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Acute-Phase Deaths from Murine Polymicrobial Sepsis Are Characterized by Innate Immune Suppression Rather Than Exhaustion

Evan L. Chiswick,* Juan R. Mella, †John Bernardo,‡ and Daniel G. Remick*

Sepsis, a leading cause of death in the United States, has poorly understood mechanisms of mortality. To address this, our model of cecal ligation and puncture (CLP) induced sepsis stratifies mice as predicted to Live (Live-P) or Die (Die-P) based on plasma IL-6. Six hours post-CLP, both Live-P and Die-P groups have equivalent peritoneal bacterial colony forming units and recruitment of phagocytes. By 24 h, however, Die-P mice have increased bacterial burden, despite increased neutrophil recruitment, suggesting Die-P phagocytes have impaired bacterial killing. Peritoneal cells were used to study multiple bactericidal processes: bacterial killing, reactive oxygen species (ROS) generation, and phagocytosis. Total phagocytosis and intraphagosomal processes were determined with triple-labeled *Escherichia coli*, covalently labeled with ROS- and pH-sensitive probes, and an ROS/pH-insensitive probe for normalization. Although similar proportions of Live-P and Die-P phagocytes responded to exogenous stimuli, Die-P phagocytes showed marked deficits in all parameters measured, thus suggesting immunosuppression rather than exhaustion. This contradicts the prevailing sepsis paradigm that acute-phase sepsis deaths (<5 d) result from excessive inflammation, whereas chronic-phase deaths (>5 d) are characterized by insufficient inflammation and immunosuppression. These data suggest that suppression of cellular innate immunity in sepsis occurs within the first 6 h. The Journal of Immunology, 2015, 195: 3793–3802.

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E.L.C. performed experiments and analyzed data; J.R.M. cultured bacteria from peritoneal cavity and prepared data; J.B. provided equipment, reagents, technical expertise, and intellectual support; and E.L.C. and D.G.R. conceived the study and wrote the manuscript.

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Abbreviations used in this article: CLP, cecal ligation and puncture; DHR-123, dihydrodorodamine 1,2,3; Fsc, forward light scatter; MDSC, myeloid-derived suppressor cell; NET, neutrophil extracellular trap; NOX, NADPH oxidase; OpH-E. coli, opsonized pHrodo–labeled E. coli; ROS, reactive oxygen species; TB, trypan blue.

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Materials and Methods

Animals
Female ICR (CD-1) mice (Harlan-Sprague Dawley, South Easton, MA) 24–28 g were used for all studies. Mice were received and acclimated to our housing room for at least 96 h prior to surgery and cared for as described previously (16). The experiments were approved by the Boston University Animal Care and Use Committee.

Sepsis model
Cecal ligation and puncture was performed as first described (17) with minor modifications (16, 18). Approximately two-thirds the length of the cecum from the terminal tip was ligated and then double punctured longitudinally with 16-gauge needle. Mice were resuscitated with 1 ml saline (37˚C) with buprenorphine (0.05 mg/kg) for pain management (one injection every 12 h for 2 d). Antibiotics (25mg/kg imipenem) were administered 2 h post-CLP and then once every 12 h for 5 d.

Sampling
Blood sampling was performed initially at 6 and 24 h to generate IL-6 receiver operator characteristic curves and discrimination levels for prediction. Blood (20 μl) was collected by facial vein puncture and diluted 1/10 in PBS containing 3.38 mM EDTA trisodium salt. Blood was centrifuged for 5 min at 1000 × g, and the plasma was frozen at −20˚C. For mice that were sacrificed, blood was collected from the retro-orbital venous plexus under anesthesia (ketamine/xylazine), followed by euthanasia via cervical dislocation. Samples collected at 6 and/or 24 h were analyzed for IL-6 concentrations by ELISA as described previously (19).

