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# Inflammasome Activation in an In Vitro Sepsis Model Recapitulates Increased Monocyte Distribution Width Seen in Patients With Sepsis

**OBJECTIVES:** Increased monocyte distribution width (MDW) has recently been shown to be a reliable indicator of early sepsis detection. This study therefore sought to determine if inflammasome activation can be linked to monocyte size changes in sepsis.

**DESIGN:** An in vitro sepsis model using bacterial endotoxin (lipopolysaccharide [LPS]) to study the effect of inflammasome activation on monocyte cell size distribution by microscopy and MDW measurements using a standard clinical hematology analyzer.

**SETTING:** University research laboratory.

**SUBJECTS:** Healthy adult volunteers and cultured human monocyte cells in wild-type state and after clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9 knockout of key inflammasome components (apoptosis-associated speck-like protein containing a caspase recruitment domain, caspase-1, gasdermin-D).

**INTERVENTIONS:** In vitro treatment of specimens with bacterial LPS.

**MEASUREMENTS AND MAIN RESULTS:** Wild-type THP1 cells demonstrated a significant increase in cell area ( $207 \mu\text{m}^2$  [159–400  $\mu\text{m}^2$ ] vs  $160 \mu\text{m}^2$  [134–198  $\mu\text{m}^2$ ];  $p < 0.001$ ) and distribution width (198 vs  $55 \mu\text{m}^2$ ;  $p < 0.0001$ ) by microscopy following treatment with LPS. Increased MDW correlated with inflammasome activation as demonstrated by release of interleukin (IL)-1 $\beta$  and with the presence of large distended pyroptotic cells by microscopy. All of these effects were blocked in the inflammasome knockout cells. Whole blood samples treated similarly also demonstrated IL-1 $\beta$  release and increased MDW (median 24.7 U [22.2–27.2 U] vs 16.3 U [15.1–17.6 U];  $p = 0.008$ ) as measured using the Beckman-Coulter Unicel DxH900 analyzer. When peripheral blood mononuclear cells were isolated prior to treatment with LPS, microscopy confirmed the presence of large pyroptotic cells correlating to IL-1 $\beta$  release in the human subject samples as well.

**CONCLUSIONS:** The increased MDW seen in patients with sepsis can be reproduced in an in vitro sepsis model and blocked using clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9 technology to inactivate the inflammasome. These findings suggest that pyroptotic cellular swelling underlies changes in MDW in septic patients and connect MDW to early events in the inflammatory cascade of sepsis.

**KEY WORDS:** biomarkers; inflammasomes; monocytes; pyroptosis; sepsis

The early diagnosis of sepsis is a challenging and high-stakes endeavor. Sepsis accounts for approximately 20% of deaths worldwide (1) and early diagnostic uncertainty often delays initiation of antibiotic therapy, resulting in measurably increased mortality (2). As such, the need for better sepsis biomarkers is an urgent priority. Many biomarkers perform well (3) but

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clinical utility is limited by cost, availability, long processing times, and the need for pretest suspicion of sepsis to prompt ordering.

Automated determination of leukocyte cell population data using volume, conductivity, and scatter characteristics circumvents many of these issues, providing detailed information within 2 minutes as part of a routine complete blood count. In particular, increased distribution width of monocyte volume (MDW) has emerged as a viable biomarker with excellent performance for early sepsis detection in the emergency department (4–6).

The underlying mechanisms responsible for increased monocyte size and heterogeneity in sepsis are unknown. Possibilities include changes in monocyte population composition due to mobilization of precursors from the bone marrow, demargination, subset differentiation, unequal cell death, or migration into inflamed tissues (7) versus alteration of individual cell morphology such as that which occurs in pyroptosis following activation of the cellular inflammasome.

Inflammasome stimulation by pathogen-associated molecular patterns such as bacterial lipopolysaccharide (LPS) activates caspase-1 via the adaptor protein apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) (8). Caspase-1 cleaves gasdermin-D (GSDMD) to form oligomeric pores in the plasma membrane, facilitating the release of inflammatory cytokines such as interleukin (IL)-1 $\beta$  and IL-18 while also causing osmotic swelling of the cell (8). Based upon our experience with an in vitro whole-blood model of sepsis (9), we hypothesized that increased MDW in septic patients reflects the presence of swollen monocytes undergoing pyroptotic death, which we here refer to as pyrocytes.

