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Let the Cells Speak: Neutrophils as Biologic Markers of the Inflammatory Response

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Introduction

Clinical manifestations of an activated systemic inflammatory response are both common and non-specific in critically ill patients. Timely and effective therapy directed against the microbial triggers of sepsis, or against its host-derived mediators, remains unsatisfactory because of our inability to answer two questions definitively and rapidly:

1. In a given patient, is the clinical syndrome a result of invasive infection, ie tissue invasion by microorganisms or their products?
2. What is the biologic nature of the host response at a particular point in time?

These questions are inherently complex, since the septic process involves the concerted interplay of multiple dynamic biochemical and physiological cascades that manifest in an unpredictable and highly variable manner. Simplified models of the host immune response in critical illness have been proposed [1–4], but not tested or validated in a manner that permits them to be of use in clinical decision-making. The value of any proposed model is to provide mechanistic insights and to identify targets for intervention that can be manipulated in a predictable manner to achieve clinical benefit.

Trauma has been a popular model of the inflammatory response since the onset of injury is well-defined, and the inciting insult readily identifiable. Patrick et al. [1] have described a two hit model of an initial dysfunctional inflammatory response following major trauma, which contributes to the development of multiple organ dysfunction (Figure 1). Sequential neutrophil priming plays a pivotal role in the genesis of remote organ injury. Faist et al. [2] have proposed a model relating monocyte/macrophage and T-cell interactions to the development of an anergic state following trauma; disruption of the normal balance between TH1 and TH2 lymphocyte subsets which serves as both a marker, and a potential mechanism for developing anergy (Figure 2). Both models describe early and late phases of the immune response: an early phase of systemic inflammation that can be exacerbated by a secondary insult, with resultant progression to a later and potentially lethal immunosuppressed or anergic state. An important assumption of these models is that the exaggerated or prolonged early pro-inflammatory phase triggers a compensatory anti-inflammatory response which may shift the immune balance to a suppressed phenotype [5]. This anergic state not only predisposes the host to nosocomial infection, but previously immunocompetent effector cells such as granulocytes become injurious due to super activation [6–8], uncoordinated function [9–11] and/or delayed apoptosis [12].

Models of trauma which delineate the course of immune competence, provide useful parallels in other clinical scenarios such as ischemia-reperfusion, or systemic infection. A logical prediction from these models is that anti-inflammatory therapies which blunt the hysteresis of the pro-inflammatory cytokinemic phase would be beneficial at an early stage, but potentially lethal during the anergic phase. Similarly, interventions designed to augment the immune response in the anergic phase could exacerbate the cytokinemic pro-inflammatory state and contribute to a deleterious outcome for the patient. Defining the immune status prior to therapeutic intervention may therefore be of critical importance to selection of therapy.

There is a recognized need for markers which can rapidly identify: 1) the inciting insults, 2) the immunological staging of the patient and 3) the immune response to therapy. Circulating levels of inflammatory mediators of acute inflammation such as interleukin 6 [13], procalcitonin [14], or C reactive protein [15] may provide clinically useful information about the state of activation of the host septic response. Previous studies have shown that the measurement of sustained plasma pro-inflammatory cytokines such as TNF-a and IL-6 rather than their peak concentrations identify those patients who develop multiple organ dysfunction and death [16]. An alternative apporach to the use of levels of inflammatory mediators as diagnostic markers is to analyze the responses of the cellular effectors of an inflammatory response—to let the cells speak for themselves. As the foot soldiers of acute inflammation, neutrophils are ideally suited to fulfill this role.

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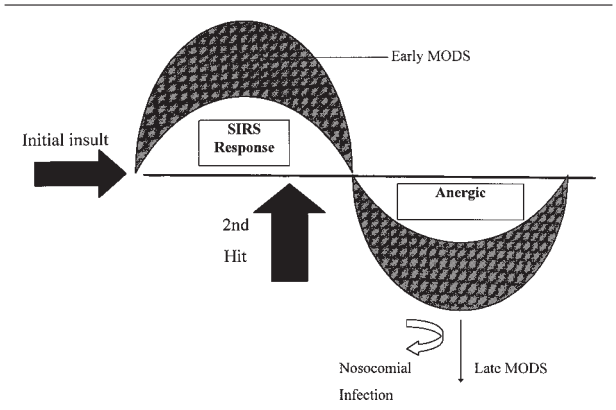

Fig. 1. *Dysfunctional inflammatory response. A biphasic inflammatory response to major trauma is shown with an early hyper-inflammatory phase. The initial insult may serve to prime PMN's and the second hit to trigger their heightened destructive potential in an unregulated manner. High levels of pro-inflammatory cytokines are detected in the early SIRS response phase followed by an exaggerated compensatory anti-inflammatory mediator cascade which swings the immune response into hyporesponsive or anergic phase with increased susceptibility to nosocomial infections. (Adapted from [1])*

