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Impact of Caspase-8 and PKA in regulating neutrophil-derived microparticle generation

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Abstract

The morbidity and mortality from sepsis continues to remain high despite extensive research into understanding this complex immunologic process. Further, while source control and antibiotic therapy have improved patient outcomes, many immunologically based therapies have fallen short. Microparticles (MPs) are intact vesicles that serve as mediators of intercellular communication as well as markers of inflammation in various disease processes. We have previously demonstrated that MPs can be produced at the infected foci during sepsis, are predominantly of neutrophil derivation (NDMPs) and can modulate immune cells. In this study, we sought to elucidate the molecular mechanisms underlying NDMP generation. Using thioglycolate (TGA) to recruit and activate neutrophils, we first determined that intra-peritoneal TGA increase NDMP accumulation. We next utilized TGA-elicited neutrophils *in vitro* to investigate signaling intermediates involved in NDMP production, including the intrinsic and extrinsic caspase pathways, cAMP dependent PKA and Epac activation as well as the role myosin light chain kinase (MLCK) as a final mediator of NDMP release. We observed that NDMP generation was dependent on the extrinsic caspase apoptotic pathway (caspase 3 and caspase 8), cAMP activation of PKA but not of Epac, and on activation of MLCK. Altogether, these data contribute to an overall framework depicting the molecular mechanisms that regulate NDMP generation.

Graphical Abstract

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Keywords

Caspase; cAMP; MLCK; thioglycolate; inflammation

INTRODUCTION

Despite recent advancements in sepsis management, treatment still remains focused on supportive care and mortality remains unacceptably high [1–3]. Much research has been devoted to better understanding the pathophysiology of sepsis and the complex interactions of the inflammatory and counter-inflammatory responses that occur. While many immune modulating therapies have been investigated, there has been little success due to the inability to characterize and fully understand the mechanisms driving the inflammatory response [4–6]. This, in combination with the growing mortality rate, emphasizes the continued need for experimental and clinical research directed to understanding and characterizing the immune response to sepsis [7].

One potential mediator of sepsis is the microparticle (MP), which has been shown to play a role in various inflammatory processes including sepsis [8, 9]. Microparticles are small, intact vesicles ranging from 0.3–1.0 μm that arise from a variety of cell types such as lymphocytes, myeloid cells, and endothelial cells [10]. Cells can release MPs during activation and apoptosis [10], and previous literature has demonstrated that MP generation is the result of cytoskeleton rearrangement and plasma membrane budding. Further, this process requires increased intracellular calcium release, increased calpain activity and increased superoxide production to occur [11, 12]. Recently, we have demonstrated that during sepsis MPs accumulate at the infected foci in critically ill patients, that these MPs are predominantly of neutrophil derivation (NDMPs), and that they have a divergent impact on the immune response [8].

Various inflammatory states have been associated with increased circulating MPs *in vivo* [11]. In addition, MPs display a variety of pro-inflammatory activities, such as promoting adhesion and rolling of leukocytes, expressing pro-inflammatory cytokines, and triggering the release of MPs from other cell types *in vitro* [11]. Microparticles may also exert anti-inflammatory effects by inducing apoptosis in immunocompetent cells [13, 14]. In this

regard, the pro-apoptotic effects of MPs could provide a mechanism to control overwhelming inflammatory responses [11].

In the current study, we sought to elucidate the molecular mechanisms underlying NDMP generation in the inflammatory state. We utilized thioglycolate (TGA) to attract and activate peritoneal neutrophils in a model of sterile abdominal inflammation. Given that apoptosis induces cytoskeleton rearrangement and MP generation via plasma membrane blebbing [15], we investigated the role of proteins involved in caspase-dependent actin rearrangement. We also evaluated cAMP dependent PKA and Epac pathway as they also influence apoptosis [16]. We hypothesized that both caspase dependent actin rearrangement and cAMP activation would play important roles in NDMP production.

MATERIALS AND METHODS

Mice

Male C57BL/6 mice between ages six and eight weeks and 20 to 28 grams obtained from Taconic Labs (Hudson, NY, USA) were used for all experiments. All murine experiments were performed between 8am and 10am and under protocols approved by the Institutional Animal Care and Use Committee (IACUC # 08-09-19-01) of the University of Cincinnati.