## MATERIALS AND METHODS

Inflammasome activation was induced using LPS at 1  $\mu\text{g}/\text{mL}$  in either THP1 cells (a human monocytic cell line) or whole blood collected in K2 EDTA tubes from healthy volunteers ( $n = 8$ ; median age 31; 75% female). THP1 cells were activated in either their wild-type (WT) state or with clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) knockout of key inflammasome components (ASC, caspase-1, or GSDMD) produced as previously described (10). Samples were incubated for 4 hours at 37°C, 5% CO<sub>2</sub>. In THP1 cells, 5 mM

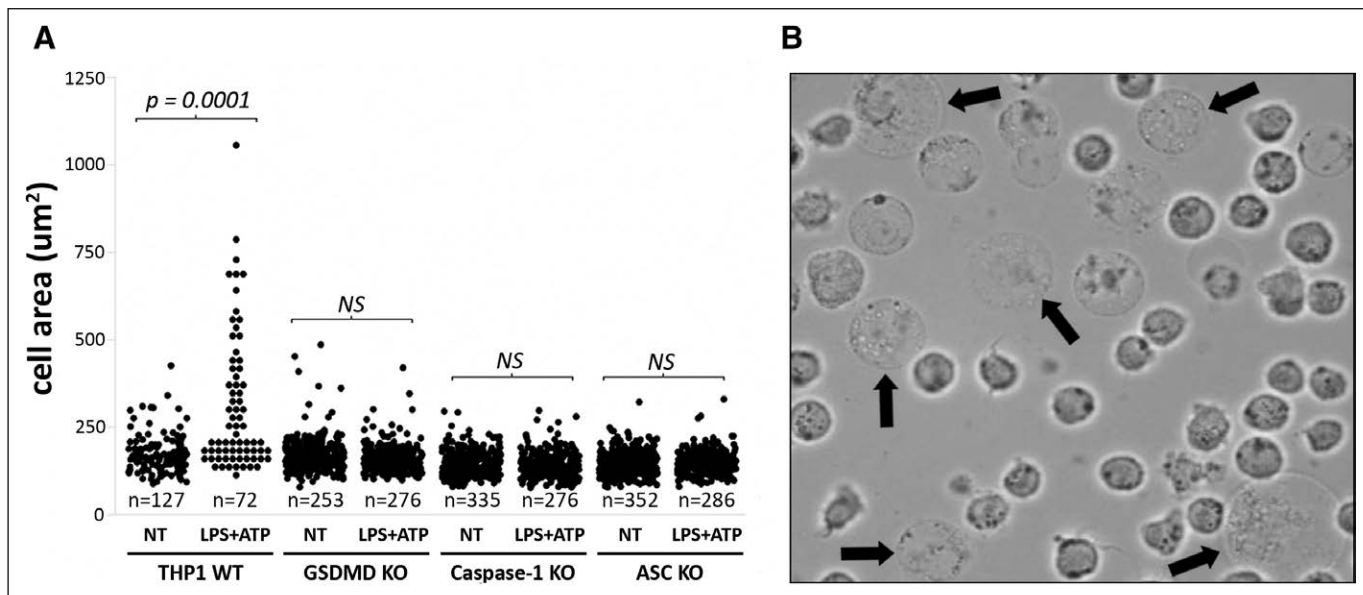
adenosine triphosphate (ATP) was added after 3.5 hours as a necessary second stimulus for inflammatory activation.

MDW was measured in blood samples using the Unicel DxH900 analyzer (Beckman-Coulter, Brea, CA). Due to differences in their baseline size and morphology compared to human monocytes, the DxH900 analyzer cannot provide reliable measurements of distribution width in THP1 cells. As such, THP1 cell diameter was measured manually in two orthogonal planes for each cell under light microscopy in three separate 60 $\times$  fields for each treatment condition. These measurements were used to calculate cell area and the SD for cell area in each treatment condition was used as a surrogate for MDW in the THP1 cells. The presence of cells with swollen morphology (pyrocytes) was also evaluated by light microscopy. While microscopic evaluation is straightforward in cultured cells, it is challenging in whole blood due to the interference of RBCs, the scarcity of monocytes, and the likelihood that many pyrocytes do not survive preparation of a blood smear due to the fragility of their membranes. As such, in a subset of our subjects ( $n = 2$ ), we also purified peripheral blood mononuclear cells (PBMCs) by Ficoll gradients prior to treatment with LPS for the purpose of microscopic evaluation for pyrocytes. IL-1 $\beta$  was measured by sandwich enzyme-linked immunosorbent assay as a marker of inflammasome activation.