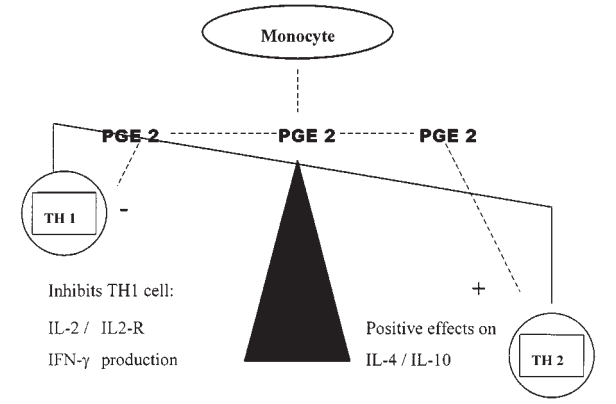

Fig. 2. *Polarization of T-helper cells to a predominant TH_2 immunosuppressive phenotype by monocyte PGE_2 secretion. Monocytes activated by phagocytosis, immune complexes, complement products and endotoxin are stimulated to release the immunosuppressive prostaglandin PGE_2 which shifts the T Helper cell balance. Adapted from reference 2.*

Quantification of Circulating Neutrophils: Neutrophilia and Neutropenia

The classic criteria for SIRS include either neutropenia or neutrophilia [17]. Neutropenia occuring a a consequence of malignancy or chemotherapy carries an increased risk of mortality, and identifies a clinical state that can be treated with recombinant G-CSF [18]. The risk of ICU [19] or hospital [20] mortality also increases with increasing degrees of neutrophilia, although neutrophilia is neither specific for infection, nor diagnostic of a particular pattern of inflammatory mediator response.

Although elevation of the white blood cell count is commonly interpreted as evidence of possible infection, neutrophilia is neither a sensitive nor specific marker of infection. The neutrophil count may be elevated, for example following upper gastrointestinal hemorrhage [21] or blood transfusion [22]. Moreover the diagnostic value of a left shift, reflecting release of newly-formed white cells from the bone marrow, is similarly limited as a marker of systemic inflammation [23].

Viable Neutrophils as Biologic Markers

While quantification of the number of circulating neutrophils provides little information about the nature or evolution of an inflammatory response, evaluation of their functional status may be more informative, both as a measure of the degree of activation or deactivation through the process of programmed cell death, or as a means for the dynamic assay of antigens such as bacterial lipopolysaccharide in the blood. We present here a novel method that uses the cellular response repertoire of neutrophils, as measured by whole blood chemiluminescence, to identify bacterial products and to assess the state of neutrophil activation within the milieu of the patients immunologic profile. Measurement of bacterial products (endotoxin) provides a measure of a major stimulus for the septic inflammatory response. The immune status of the patient may be reflected in the degree of neutrophil activation and their ability to respond to agonists and immune complexes. Repeated measures can be used to observe the influence of therapeutic intervention on neutrophil activity as a surrogate measure of efficacy. Further benefits of this approach include rapidity and simplicity of analysis using an analytical protocol which does not require cell purification and mimics the in vivo cellular milieu. The approach is simple and minimizes factitious cell activation during cell purification and possible contamination from exogenous LPS.

Measurement of neutrophil response capacity by chemiluminescence

The measurement of neutrophil chemiluminescence has been pioneered by Allen [24,25]. Neutrophil respiratory burst activity can be measured in the presence of a lumiphor (light emitting reporter molecule) by the production of NADPH oxidase dependent oxidants such as H_2O_2, O_2- (using lucigenin as the lumiphor) and myeloperoxidase dependent HOCL production (using luminol as the lumiphor). Other lumiphors such as pholasin allow detection of chloramines produced secondary to HOCL release [26]. The respiratory burst activity of normal unstimulated non-inflammatory neutrophils is

minimal and its induction requires stimulation with particulate or soluble agonists such as zymosan or phorbol myristate acetate (PMA). Using particulate stimulants such as zymosan, the choice of ligand dependent stimulation of respiratory burst activity and degranulation in PMN's can be achieved by varying the ligands bound to zymosan. Zymosan A alone stimulates both degranulation and respiratory burst activity via interaction with the glucan binding site on CD11b/CD18 (CR3)[27]. Opsonization of Zymosan A with complement fragments such as iC3b and C3b, leads to synergistic engagement of CR1 (CD35) and CR3 receptors. When IgG molecules are bound to zymosan A, engagement of neutrophil Fc receptors with stimulation of oxidative burst and degranulation occurs. Low concentrations of the soluble diglyceride analogue PMA (10 pmol/assay) can be used to activate the NADPH oxidase complex and cause degranulation of secondary granules while high doses (5 nmol/tube) activate the NADPH oxidase and trigger degranulation of both primary and secondary granules.