Cecal ligation and puncture was performed as described previously (16). Cell pellets were resuspended in 2 ml wash buffer (PBS + 0.5% BSA) and 100 μl retained for total cell counts and cytospins. The remaining cell volume was underlain with 2 ml 30% sucrose (w/v) in PBS and centrifuged for 8 min at 450 × g to remove extracellular bacteria (20). The supernatant containing bacteria was aspirated, and the cell pellet was resuspended in 3 ml wash buffer. One milliliter of 100% isotonic Percoll in PBS was added to the cell volume to generate a 25% Percoll solution. This was underlain with 1 ml Histopaque 1.19 g/ml and then centrifuged for 25 min at 500 × g. The viable leukocytes were located at the Percoll Histopaque interface. Light debris was aspirated from the 25% Percoll layer, dead cells were removed from the pellet by pipette (21), and the remaining viable cells were washed once, followed by resuspension in HBSS containing Ca2+ and Mg2+ + 1% BSA. Cells were enumerated with a Beckman-Coulter particle counter model ZF (Coulter electronics, Hialeah, FL).

Peritoneal bacterial quantification
One hundred microliters of the first milliliter of lavage fluid was serially diluted with HBSS and cultured on 5% sheep blood agar plates (Fisher Scientific) for 24 h at 37˚C in anaerobic or aerobic conditions, followed by CFU enumeration.

Bacterial killing by peritoneal cells
Streptococcal-resistant Escherichia coli (E. coli strain HB101) were grown to log phase in tryptic soy broth containing streptomycin. Bacteria were cultured overnight on streptomycin-infused tryptic soy agar plates to prevent contamination. The percentage of bacteria killed = (CFU sample + CFU control – CFU sample) / CFU control × 100.

Neutrophil and macrophage ROS burst
ROS was measured by the conversion of nonfluorescent dihydrodichlorofluorescein 1,2,3 (DHR-123) to fluorescent R-123. Cells (4 × 10^6/ml) were loaded with 2 μM DHR-123 and stimulated with opsonized heat-killed E. coli (20 bacteria/cell) or 100 nM PMA for 30 min at 37˚C, then chlored on ice, followed by extracellular marker staining for flow cytometry. The ROS burst was calculated by the percentage increase of R-123 (geometric mean fluorescence intensity) of stimulated cells over unstimulated cells. Cellular FcRs were blocked with Fc Block (BD Biosciences). Cell viability was determined with Sytox Blue stain (Life Technologies). The experiment was repeated at least 5 min before acquisition. The following Abs were used: CD11b (clone M170), CD19 (1D3), CD3e (145-2C11), Ly6G (1A8), Gr-1 (RB6-8C5), F4/80 (Ci-A13), CD11b (clone M170), CD19, CD3e, Ly6G, and Ly6C (1A3). Only Sytox Blue negative events (Live Cells) were used for gating. Doublet discrimination was performed by forward light scatter (Fsc)-A versus Fsc-H.

Peritoneal cells were loaded to white-opaque microplate wells in duplicate at a concentration of 4 × 10^6/ml. Luminol was present at 20 μM. Cells were placed in a temperature-controlled fluorescent plate reader (Tecan Infinite M1000) and warmed to 37˚C for 5 min. Stimulus was then added (100 nM PMA or 20:1 bacteria:cell), and chemiluminescence was measured every ∼10 s (0.5-s integration time) followed by mechanical shaking.

3X-Labeled bacteria preparation
Labeling was performed as published previously (23). Briefly, 2 × 10^10/ml heat-killed (80˚C at 1 h) HB101 E. coli were sequentially labeled with 500 μM dihydrodichlorofluorescein-succinimidyl ester to detect ROS, 50 μM pHrodo-SE to detect pH changes, and 159 μM Alexa Fluor-350-SE to calibrate DCF/pHrodo fluorescence (Life Technologies). Labeling was performed in PBS (pH 9), degassed, and purged with N2 to minimize auto oxidation of DCF. Bacteria (∼1.5 × 10^7/ml) were opsonized with 1/10th volume of normal mouse plasma (heparinized) and anti-E. coli Abs (50 μg/ml) (Life Technologies). The use of particles covalently labeled with multiple fluorescent indicators/substrates provides temporal, spatial, and calibrated information for phagocytosis and phagosomal events that are not readily available with most cell permeable dyes. These approaches have been validated in previous publications (24, 25).