Neutrophil and NDMP isolation

Mice underwent intra-peritoneal (IP) injections of 1 mL 3% thioglycolate (TGA) (Sigma, St. Louis, MO). Peritoneal lavage was performed 24 hours after injection using 10mL of sterile saline as previously described [17]. Lavage samples were centrifuged at $500 \times G$ for 10 minutes to pellet cells. The pelleted cells were either resuspended in FACS buffer (PBS with 1% bovine albumin and 0.1% azide) for analysis or culture media (RPMI 1640 supplemented with 5 % dialyzed FCS (heat inactivated) and 100 U/ml penicillin, 100mg/ml streptomycin, 1 mM sodium pyruvate, 1 mM HEPES, and non-essential amino acids (RP5)) for overnight culture. Cell free supernatants were further centrifuged at $10,000 \times G$ for 10 minutes. Supernatants were centrifuged at $25,000 \times G$ for 30 minutes to pellet MPs. The MP pellet was then resuspended in FACS buffer for flow analysis and Roswell Park Memorial Institute media (RPMI) for Nanoparticle Tracking Analysis (NTA) analysis.

Flow cytometry

Flow cytometry data acquisition and analysis were performed on a Beckman Coulter Epic flow cytometer using Kaluza software (Indianapolis, IN). Reagents used for microparticle labeling included PE Ly-6G (Clone: 1A8, BD Pharmingen, San Diego, CA) and FITC Annexin V (BD Pharmingen, San Diego, CA). Analysis of peritoneal MP surface antigen expression was conducted as previously described [18]. Briefly, gating for the microparticle populations was based upon the location of latex beads sized 0.5, 0.9 and 3.0 μm in the forward and side scatter channels. The rectangular gate was then situated to include the 0.5 and 1.0 μm region and used for gating upon microparticles.

Nanoparticle Tracking Analysis (NTA)

Microparticle preparations were diluted 1:10 with RPMI and stained with 10 μ l/sample PE Ly-6G (Clone: 1A8, BD Pharmingen, San Diego, CA). Nanoparticle Tracking Analysis (Nanosight, Malvern Instruments Ltd, Worcestershire, UK) was then used to enumerate NDMPs. Nanosight technology passes a laser through the sample liquid and both light scattering and Brownian motion are used to determine particle size and concentration in the sample by the NTA software. Unlike flow cytometry, which can only detect particles down to 300nm, NTA can detect particles ranging from 20nm-1000nm.

Cell culture assays

Cells were isolated from peritoneal lavage 24 hours after TGA injection as described above. Peritoneal cells were incubated with one of the following agonists or inhibitors overnight: 200 μ M pan-Caspase inhibitor (Z-VAD-FMK), 30 μ M Caspase-8 inhibitor (Z-IETD-FMK), 30 μ M Caspase-9 inhibitor (Z-LEHD-FMK), 100 μ M 8-Bromo-cAMP, 100 μ M 6-Bnz-cAMP (PKA agonist), 100 μ M 8-CPT-2Me-cAMP (EPAC agonist), or 250 μ M myosin light chain kinase inhibitor (ML7). All inhibitors were purchased from R&D Systems (Minneapolis, MN). Cells and MPs were collected 24 hours following incubation and analyzed using flow cytometry and NTA as described above.

Statistical analysis

Statistical comparisons were performed using Student *t* Test (two groups), or one-way ANOVA with Holm-Sidak post-hoc test (more than two groups) using Prism 6 software (GraphPad Software, La Jolla, CA). The mean and standard error of the mean were calculated in experiments containing multiple data points. A *p*-value of 0.05 was considered significant.