Neutrophils from patients with circulating pro-inflammatory mediators such as TNF-a, PAF, LTB4, C5a, and IL-8 are primed for enhanced respiratory burst activity and also manifest increased oxidant production under quiescent conditions. Pro-inflammatory molecule priming can result in enhancement of oxidant production which can exceed basal levels by more than 50 fold when measured by complement opsonized zymosan stimulated chemiluminescence. Such priming is associated with an increased surface expression of CR1 and CR3 receptors and conformational activation of CR3 into a higher affinity "avid" state [28]. The extent of surface CR1 and CR3 receptor expression can be estimated by comparing luminescence curves in the presence and absence of a maximal stimulatory dose of C5a (20 pmol/tube). The ratio of luminescence areas (without C5a divided by maximal C5a during the acceleration phase of neutrophil activation with complement opsonized zymosan), when subtracted from 1 gives the fraction of opsonin receptor reserve which is remaining on the neutrophil surface. In patients with persistent highly activated neutrophils this reserve approaches zero.

Clinical studies utilizing whole blood chemiluminescence

Stevens et al. [29] examined the utility of neutrophil chemiluminescence to diagnose and stage infection. Functional analysis of phagocyte activity was determined in healthy volunteers, patients with acute infections of varying severity, patients with diabetes mellitus, and those with human immunodeficiency virus (HIV) infection. The authors were able to discriminate these patient groups on the basis of defined neutrophil chemiluminescence parameters using multiple discriminate function analysis. These parameters included basal and PMA stimulated oxidase activity, oxidase driven myeloperoxidase activity, circulating and exogenously primed opsonin receptor dependent dioxygenation of luminol. Longitudinal studies in patients with soft tissue infection and HIV indicated that chemiluminescence parameters correlated with the degree of disease progression or stage of infection.

In a study of 22 patients following elective repair of an abdominal aortic aneurysm (AAA), and 15 patients with a ruptured AAA [30], we demonstrated that neutrophils from patients with a ruptured AAA were significantly primed in response to both PMA and zymosan by chemiluminescence analysis; these neutrophils were significantly primed before surgery and maintained a high level of activation for up to 4 days following aneurysm repair. In contrast, neutrophils from patients who had undergone elective AAA repair demonstrated only a modest increase in priming with regard to NADPH oxidase and myeloperoxidase activity when stimulated with high dose PMA. This increase occurred 24 hours post-surgery but also persisted for up to 4 days. These studies indicate that neutrophils from patients with ruptured AAA's are highly primed prior to surgery, presumably as a consequence of hypotension and associated gut ischemia. The mortality in this patient population often exceeds 40% and is associated with multiple organ failure. The high level of neutrophil priming which persists for days post surgery in these patients makes them vulnerable to a "second hit which could provoke dysregulated and injurious neutrophil activity, and perhaps contribute to the development of organ failure.

We have also used whole blood chemiluminescence to identify foci of inflammation by evaluating neutrophil priming across various tissue beds. By comparing neutrophil priming across the lung in blood taken from the right atrium (mixed venous) and relating the values with arterial (radial arterial line) and femoral venous blood (percutaneous stab) we have been able to distinguish ARDS, or inflammatory sources above the diaphragm from those with intraabdominal sources of infection and lower limb ischemia.

Neutrophils as a biological reporter system to detect bacterial products

The rapid detection of lipopolysaccharide (endotoxin) in whole blood has been a daunting analytical problem and a major diagnostic limitation in the *a priori* selection of patients for treatment with anti-endotoxin agents. Many clinical studies [31–35] base patient selection predominantly on the suspicion of infection and the presence of physiological signs of the systemic inflammatory response syndrome (SIRS). Retrospective subset analysis of data from a failed clinical trial using an anti-endotoxin agent [36] showed statistically significant mortality benefit for patients who were endotoxemic at the time of randomization and received the study drug when compared with endotoxemic patients who received placebo. In contrast, a planned confirmatory phase III trial of an anti-endotoxin mono-

clonal antibody was terminated prematurely because of evidence of increased mortality in patients with Gram positive infections [34]. A major obstacle to the successful implementation of clinical trials of immunologic therapy for patients with sepsis has been lack of diagnostic markers for the activity of the components of the inflammatory cascade that are targetted by the experimental intervention [37].

