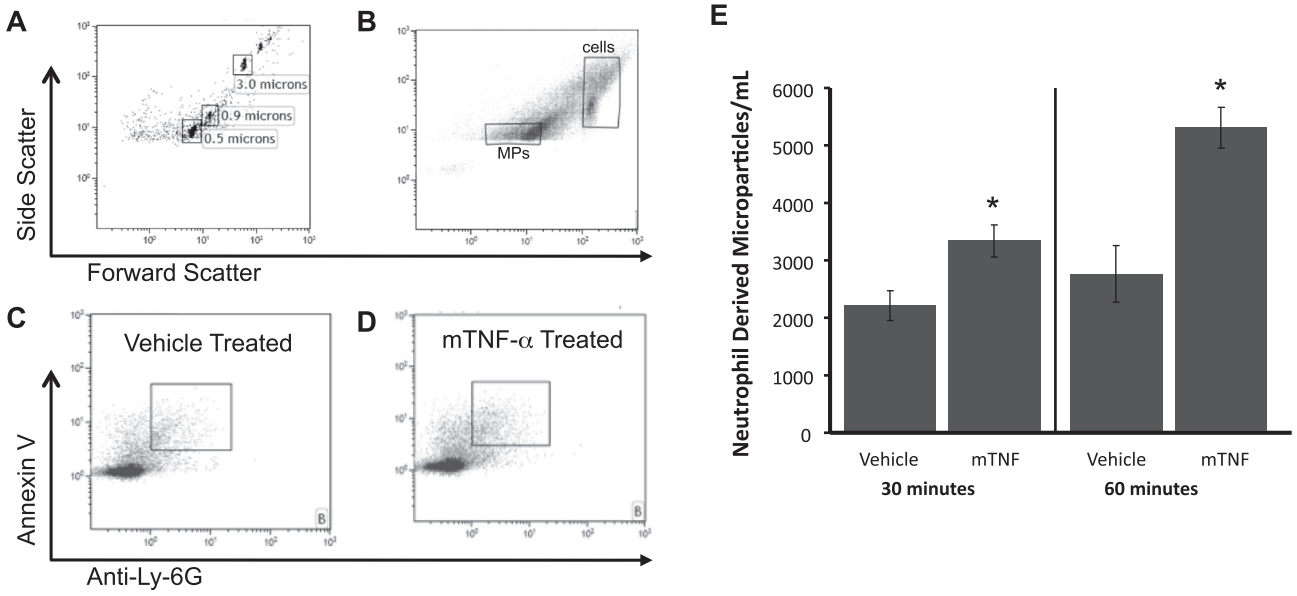

Fig. 1. TNF-α treatment *in vitro* increases NDMP numbers. Neutrophils were isolated from bone marrow as described in the methods. (A) Sizing for the microparticle populations was based upon latex beads sized between 0.5 and 3.0 μm. (B) Representative flow cytometric analysis of the forward and side scatter of a cell microparticle mixture obtained from culture. MP (microparticle gate) (C) Representative flow cytometric analysis using anti-Ly-6G (mouse neutrophil specific marker) and Annexin V (apoptosis marker) treated with vehicle. (D) with 25 ng/mL rmTNF-α. (E) Neutrophils were treated with rmTNF-α (25 ng/mL) and enumerated by flow cytometry at the indicated times. Each bone marrow was isolated and processed separately, the sample size = 4 mice. Data are expressed as the mean ± the SEM. The significance was determined using Student *T*-test at respective time points.*$p < 0.05$ compared vehicle. MP – microparticles, mTNF – murine tumor necrosis factor.

scatter gate for microparticles was then set based upon size (Fig. 1B). To enumerate NDMPs, we analyzed neutrophil and NDMP mixture with the neutrophil-specific marker, Ly6G, and the apoptosis marker, annexin V. Vesicles within the 0.3–1.0 μm range that expressed both Ly6G and phosphatidylserine were identified as NDMPs. (Fig. 1C) is representative of NDMP generation after vehicle treatment and (Fig. 1D) is representative NDMP generation after rmTNF-α treatment. In (Fig. 1E), we observed that 25 ng/mL of rmTNF-α increased the number of NDMPs both at 30 min and at 1 h. Viability of neutrophils was also assessed at these time points and in all subsequent experiments and no significant difference in cell viability was found in regard to treatment (data not shown). Thus, the one-hour time point was used for all future experiments.

3.2. Activation of both TNFr1 and TNFr2 results in NDMP generation

We next postulated that activation of TNFr1, TNFr2 or both would result in increased NDMP numbers. To determine the role of these two TNF receptors in NDMP generation, we took advantage that there are two forms of TNF-α, (1) rmTNF-α which binds to both TNFr1 and TNFr2 in mice and, (2) recombinant human TNF-α (rhTNF-α) which binds only to TNFr1 in mice [28,29]. We first observed that rhTNF-α treatment generated a significantly higher number of NDMPs compared to untreated neutrophils. Further, rmTNF-α generated a significantly higher number of NDMPs compared to rhTNF-α (Fig. 2A).