RESULTS

Thioglycolate can be used to generate microparticles *in vivo*

To determine whether of thioglycolate treatment would result in microparticle accumulation *in vivo*, we harvested peritoneal fluid from mice 24 hours after having received an intra-peritoneal injection of either 3% TGA or vehicle. We first used flow cytometry to characterize and enumerate NDMPs. Figure 1a depicts a representative forward (FSC) and side scatter (SSC) plot of the lavage with appropriate cellular and microparticle gates using 0.3–1.1 μ m beads. Figure 1b depicts a representative graph of isolated NDMPs identified using anti-mouse Ly6G and Annexin V. Intra-peritoneal NDMP enumeration from TGA-treated and vehicle mice are demonstrated in Figure 1c. We next accessed NDMP enumeration and characterization using Nanoparticle Tracking Analysis (NTA). A video frame sample from the NTA (Figure 1d), a representative histogram demonstrating the average size and concentration of the NDMPs (Figure 1e) are presented. Enumeration of intra-peritoneal NDMPs by NTA (Figure 1f) demonstrated a robust accumulation of NDMPs in the TGA-treated mice compared to untreated mice, which was consistent with FACS based NDMP characterization. Altogether, we observed that TGA treatment leads to NDMP accumulation *in vivo*.

NDMP generation is dependent on the extrinsic apoptotic caspase pathway

The caspase proteases are key regulators of apoptosis and their role in microparticle generation is not completely understood. We therefore sought to elucidate the involvement of active caspases upon NDMP generation. Peritoneal neutrophils were activated overnight in the presence of caspase inhibitors and enumerated by both flow cytometry and by NTA. Decreased NDMP production was seen in the presence of a pan-caspase inhibitor by flow cytometry (Figure 2a). To further determine if NDMP generation was due to activation of either active caspase 8 or caspase 9, we treated with cells with the indicated caspase inhibitor and enumerated NDMP numbers by FACS (Figure 2b and 2c). We further evaluated the role of the caspase inhibitors and observed decreased NDMP numbers after pan-caspase inhibition (Figure 2d) and caspase 8 inhibition (Figure 2e), and no change with caspase 9 inhibition (Figure 2f). Together, these data suggests that NDMP production is influenced by active caspase 8, but not active caspase 9.

Activation of the cAMP dependent PKA pathway can regulate NDMP formation

Cyclic AMP has been shown regulate cell activation and homeostasis through both protein kinase A (PKA) and cAMP-regulated guanine nucleotide exchange factor (Epac) [16]. Therefore, we next sought to investigate whether NDMP generation was modulated through activation of these cAMP mediated signaling pathways. First, TGA-elicited neutrophils were activated overnight in the presence of 8-Bromo-cAMP, a non-hydrolysable cAMP analogue. Enumeration of NDMPs by both FACS and NTA demonstrated that increased intracellular cAMP reduced NDMP generation (Figure 3). To further dissect the influence of cAMP-mediated signaling, neutrophils were cultured with either 6-Bnz-cAMP (a PKA agonist) or 8-pCPT-2Me-cAMP (an Epac agonist). We observed that activation of PKA by 6-Bnz-cAMP led to decreased NDMP production as measured by both flow cytometry and NTA (Figure 3). In contrast, Epac activation by 8-pCPT-2Me-cAMP did not affect NDMP formation when compared to vehicle. We therefore conclude that NDMP formation is regulated by the cAMP signaling pathway through activation of PKA but not Epac.

Myosin light chain kinase mediates NDMP generation

Finally, we next examined the role of myosin light chain kinase (MLCK) in NDMP generation. Peritoneal neutrophils were incubated overnight with ML7, an MLCK inhibitor. Decreased NDMP generation was seen after incubation with ML7 compared to vehicle on both flow cytometry and NTA (Figure 4).

DISCUSSION

This study demonstrates that NDMPs can be generated in a sterile inflammatory response. In addition, we show that NDMP generation is dependent on active caspase 8, and not caspase 9. We also demonstrated that cAMP activation of PKA, but not Epac, can inhibit NDMP formation. Altogether, we have demonstrated a novel way to generate NDMPs, compared 2 enumeration methods and have further elucidated key signaling intermediates underlying NDMP formation.