Phagocytosis and phagosomal ROS/acidification of 3X-labeled bacteria
Peritoneal cells were loaded into polypropylene cryovials at 4 × 10^6/ml. While on ice, ∼150 bacteria/cell were added to cells. Tubes were transferred to a water bath at 37˚C, placed on a stir plate, and agitated at lowest speed for 30 min with 7 × 2-mm micro stir bars. For controls, samples were also incubated on ice for 30 min. Following incubation, cells were kept on ice until data acquisition with an LSRII flow cytometer (BD Biosciences).

Ice controls were acquired first. Approximately 30,000 events were collected to measure attachment. Then, trypan blue (TB) was added to quench extracellular fluorescence (0.25% final concentration) and incubated on ice for 1 min, and then, ∼30,000 gated events were collected. Cells that were allowed to phagocytize were only acquired with TB present. Non-debris cells were gated on by Fsc/side scatter (of light). Doublets were removed by Fsc-h versus Fsc-w. Phagocytosis* gates were curated for each sample based off its own ice control (no phagocytosis occurs).

DCF, pHrodo, and Alexa Fluor 350 fluorescence was excited by 488-, 561-, and 355-nm lasers, respectively, and their emission was collected by (wavelength/band pass) 530/15, 590/10, and 450/50 filters, respectively. It was important to use a custom 590/10 filter for pHrodo because TB autofluoresces when bound to protein and its emission begins at ∼615 nm.

Data were analyzed with Flowjo (Tree Star). Derived parameters (i.e., calibrated fluorescence) were constructed so that DCF or pHrodo fluorescence for each event was divided by the fluorescence of Alexa 350 for that event. The ROS index was calculated by dividing the DCF/Alexa ratio of the cell by the DCF/Alexa ratio of the bacteria. Similar calculations were performed for the acidification index.

Statistical analysis
Statistics were performed using Prism 5 software (GraphPad Software, San Diego, CA). For comparison between two groups, an unpaired Student t test was used. For survival analysis, a Kaplan–Meier curve and log-rank survival were performed with a 95% confidence interval. All values were expressed as mean ± SE.

Results
Characterization of CLP-induced sepsis: similar beginning, dissimilar fate
To study the mechanisms of mortality, our laboratory uses a murine model of peritoneal sepsis induced by CLP. This model, which includes fluid resuscitation and antibiotic treatment, produces ∼50% mortality within the first 5 d of sepsis (acute phase). Mice were subjected to CLP, a small sample of blood was collected at 6 and 24 h post-CLP, and the mice were followed for survival. Monitoring plasma IL-6 levels of survivors/non-survivors shows that mice that succumb to sepsis have significantly elevated IL-6 24 h post-CLP (Fig. 1A) as well as at 6 h post-CLP (data not shown). Receiver operator characteristic curves were used to
generate IL-6 discrimination values to stratify mice as predicted to live (Live-P) or predicted to die (Die-P), as described previously (13). This discrimination value accurately predicted survival (Fig. 1B). Using the discrimination value, mice were sacrificed in subsequent experiments at 6 or 24 h post-CLP and posthumously stratified into Die-P and Live-P.

As reported previously by this laboratory, Die-P mice have similar numbers of cells in their peritoneum as compared with Live-P mice at 6 h post-CLP (Fig. 1C). Similarly, there is no difference in the number of bacteria within the peritoneal cavity at 6 h post-CLP (Fig. 1D) (16). This demonstrates that our CLP model is consistent, but more importantly, that both Live-P and Die-P groups were subjected to a similar initial bacterial insult with a similar initial cellular response. Despite a nearly identical 6-h response, by 24 h post-CLP Die-P mice have increased peritoneal cells (Fig. 1C) and bacteria (Fig. 1D). The divergence from similar phagocyte recruitment and bacterial burden at 6 h post-CLP to increased phagocytes and bacterial numbers at 24 h post-CLP suggests an early defect in phagocyte function in Die-P mice.