To determine if rhTNF-α was specific for TNFr1 activation, we treated neutrophils from TNFr1−/− mice with either vehicle or rhTNF-α (Fig. 2B). We demonstrate that rhTNF-α produces no significant difference in NDMP generation compared to vehicle in TNFr1−/− mice, suggesting that rhTNF-α activates only TNFR1.

Due to rmTNF-α generating a significantly higher number of NDMPs compared to rhTNF-α in WT mice, we hypothesized that activation of TNFr2 alone could generate microparticles. To test this hypothesis, we took TNFr1−/− mice and treated with rmTNF-α. We observed in (Fig. 2C), neutrophils from the TNFr1−/− mice treated with rmTNF-α still generated a significant number of microparticles above vehicle treatment. Additionally, rmTNF-α treatment in the WT mice generated significantly more microparticles than the TNFr1−/− mouse. Altogether, we observed that NDMP can be produced after activation of TNFr1 alone or TNFr2 alone, but is robustly increased by the combined activation of TNFr1 and TNFr2.

3.3. Inhibition of Caspase 8 diminishes NDMPs generated through activation of TNFR1

It is known that TNF-α can activate caspase 8, an enzyme known to be involved in cell apoptosis and activation [30]. As microparticles can be produced during cellular activation or apoptosis [10], we hypothesized that caspase 8 activation would increase NDMP generation. To test this, we utilized genetically modified mice and specific pharmacological agents with and without caspase 8 inhibitor (Fig. 3). Activation of TNFr1 alone (rhTNF-α) or TNFr1 and TNFr2 (rmTNF-α) coupled with caspase 8 inhibitor resulted in a significant decrease in NDMPs. Significantly, there was no decrease in microparticle generation from addition of Caspase 8 inhibitor to neutrophils isolated from TNFr1-/- mice. Thus, we observe that TNFr1 activation generates microparticles partially through a caspase-8 dependent pathway and that microparticle generation through activation TNFr2 is independent of the caspase-8 pathway.