All variables reported were continuous and treated as nonparametric. Central tendency is described by median (interquartile range) and compared using Wilcoxon signed rank tests. SDs for cell size were compared using the Levene  $F$  test. Two-tailed  $p$  values are reported. Alpha was set at 0.05. The human subjects protocol was approved by The Ohio State University's Biomedical Sciences Institutional Review Board (study number 2011H0059).

## RESULTS

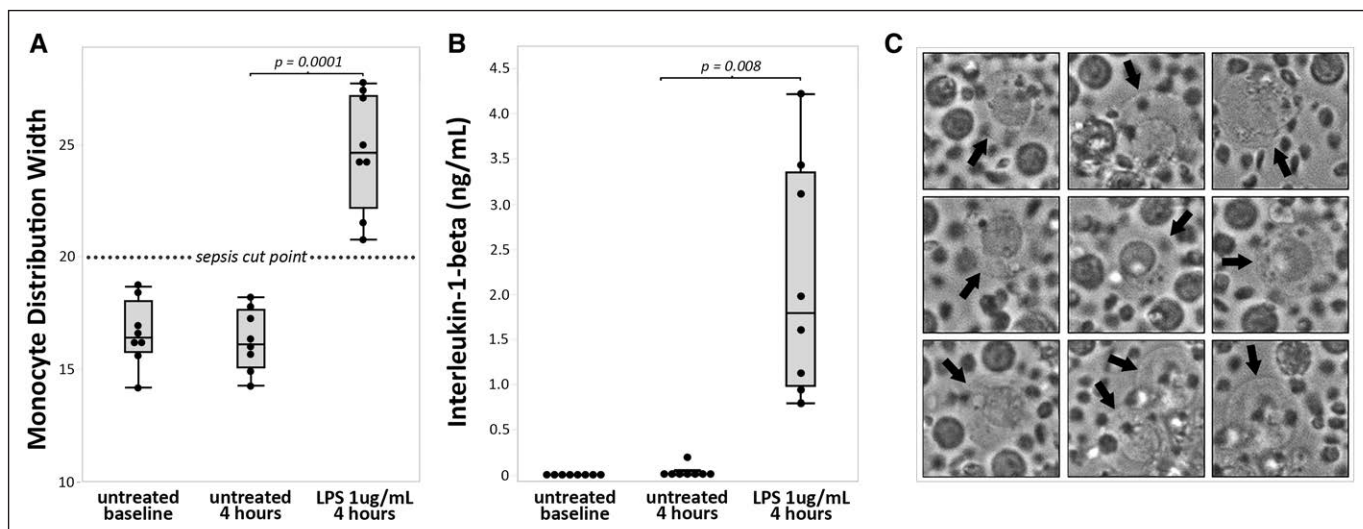
WT THP1 cells treated with LPS/ATP showed a significant increase in median cell area (207  $\mu\text{m}^2$  [159–400  $\mu\text{m}^2$ ] vs 160  $\mu\text{m}^2$  [134–198  $\mu\text{m}^2$ ];  $p < 0.001$ ) and SD for cell area (a surrogate for MDW; 198 vs 55  $\mu\text{m}^2$ ;  $p < 0.0001$ ) compared to untreated cells (**Fig. 1A**). No increase in size or distribution width was seen with LPS/ATP treatment in the inflammasome knockout cells. Pyrocytes were present in WT samples treated with LPS/ATP and were absent in all other groups (**Fig. 1B**).



**Figure 1.** Results of THP-1 experiments. Wild-type (WT) THP1 cells treated with lipopolysaccharide (LPS) and adenosine triphosphate (ATP) showed a dramatic increase in size and distribution width compared to those not treated (NT) (A). These changes are analogous to the increased monocyte distribution width seen in our whole blood samples treated with LPS and in patients with sepsis. Similar LPS-induced changes were not seen in cells in which key inflammasome components (gasdermin-D [GSDMD], caspase-1, or apoptosis-associated speck-like protein containing a caspase recruitment domain [ASC]) were knocked out using clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9 technology. B, The enlarged, transparent morphology of pyrocytes (e.g., arrows) as compared with surrounding unaffected THP1 cells. KO = knockout, NS = not significant.