Previous investigations demonstrate that MP release is often associated with cell activation or initiation of apoptotic pathways [11], both processes that are increased during infection or sepsis. Neutrophil derived MPs have been reported to be the predominant MP population at the site of infection [8] as well as enhancing neutrophil recruitment to inflammatory sites [12]. Of note, it was recently observed that septic patients had increased levels of alpha-2-macroglobulin (A2M)-expressing neutrophil microparticles [19]. Further research demonstrated that A2M-expressing NDMPs enhanced survival and decreased bacterial loads in a severe murine model of sepsis (100% mortality in 36 hours) [20]. Elevated NDMPs have been shown to result in increased coagulation [21] and platelet adhesion [22] leading to micro-thrombosis, increased inflammation of the vasculature [21, 23], and increased reactive oxygen species [23, 24] leading to impaired vascular function. Thus, NDMPs may have either beneficial or detrimental effects during sepsis. We speculate that these divergent effects may be influenced by the underlying signaling pathways activated during generation as well as the degree of sepsis severity (reviewed in [25]).

Previous reports have demonstrated that the activation of neutrophils by mediators such as TNF- α [18], endotoxin [26] can lead to the activation of caspase 8. Key to these pathways is increased intracellular calcium concentrations, which impact signaling cascades that result in calpain activation, cytoskeletal rearrangement, and vesicle release [11, 12, 27]. A mediator of calpain is caspase 3, which is the point of convergence of both the intrinsic and extrinsic apoptotic pathways [28], and this pathway has been shown to impact MLCK activation resulting in membrane blebbing [15]. Other calcium dependent apoptotic and anti-apoptotic pathways have also been implicated in altered exosome release including cAMP dependent PKA activation [29].

Combining these known mechanisms of MP generation, and more specifically of NDMP generation, with the results of this study, we propose that there are activating and regulating mechanisms in place to alter generation of microparticles. We postulate that under activating or apoptotic conditions and through the caspase 8 pathway, NDMP numbers are increased. Surrounding immune cells then phagocytose the released NDMPs. When a critical concentration of NDMPs is present, these phagocytes begin producing immuno-regulatory cytokines, such as PGE₂ that can increase intracellular cAMP within inflammatory cells. Cyclic AMP then activates PKA, which subsequently down-regulates the formation and generation of NDMPs. Both of these pathways likely converge with the activation of MLCK in the case of the extrinsic caspase pathway, and inhibition of MLCK with the activation of the cAMP dependent PKA pathway, thus accounting for alterations to cytoskeletal rearrangement, plasma membrane blebbing and microparticle release. These pathways together serve as an auto-regulatory feedback mechanism for the immune system. We further hypothesize that in the septic patient, this system of NDMP generation and regulation is out of balance leading to increased inflammation and resulting morbidity and mortality.

It is important to understand both the mechanisms and stimuli for the release of NDMPs during infection and inflammation as NDMPs are known to modulate the immune response at the site of infection [8]. These particles contain a variety of biologic material including DNA fragments, micro-RNA, and cytokines and express a variety of receptors that define

their signaling role [10, 30]. These particles can act on target cells by transferring their contents inside the cell or by activating surface receptors [30], and based on their content and surface receptors can have differing effects on target cells [12, 19, 31]. While this study advances the understanding of the mechanisms behind NDMP generation, future studies are required to explore whether these mechanisms are specific to certain subpopulations. In addition, these findings also will need to be applied to an *in vivo* model to better elucidate the immune pathways affected.

Another unique aspect of this study is that it utilizes both flow cytometry and NTA to enumerate and characterize MP populations. We believe these two techniques are complementary as both have distinct strengths. Flow cytometry has traditionally been used to characterize MPs with a chief advantage of detecting multiple proteins [32]. The chief advantage of the NTA is its ability to detect a broad range of particle sizes, especially those of smaller size [33, 34]. In fact, a recent comparison of MP characterization techniques demonstrated that NTA could reliably detect vesicles down to 70–90 nm in size whereas conventional flow cytometry was only able to detect reference beads as small as 270nm [35]. In terms of characterizing different MP populations, fluorescent markers can be utilized on the NTA [34], however they are limited to use of a single markers. For this study, we utilized both methods of detection and found that similar trends upon inhibition of select signaling intermediates. However, we found a two-fold higher NDMP number values when using the NTA suggesting that the flow cytometer is only enumerating 1% of the vesicles being produced. Further studies are required to determine whether NDMPs of varying size can impact their content and subsequent messenger role.