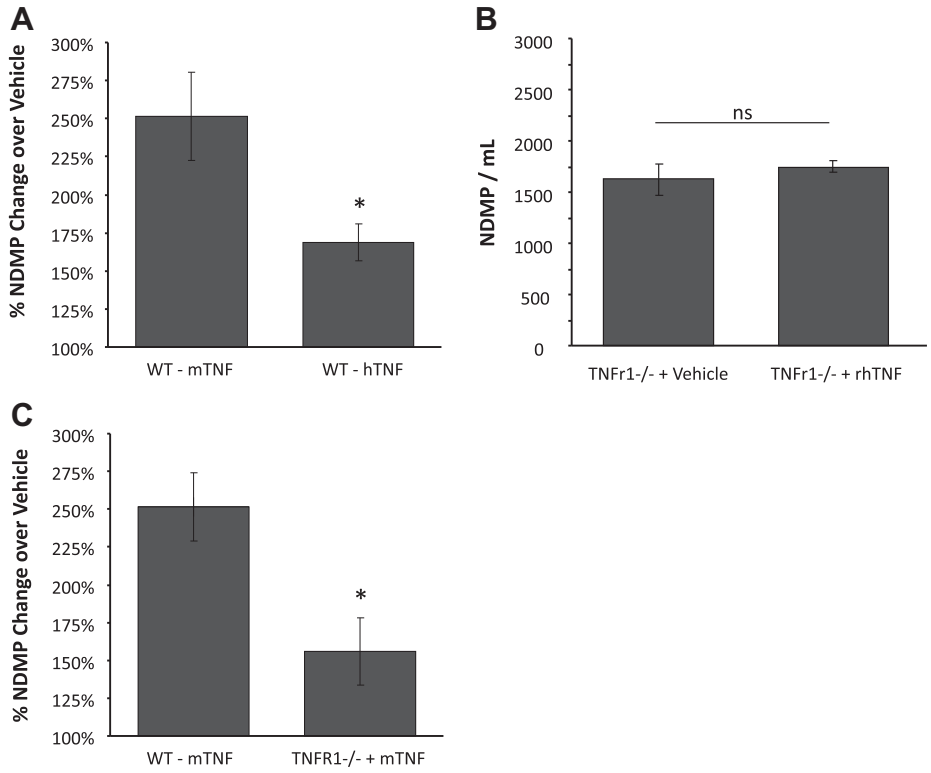

Fig. 2. The production of NDMPs is additive upon TNFr1 and TNFr2 activation. Mouse neutrophils were isolated from bone marrow and cultured *in vitro* as described in the methods. (A) Neutrophils from WT mice were treated with either 25 ng/mL of rmTNF-α (activates both TNFr1 and TNFr2) or 25 ng/mL of rhTNF-α (TNFR1 specific). After 1 h, NDMPs were analyzed by flow cytometry. The sample size = 12 mice. (B) Neutrophils from TNFr1−/− mice treated with vehicle or 25 ng/mL of rhTNF-α. After 1 h, NDMPs were analyzed by flow cytometry. The sample size = 8 mice. (C) Neutrophils from either WT mice or TNFr1−/− mice were treated with 25 ng/mL of rmTNF-α. After 1 h, NDMPs were analyzed by flow cytometry. The sample size = 8 mice. Each bone marrow was isolated and processed separately. All data are expressed in fold increase above vehicle and as means ± SEM. The significance was determined using ANOVA analysis and Tukey post hoc test. $^{*}p < 0.05$ compared vehicle.

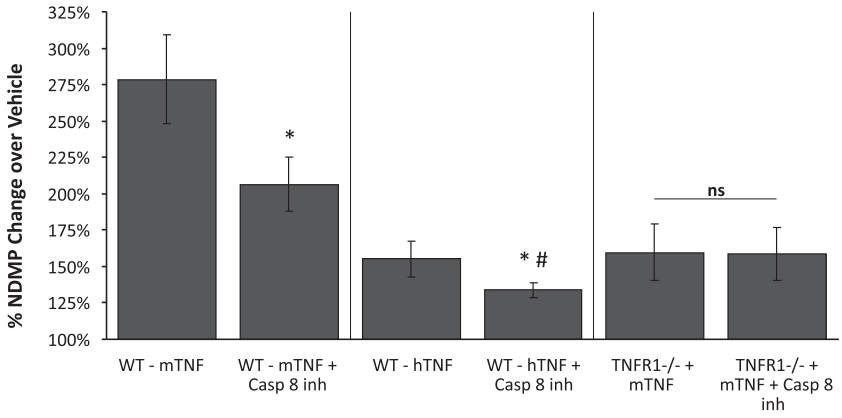

Fig. 3. TNFr1-specific generation of NDMPs is partially dependent upon caspase-8 activation. Mouse neutrophils were isolated from bone marrow from either WT or TNFr1−/− mice and cultured *in vitro* as described in the methods. Neutrophils were treated as indicated with 25 ng / mL rmTNF-α or 25 ng / mL rhTNF-α, or ± 30 μM of Caspase-8 inhibitor (Z-IETD-FMK). After 1 h, NDMPs were enumerated by flow cytometry. The sample size = 6 mice for each treatment group. Each bone marrow was isolated and processed separately. The data are expressed in fold increase above vehicle and as means ± SEM. The significance was determined using ANOVA analysis and Tukey post hoc test. $^{*}p < 0.05$ compared rm/rhTNF-α alone. # $p < 0.05$ compared to vehicle.

3.4. Inhibition of NF-κB abrogates NDMPs generated through activation of TNFr1 and TNFr2

As the NF-κB pathway is activated after cellular TNF-α stimulation [31], we hypothesized that NF-κB activation would increase NDMP numbers through TNFr1 or TNFr2. To test this, we utilized BAY 11-7085, which inhibits the activation of NF kappa B and the phosphorylation of Iκ Bα [32]. As shown in fig. 4, we observed that BAY 11-7085 completely abrogates NDMP generation after activation of TNFr1, TNFr2 or both. Altogether, we conclude NDMP generation through TNFr1 and TNFr2 activation is NF-κB dependent.

4. Discussion

In this study, we observed that treatment of neutrophils with TNF-α within 30 min (Fig. 1). Additionally we conclude that activation of either TNFr1 or TNFr2 is sufficient for NDMP generation, and that activation of both is additive (Fig. 2). Upon the selective activation of TNFr1, NDMP numbers were sharply decreased but not entirely eliminated with addition of Caspase 8 inhibitor. In contrast, caspase 8 inhibition did not alter NDMP numbers when only TNFr2 was activated (Fig 3). Finally, we demonstrate that upon activation of either TNFr1 or TNFr2, the inhibition of NF-κB activity significantly ameliorates NDMP generation (Fig. 4).