Die-P phagocytes show impaired bactericidal activity

To test the idea that impaired phagocyte function was responsible for the increased bacterial burden observed in Die-P mice, peritoneal cells were carefully processed to remove excess bacteria and dead cells, and the cells were then used in a bacterial killing assay. Interestingly, at 6 h post-CLP when cellular recruitment and bacterial burden were similar, differences in cellular function were already present. When bacterial killing was examined using live E. coli, Die-P mice showed a marked reduction in bacteria killed compared with Live-P mice within 6 h post-CLP and this persisted through 24 h (Fig. 2). Previous studies have shown that increased bacterial burden correlates with mortality (26–28) and that source control (removal of infectious foci) increases survival (13). In conjunction with those studies, the current data strongly suggest that the reduced bacterial killing within the first 6 h of sepsis leads to subsequent mortality.

Mechanisms of reduced bacterial killing

To determine why Die-P peritoneal phagocytes kill fewer bacteria than Live-P, bactericidal mechanisms were further examined. While multiple mechanisms are important in the clearance of pathogens, ROS generation is integral to the microbial killing process, as evidenced by humans with chronic granulomatous disease, a condition where patients are prone to recurrent bacterial infections because of a mutation that renders the ROS producing NADPH oxidase (NOX)
complex inoperable. To assess the ROS burst, peritoneal cells were loaded with DHR-1,2,3, stimulated with opsonized pHrodo–labeled E. coli (OpH–E. coli) or PMA, and analyzed by flow cytometry.

Phagocytes consist primarily of neutrophils and macrophages. To determine whether impairments in either cell type were responsible for the decreased bacterial killing, both neutrophil and macrophage function were distinguished through the use of cell-specific markers.

Neutrophils were defined as CD11b+, LY6G Hi and monocytes/macrophages were defined as CD11b+, LY6G lo/neg (Supplemental Fig. 1) (29, 30). Although the analyzed macrophage population expressed the classical macrophage marker F480, its variable level of expression in Die-P mice made it unsuitable for gating purposes (Supplemental Fig. 1G). Recent work has also shown that eosinophils express F480, and inflammatory conditions induced by thioglycollate result in decreased F480 expression by macrophages (31). This is likely due in part to newly recruited monocytes/macrophages that express little F480 (32).

Distinguishing bactericidal activity between neutrophils and macrophages showed that both populations likely contributed to the impaired bacterial killing observed in Die-P peritoneal cells. Following incubation with OpH–E. coli, there is a marked increase in ROS production measured by R-1,2,3 fluorescence in Live-P neutrophils, whereas the histograms for stimulated/unstimulated Die-P neutrophils are virtually superimposable (Fig. 3A). Die-P neutrophils produce significantly less ROS than their Live-P counterparts in response to OpH–E. coli within 6 h after induction of sepsis (Fig. 3B, left panel), and this deficiency persists through 24 h post-CLP. The use of OpH–E. coli as a stimulus is physiologically relevant; it measures how a cell would likely respond in vivo when encountering cecal bacteria. This process leads to ROS production subsequent to cell surface receptor ligature and signaling. However, if there were decreased receptor expression, desensitization, or other means of blunted receptor signaling, the ROS burst may fail to occur despite the ROS machinery (e.g., NOX) remaining fully functional. To determine whether the differences in ROS production were due to impaired pattern recognition receptor signaling, or a decreased capacity to generate ROS, PMA was used to stimulate the cells. PMA, as

**FIGURE 2.** Die-P mice exhibit impaired bacterial killing of E. coli at 6 and 24 h post-CLP. E. coli were incubated with or without peritoneal cells for 1 h and then plated overnight for CFU counts. Data from at least three independent experiments. n = 7–13/group at 6 h, 7–8/group at 24 h, where N represents an individual mouse. ***p < 0.0001 comparing the indicated groups.