IL-1 $\beta$  release was increased in treated WT cells (median, 2.2 ng/mL [2.29–2.3 ng/mL]) and undetectable in all other groups, indicating effective neutralization of inflammasome machinery in the knockout cells (trace release was seen in LPS/ATP-treated GSDMD knockout cells; median 0.01 ng/mL).

In whole blood, LPS treatment caused a significant increase in MDW (median 24.7 U [22.2–27.2 U] vs 16.3 U [15.1–17.6 U];  $p = 0.008$ ), crossing the sepsis cut-point of 20 U defined by a recent clinical trial (5) (Fig. 2A). IL-1 $\beta$  was released in LPS-treated samples (Fig. 2B), reflecting inflammasome activation (1.79 ng/



**Figure 2.** Results of whole blood experiments. Whole blood from healthy volunteers treated with lipopolysaccharide (LPS) showed a dramatic increase in monocyte distribution width, surpassing the sepsis cut-point established in a recent clinical trial (5) (A). Specimens treated with LPS also showed increased interleukin-1 $\beta$  release, confirming inflammasome activation (B). Microscopy of peripheral blood mononuclear cells (PBMCs) treated with LPS (C) confirmed the presence of pyrocytes (arrows) with balloon-like swollen morphology, seen here surrounded by unaffected PBMCs and platelets. Pyrocytes were absent in untreated samples.

mL [0.98–3.35 ng/mL] vs 0 ng/mL [0–0.04 ng/mL];  $p = 0.008$ ). Absolute monocyte counts (i.e., cell viability) did not significantly differ between the LPS-treated and untreated samples (450 [300–500] vs 384 [300–650];  $p = 1$ ). Microscopy confirmed the presence of pyrocytes in PBMC samples treated with LPS but not in untreated samples (Fig. 2C). Treatment with LPS caused increased MDW as measured on the DxH900 (median 32.8 vs 20.3 U) and IL-1 $\beta$  (median 4.6 vs 0.07 ng/mL) in PBMC samples as was observed in whole blood, although baseline MDW values were higher.

## DISCUSSION

These data show that increased MDW can be reproduced in an in vitro sepsis model using LPS to induce inflammasome-mediated monocyte swelling. Changes in cell size and morphology by microscopy mirror the increase in MDW suggesting that the effect is caused by changes in individual cells rather than an alteration in circulating monocyte populations. The observed inhibition of the LPS effect on size heterogeneity in the knockout cells strongly supports inflammasome activation as a major driver of these events.

Our findings link MDW to early events in the dysregulated host response to infection, supporting its potential to predict progression to sepsis in patients without overt clinical manifestations. In a recent study, elevated MDW at the time of presentation predicted progression from localized infection to Sepsis-3 within 72 hours in 71% of cases (5). Furthermore, since inflammasome activation in circulating leukocytes is a pivotal event in the transition from localized infection to sepsis, a biomarker that directly reflects this process and is rapidly available at the bedside could prove useful in future definitions of sepsis.

Our study does have several limitations. First, in vitro inflammasome induction is an over-simplified model for sepsis in a human patient. Additionally, sepsis caused by many different types of infections (Gram-positive, Gram-negative, viral, fungal, etc.) has been associated with increased MDW, and our model only tests the effect of one stimulus (bacterial endotoxin). Future studies should confirm our findings using actual organisms and other damage- and pathogen-associated molecular patterns to activate the inflammasome.

While our data support the role of inflammasome activation as a cause of increased MDW, it does not allow us to rule out the possibility that clinically observed changes in MDW are multifactorial with contribution from other mechanisms as well. For example, LPS-induced phenotypic differentiation or unequal cell death of monocyte subsets could also account for some degree of the changes in cell size heterogeneity. Future studies should use flow cytometry to monitor in vitro changes in CD14 and CD16 expression with LPS treatment and seek to demonstrate the presence of pyrocytes showing markers of inflammasome activation in the blood of septic patients.

## CONCLUSIONS

The increased MDW seen in patients with sepsis can be reproduced in an in vitro sepsis model and blocked using CRISPR/Cas9 technology to inactivate the inflammasome. These findings suggest that pyroptotic cellular swelling underlies changes in MDW in septic patients and connect MDW to early events in the inflammatory cascade of sepsis.

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