Microparticles, while present in healthy individuals, are elevated in patients with inflammatory conditions [22,33] and sepsis [34], and is associated with increased morbidity and morality [35]. In sepsis and systemic inflammatory response syndrome, increases in a number of cell-derived microparticles are observed [33]. It has been observed that microparticles can contain bioactive effector molecules, to include cytokines [36], mRNA or microRNA [37]. In their bioactive role, microparticles have been reported to interact with target cells to increase production of inflammatory molecules [38–41]. Interestingly, bacterial infected macrophages generate microparticles which stimulate non-infected neutrophils and macrophages to secrete TNF-α and induce production of nitric oxide synthase [42]. Finally, NDMPs have been shown to interact with endothelial cells to release IL-6 and IL-8 in addition to causing an up-regulation of gene expression of ICAM-1, VCAM-1, and E selectin [18]. Altogether, increased microparticle production during sepsis has the potential to influence the host response to sepsis.

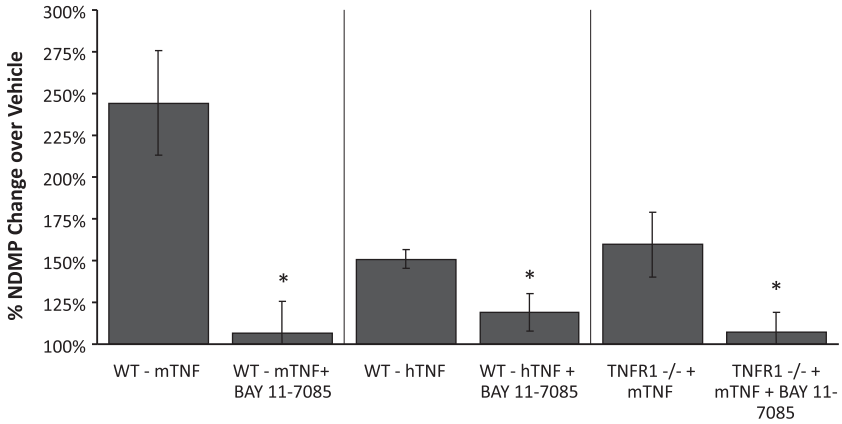

Fig. 4. Treatment with an NF-κB inhibitor decreases NDMP production induced by either TNFr1 or TNF-r2 activation. Mouse neutrophils were isolated from bone marrow of WT or TNFr1 -/- mice and cultured *in vitro* as described in the methods. Neutrophils were treated as indicated with 25 ng / mL rmTNF-α or 25 ng/mL rhTNF-α ± 20 μM of NF-κB inhibitor (BAY 11–7085). After 1 h, NDMPs were enumerated by flow cytometry. The sample size = 6 mice for each treatment group. Each bone marrow was isolated and processed separately. Data are expressed in fold increase above vehicle and as means ± SEM. The significance was determined using ANOVA analysis and Tukey post hoc test. $^{*}p < 0.05$ compared to rh/rmTNF-α treatment samples.

Previously, we reported that NDMP numbers were increased in the inflamed lung or peritoneum of critically ill patients when compared to uninfected patients [22]. We observed that these NDMPs expressed extracellularly oriented phosphatidylserine (PS). Extracellular PS acts as a signal for ingestion by phagocytes [43]. Upon uptake, it has been reported that phagocytes produce anti-inflammatory mediators, such as PGE-2, IL-10 and TGF-β [44]. In combination, these reports suggest that ingestion of PS expressing NDMPs may increase immune suppression with a resulting increase in morbidity and mortality [35]. In contrast, platelet-derived MPs have been observed to increase leukocyte-leukocyte interaction [45] as well as enhance monocyte chemotaxis [46]. Further, it has been reported that NDMPs can enhance neutrophil migration to sites of inflammation and injury [47]. These reported divergent effects of microparticles upon inflammation pose new questions and illustrate the importance of future study into the different ways microparticles modulate the immune system.

Since NDMPs have been shown to modulate the immune response, understanding the different mechanisms by which NDMPs are generated and better elucidating the specific effects they have on the immune system or target cells, will lead to identification of molecular targets for therapies directed at modulating the immune response during disease like sepsis. In this study, we demonstrate that NDMP numbers are increased in response to TNF- treatment, which can be mediated by TNFr1 and/or TNFr2. Additionally, Caspase 8 as well NF-κB activation support NDMP generation. Our study adds to our understanding of how microparticles are generated specifically after TNF- activation, and thus is just one piece of the puzzle dealing with the molecular mechanisms of NDMP generation. Altogether, further *in vivo* studies will be needed to confirm the significance of our *in vitro* findings. Additional investigations determining the impact of other key sepsis-related molecules (such as endotoxin or complement) and their mechanisms involved with NDMP generation will need to be done to create a broader picture of how NDMP generation could be modulated. Additionally, identification of the targets of NDMPs and specifically how the NDMPs interact with these target cells adds an additional object for modulation. By understanding the effects NDMPs have on the host cells and identifying molecular targets involved with NDMP generation, perhaps the future clinician will be able to modulate NDMP production, which in turn will allow for more proactive, specific, and directed treatments of sepsis.

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