**FIGURE 3.** Die-P peritoneal phagocytes have decreased ROS burst. (A) Live-P and Die-P peritoneal cells loaded with DHR-1,2,3 were incubated with OpH–E. coli or PMA to delineate physiological-response ROS or cellular ROS capacity via flow cytometry. Histograms show representative staining of PMN incubated with (red histogram) or without OpH–E. coli (blue). (B and C) Bar graphs show results from three independent experiments (n = 4–7 mice per group). (D and E) Representative ROS Kinetics for unstimulated (basal) (D) or Opsonized–E. coli (E) stimulated cells for Live-P and Die-P mice, as measured by Luminol based chemiluminescence (relative light unit [RLU]). (F) Bar graphs show the area under the curve (AUC) calculated for samples in unstimulated and stimulated conditions. (n = 3 mice/group). For all graphs, black filled symbols/bars represent Die-P mice and open symbols/bars represent Live-P mice. *p < 0.05, **p < 0.01, ***p < 0.001 between LIVE-P and DIE-P.
a nonspecific activator of ROS production, bypasses the requirement of pattern recognition receptor signaling for NOX activation. Following PMA stimulation, Die-P neutrophils were shown to be capable of generating ROS, albeit significantly less so than Live-P at both time points (Fig. 3B, right panel). Similar differences in ROS production in response to either stimuli were also evident in macrophages (Fig. 3C).

Diminished ROS generation alone does not explain decreased bacterial killing because macrophages with defective NOX and inducible NO synthase (iNOS) enzymes are as bactericidal as their NOX/inducible NO synthase competent counterparts (33). This occurs because, following internalization, the phagosome acidifies and enables pH-sensitive antimicrobial products to destroy the phagosome’s microbial cargo. Because pHrodo is a pH-sensitive fluorophore, the term pHrodocytosis was used to reflect the fact that differences in fluorescence could be due to differences in the number of attached and/or internalized bacteria or due to differences in pH. Comparing pHrodo (OpH–E. coli) fluorescence showed decreased pHrodocytosis for neutrophils and macrophages at both time points (data not shown).

Interestingly, Die-P phagocytes produce less ROS in response to OpH–E. coli stimulation compared with the unstimulated state (Fig. 3A, right histogram, 3B, 3C, black bars). It is important to note that unstimulated control cells are not naive cells; they were harvested from the peritoneums of mice with extreme peritonitis. Although these cells are already stimulated, there were significantly different responses at 6 h post-CLP, a time at which bacterial loads (i.e., preassay stimulation) were similar (Fig. 1D). A caveat to end-point measurements for ROS lies in that our analysis, and many others’ (34–37), employ normalization to an unstimulated control that is producing basal ROS. Because these cells were procured from inflamed tissue, it may be that Die-P phagocytes appear to generate less ROS than Live-P phagocytes in response to stimuli because the Die-P cells are already generating maximal ROS (i.e., hyperstimulated). If the Die-P mice were hyperstimulated, it would be difficult to detect any increase in ROS upon further stimulation. To determine whether Die-P cells were hyperstimulated, a chemiluminescence-based kinetic assay was used to measure ROS. Basal ROS data show that Die-P peritoneal cells produce less ROS prior to stimulation, thus arguing against the hyperstimulation hypothesis (Fig. 3D). Similar to what was observed with the flow cytometric ROS measurements, opsonized–E. coli (Fig. 3E) resulted in less ROS production in Die-P cells. The sum of the cellular response to either opsonized–E. coli or PMA was calculated as the total area under the curve (Fig. 3E, 3F). Importantly, luminol chemiluminescence shows that Die-P cells do generate more ROS from opsonized–E. coli as compared with basal conditions, unlike what was observed