In conclusion, as these data better elucidate the mechanisms that result in NDMP production, they also bring us closer to targeting NDMPs as a therapeutic intervention. It is clear that NDMPs are released at the site of infection and assist in recruitment of other immune cells, and if we can target NDMPs at different time-points during sepsis, we may be able to control the immune response and decrease sepsis-induced morbidity and mortality. Future research will focus on identifying the specific cellular targets of NDMPs in *in vivo* models and use these targets to modulate the immune response during sepsis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

MP	microparticle
NDMP	neutrophil-derived microparticle

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Highlights

- Thioglycolate treatment results in neutrophil-derived microparticle accumulation in the peritoneum
- Neutrophil-derived microparticle generation is dependent on caspase 3 and caspase 8
- PKA, but not EPAC, impact neutrophil-derived microparticle generation
- Myosin light chain kinase activation is key to neutrophil-derived microparticle generation

Figure 1. Thioglycolate can be used to generate microparticles in vivo

Naïve mice were injected with 3% thioglycolate IP and peritoneal lavage samples collected 24 hours after injection. Microparticles were isolated from the peritoneal lavage samples and enumerated and characterized via flow cytometry and NTA. **(A)** Representative forward and side-scatter flow cytometry plot with appropriate MP and cellular gates based on size. **(B)** Representative flow cytometry plot of Ly6G and Annexin V staining. **(C)** NDMP enumeration based on Ly6G+ and Annexin V+ staining on flow cytometry. **(D)** Representative video frame sample from NTA analysis. **(E)** Representative histogram of microparticle size plotted as a function of concentration. **(F)** NDMP enumeration based on Ly6G+ staining on NTA. Sample sizes are n= 4–5. Data are expressed as the mean $\pm$ SEM. * $p < 0.05$ when compared to untouched on Student's t-test.

Figure 2. NDMP generation is dependent on the extrinsic apoptotic caspase pathway

Naïve mice were injected with 3% thioglycolate IP, peritoneal lavage samples collected 24 hours after injection and neutrophils incubated overnight with various inhibitors. Flow cytometry and NTA were used to enumerate NDMPs. Analysis via flow cytometry demonstrated decreased NDMP populations with (A) pan-caspase inhibition and (B) caspase 8 inhibition. (C) Caspase 9 inhibition does not alter NDMP populations. Similarly, NTA analysis demonstrated decreased NDMP populations with (D) pan-caspase inhibition and (E) caspase 8 inhibition, and similar NDMP populations with (F) caspase 8 inhibition. Sample sizes are n= 4–6. Data are expressed as the mean $\pm$ SEM. *p < 0.05 when compared to vehicle on Student's t-test.

Figure 3. Activation of the cAMP dependent PKA pathway can regulate NDMP formation

Naïve mice were injected with 3% thioglycolate IP, peritoneal lavage samples collected 24 hours after injection and neutrophils incubated overnight with various inhibitors. Flow cytometry and NTA were used to enumerate NDMPs. Activation of PKA by 8-Bromo-cAMP and 6-Bnz-cAMP led to decrease NDMP production whereas Epac activation by 8-pCPT-2Me-cAMP did not affect NDMP formation compared to vehicle on both (A) flow cytometry and (B) NTA. Sample sizes are n= 4-6. Data are expressed as the mean $\pm$ SEM. *p < 0.05 on one-way ANOVA when compared to vehicle.

Figure 4. Myosin light chain kinase mediates NDMP generation

Naïve mice were injected with 3% thioglycolate IP, peritoneal lavage samples collected 24 hours after injection and neutrophils incubated overnight with an MLC-K inhibitor, ML7. Flow cytometry and NTA were used to enumerate NDMPs. Decreased NDMP generation was seen on both (A) flow cytometry (B) NTA after ML7 treatment. Sample sizes are n= 4–6. Data are expressed as the mean $\pm$ SEM. *p < 0.05 when compared to vehicle on Student's t-test.