FIGURE 4. Die-P phagocytes are suppressed in phagocytosis. 3×-Labeled E. coli were incubated with peritoneal cells for 30 min. Gating for Phagocytosis+ events: (A) Ice control (left), 37˚C incubation (right). TB was used to quench extracellular fluorescence. The percentage of Peritoneal cells from Live-P and Die-P mice that phagocytize bacteria at 6 h (B) and 24 h post-CLP (C), and the overall amount of bacteria internalized at 6 h (D) and 24 h (E). Data collected from three to four independent experiments. n = 4/group for 6 h, n = 5–7/group for 24 h. *p < 0.05, **p < 0.0001 Live-P versus Die-P.
by flow cytometry (Fig. 3B, 3C, leftmost panels). It was determined that the increased fluorescence seen in Die-P cells prior to exogenous stimulation (Fig. 3A) was due to increased autofluorescence (data not shown). Importantly, luminol chemiluminescence is not confounded by cellular autofluorescence. Furthermore, whereas opsonized-E. coli incubation produces a minuscule ROS burst in Die-P cells, PMA stimulation results in a marked increase in ROS production (Fig. 3F). This suggests that although Die-P cells retain the capacity to respond more vigorously to bacteria with ROS, they fail to do so. Taken together, these data suggest that Die-P cells are not hyperstimulated but may instead be exhausted or suppressed, resulting in decreased bacterial killing (Fig. 2), increased bacterial burden, and increased mortality (Fig. 1).

**Die-P phagocytes are immunosuppressed**

The preceding data suggest that Die-P phagocytes are impaired compared with Live-P, but the data do not indicate the mechanism. Die-P cells may exhibit impaired bactericidal activity because fewer cells have the capacity to respond to stimuli (i.e., exhausted cells). Alternatively, a similar fraction of stimuli-responsive cells may exist between groups, but the Die-P response is significantly weaker than Live-P (i.e., suppressed cells).

To address this, a saturating dose of labeled bacteria was used to stimulate the peritoneal phagocytes. A saturating dose is defined as the number of bacteria per cell needed to stimulate the maximum number of cells to phagocytize the bacteria. This presumably occurs due to ligation of all available receptors, as suggested by a study in human PMNs which showed that despite equal binding of ligand by all cells, only a subset of cells will actually respond (i.e., phagocytize) (38). In conjunction with TB to quench extracellular fluorescence, cells that have internalized bacteria can be discriminated from those that have not by comparing cells incubated with bacteria on ice versus 37°C (Fig. 4A).

Using a saturating dose, similar proportions of cells phagocytized the bacteria at both 6 h (Fig. 4B) and 24 h (Fig. 4C). However, Die-P cells phagocytized significantly less bacteria overall than Live-P at both the early and later time points (Fig. 4D, 4E). Decreased phagocytosis could be due to decreased attachment of the bacteria to the cell surface. However, this does not seem likely as both groups exhibit similar levels of bacterial fluorescence when...
incubated on ice and without addition of TB quenching agent (data not shown). These data support the differences observed in bacterial killing and suggest that Die-P peritoneal cells are suppressed in their function but are not exhausted because a similar percentage of cells do phagocytize, albeit significantly less than seen in Live-P.

Quantifying phagosomal responses

Intraphagosomal processes were examined to further define the mechanism of suppression and ascertain whether bacteria were still exposed to the caustic microbicidal environment following internalization. This was accomplished with a recently developed technique in which E. coli were triple labeled with Dichloro-fluorescein (ROS sensor), pHrodo (phagolysosome fusion, i.e., acidification), and Alexa Fluor 350 (ROS/pH insensitive). A specific gating strategy was used to determine the relative oxidation and/or acidification of the phagosomes. For all phagocytizing cells (Fig. 5A), DCF/pHrodo fluorescence (Fig. 5B) was normalized to Alexa Fluor 350 fluorescence. In this manner, DCF/pHrodo fluorescence can be attributed to the amount of oxidation/acidification and not to differences in the amount of labeled bacteria within the cell. This approach identified two distinct populations of high and low ROS cells (Fig. 5C). Furthermore, cell sorting of high ROS and low ROS cells revealed the cells to be PMNs or macrophages, respectively (Fig. 5D). This agrees with previous literature that showed the proclivity of PMNs to produce increased ROS (39), whereas macrophages have more phagosomal acidification (40) and an increased capacity for phagocytosis (41).

To determine whether the decreased phagocytosis of Die-P cells was due to decreased phagocytosis by either macrophages or neutrophils, the peritoneal phagocytosis data (Fig. 4D, 4E) were re-examined by first stratifying the cell populations based on high or low phagosomal ROS production (Fig. 5C). This approach showed that both Die-P neutrophils and macrophages are suppressed in their ability to phagocytize within 6 h post-CLP, and this continues through 24 h (Fig. 5E), thus suggesting why Die-P cells kill fewer bacteria than Live-P.

Similar to their impaired ability to phagocytize (Figs. 4, 5), Die-P peritoneal phagocytes also show decreased phagosomal maturation (i.e., ROS/acidification) as compared with Live-P.

Differences in ROS production and phagosomal acidification

Although significant differences in PMN phagocytosis between Live-P and Die-P existed by 6 h (Fig 5E), there were not significant differences in the generation of phagosomal ROS, the hallmark of PMN bacterial killing (Fig. 6A). However, by 24 h, there were significant differences in phagosomal ROS (Fig. 6B), with Live-P demonstrating an increased burst as compared with 6 h, whereas Die-P PMNs did not show this improvement. Although less pronounced in PMNs, phagosomal acidification and fusion with lysosomes contributes significantly to bacterial killing (42). Similar to PMN ROS, no significant differences in PMN phagosomal

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**FIGURE 6.** Die-P peritoneal phagocytes are suppressed in phagosomal maturation. 3×-Labeled E. coli were incubated with peritoneal cells for 30 min and TB added to quench extracellular fluorescence. PMN phagosomal ROS was calculated by calibrating DCF (ROS sensitive) fluorescence to Alexa 350 fluorescence (ROS/pH insensitive) at 6 h (A) and 24 h post-CLP (B). PMN phagosomal acidification was calculated by normalizing pHrodo fluorescence to Alexa 350 at 6 h (C) and 24 h (D). Macrophage phagosome acidification at 6 h (E) and 24 h (F), n = 5–7 from three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001 Live-P versus Die-P.
Acidification existed within 6 h post-CLP (Fig. 6C), but significant differences did emerge by 24 h (Fig. 6D). In contrast to Die-P PMNs, macrophage phagosomal acidification was significantly decreased within 6 h post-CLP (Fig. 6E) and this continued through 24 hr (Fig. 6F).

Taken together, these data strongly support that Die-P phagocytes are suppressed in their ability to internalize bacteria and to create the caustic conditions necessary to kill internalized bacteria. This suppression was evident within 6 h after the onset of sepsis. This in turn explains why despite increased phagocyte recruitment, Die-P mice fail to eliminate their infection and subsequently die.

Discussion

The most important finding in this study is that despite receiving equivalent inoculums and recruiting similar numbers of phagocytes in response to the inoculum, Die-P peritoneal phagocytes are impaired in their ability to kill bacteria. These cellular defects were present in the very first hours of sepsis and became progressively worse. Less obvious but of equal concern is that without proper stratification of mice according to their likelihood of survival (i.e., mild versus severe sepsis), considerable variability may confound studies that compare sham operated mice to septic mice. For example, our laboratory has published that cell function, renal function, bacteria, and cytokines differ significantly between survivors and nonsurvivors (13, 43). Our laboratory has also published that high IL-6 levels merely correlate with, but do not mediate mortality from CLP (44). These studies suggest that stratification would be of considerable benefit in human studies where heterogeneity in individuals and their responses are more pronounced. In further support of stratification and our findings of decreased bacterial killing preceding death, Danikas et al. (45) showed phagocytic activity as prognostic for outcome in human sepsis.

Although numerous studies have argued for and against organ injury preceding death from sepsis (43, 46, 47), this study strongly suggests that early phagocytic impairment (within 6 h) ultimately leads to uncontrolled microbial growth and death. This occurs even with the use of effective broad spectrum antibiotics. It may be that failure to contain the initial infection allows the intense inflammatory reaction to ripple throughout the organism, leading to organ injury. The successful eradication of microbial pathogens requires recruitment of myeloid cells to the site of infection (48, 49). To that end, other laboratories have shown that increasing or inhibiting neutrophil recruitment following CLP (50, 51) affects bacterial burden and animal survival. Our laboratory has shown that augmented recruitment of neutrophils also increases survival (16). However, that study and this one demonstrate that the mice that are predicted to die do not fail to recruit neutrophils or monocytes/macrophages as compared with the mice predicted to live. In fact, Die-P mice recruit more cells by 24 h post-CLP. Similar findings were recently reported that used CLP to produce ≥60% mortality in control mice (52) and demonstrated that without GM-CSF–producing B cells, CLP resulted in 100% mortality. Most importantly, as it relates to this study, the B cell GM-CSF−/− mice recruited significantly more neutrophils to the peritoneum yet still had increased bacterial burden, increased inflammatory mediators, and decreased phagocytosis, as observed in our model. Taken all together, this suggests that although phagocyte recruitment is important, their functional status is more important.

Although this study strongly argues for impaired phagocyte function as contributing to death, it does not address the role of lymphocytes, which are fundamental to an immune response. Others have shown that treatments affecting lymphocyte apoptosis and function result in increased survival (53, 54). Although these studies did not stratify by survival likelihood, it is interesting to speculate that impaired lymphocyte function or death drives the phagocyte dysfunction observed in our model. Certainly, the report by Rauch et al. (52) detailing GM-CSF–producing B cells supports this. Also not addressed is the role endotoxin tolerance may have in phagocytic suppression. The endotoxin tolerance phenotype of monocytes/macrophages is one of epigenetic re-modeling (55) and decreased cytokine production with increased phagocytosis (56). It is plausible that Live-P phagocytes experience similar rewiring that promotes bacterial clearance along with decreased cytokine production (i.e., IL-6).

Another limitation to this study is that only peritoneal cells were evaluated for performance. It would be interesting to know whether Die-P phagocytes become impaired upon their arrival to the peritoneum or whether their impairment exists prior to their arrival. Myeloid-derived suppressor cells (MDSCs) may play a role if the phagocytes are suppressed prior to recruitment to the site of infection. Delano et al. showed that CLP results in suppressed ROS burst by monocytic splenocytes and bone marrow neutrophils, suggesting that phagocytic suppression is systemic. It will be important to determine to what extent systemic alterations are implicated in a survivor/non-survivor model of sepsis. MDSCs have been shown to improve or hinder survival from CLP, depending on the maturity of the MDSCs (57, 58). Unfortunately, the phenotypic markers for MDSCs (e.g., Cd11b+Gr-1+) are insufficiently specific to readily distinguish them from the peritoneal neutrophils or macrophages/monocytes in this study.

There is considerable evidence that suggests that overstimulation of cells with contradictory stimuli (e.g., pro- and antiapoptosis agents) can result in a terminally nonresponsive state, affectionately termed zombie cells (59). Along similar lines, Die-P mice demonstrate a significantly larger surge in pro and anti-inflammatory mediators in their peritoneum and plasma (16), potentially creating the conditions for terminally nonresponsive cells that render them incapable of defending the host. In light of this, perhaps the most hopeful finding of this study is that Die-P cells are functionally intact and equally capable of responding to bacteria, although they are deficient in both the amount they can phagocytize and how they process their phagosomal cargo. Because they are responsive, phagocytes would be attractive targets for functional modulators, such as antagonists of adenosine receptors or programmed death receptor-1, both of which have been suggested to increase phagocyte function and improve survival from sepsis (15, 60).

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References


Figure S1: Flow Cytometry Gating Strategy for Peritoneal Phagocytes. A-C: Removal of debris, doublets, and dead cells. D-F: Neutrophil & Monocyte/Macrophages (Macs) identified as (Cd11b+, Cd3e/Cd19-) LY6G-Hi or LY6G-Low, respectively. G: Fluorescence Minus One (FMO) staining of LY6G-low cells shows variable F480 staining on LY6G-low cells (Die-P, Red; Live-P, Blue; Naïve Resident Macs, Orange; FMO controls shaded).