Using neutrophil dependent whole blood chemiluminescence as a platform, we have developed a rapid whole blood assay for endotoxin [38]. The specificity of the assay is dependent upon the binding of the lipid A moiety of endotoxin with a monoclonal antibody against this antigenic determinant. The assay sensitivity is dependent on the differential priming of neutrophils by LPS-anti-LPS antibody complexes opsonized with complement. Each patient assay is calibrated with an internal LPS standard to compensate for inter-individual differences in neutrophil concentration and oxidative activity allowing standardized endotoxin measurement using the patients own neutrophils and complement proteins as reagents.

Neutrophils as Indicators of Host Response

An essential element of the assay technique for the quantitation of endotoxin by chemiluminescent assay is measurement of the priming of the patient's neutrophils to a maximal stimulatory concentration of endotoxin. This component of the assay constitutes the determination of the "maximal gain" of the neutrophil priming response to maximal stimulation with LPS-anti-LPS antibody complexes. This parameter, that we have termed "responsiveness," is measured by calculating the light integral difference between the control tube (with maximal exogenous LPS) and the antibody containing tube (with maximal exogenous LPS), and reflects the ability of neutrophils to respond to opsonized immune complexes. Another parameter relating the mathematical product of neutrophil priming and neutrophil concentration termed the "maximum chemiluminescence" (Clmax) provides a measure of the destructive potential of neutrophils in the circulation. As demonstrated in Table 1 a combination of "responsiveness" and "Clmax" can be used to distinguish septic from non-septic patients when combined with LPS measurement. All of these parameters are derived from the chemiluminescent endotoxin assay, and can be measured within the 20 minutes required to perform the assay.

We have evaluated the chemiluminescent endotoxin assay as a screening tool to rule out Gram negative infection in a population of ICU patients including all admissions (study 1, n=74) and patients with suspected sepsis (study 2, n=104) Table 2. In a subgroup of 52 septic patients with pneumonia, neither Clmax nor endotoxin levels alone were predictive of mortality. Responsiveness, however, was able to stratify patients with respect to outcome. Those patients whose neutrophils were not significantly primed by maximally stimulatory concentrations of immune complexes were at statistically higher risk of mortality, perhaps because of an inability to resolve invasive infections. Patients who manifested low neutrophil responsiveness and elevated Clmax were at higher risk of multi-organ failure and mortality (unpublished observations).

Table 1. *Mean concentration of LPS and neutrophil responsiveness in healthy ambulatory controls and septic versus non-septic critically ill patients*

Category	N	LPS (pg/ml)	Clmax (cpm)	PMN responsiveness
control	30	—	1.2 (± 0.8)	47.0 (± 15)
non-sepsis	20	226.0 (± 345)	7.1 (± 5.6)*	43.5 (± 17)
sepsis	64	404.2 (+ 353.8)**	12.0 (+ 11.8)*^	29.0 (+ 24)*^

*p= 0.0001 vs control, ^p= 0.001 vs non-sepsis, **p= 0.05 vs non-sepsis LPS; lipopolysaccharide, Clmax, maximal chemiluminescence measured in counts per minute, PMN polymorphonuclear leukocyte. All measured values are given as a mean ± 1 SD. For septic and non-septic patients all chemiluminescent measurement were made within 12 hours of ICU admission. Statistical significance was evaluated by student "t" test.

Inhibition of neutrophil apoptosis: a marker of pro-inflammatory activation

Neutrophil activation in vivo is reflected not only in augmented respiratory burst activity, but also in alterations in the process through which neutrophils die. Under normal circumstances, neutrophils are continuously formed from precursor cells in bone marrow stores, and released into the systemic circulation. Here their survival is brief. The life span of the quiescent neutrophil *in vivo* is no more than five or six hours, terminated through the activation of a constitutive

Table 2. *Performance characteristics of the CL assay for endotoxin against cultures for the diagnosis of Gram negative infection*

Dataset	Study 1	Study 2	Combined
Sensitivity	100%	94%	96%
Specificity	56%	30%	40%
$^{+}$Pred. Value	48%	46%	47%
$^{-}$Pred Value	100%	89%	95%

$^{+}$Pred; positive predictive, $^{-}$Pred; negative predictive. In study 1 all admissions to the ICU were studied over a period of 6 weeks (n = 74). Patients had LPS levels measured twice consecutively over the first two days of admission. LPS results were correlated to bacterial cultures drawn within a 24 hour window of the LPS assay. In study 2, 104 patients with suspected sepsis were studied longitudinally, until discharge from the ICU or death. LPS assays were correlated to cultures drawn within 24 hours of chemiluminescent assay.

process of programmed cell death or apoptosis [39]. Apoptosis is a highly regulated intracellular process, mediated through the activation of a cascade of cysteine proteases known as *caspases*, that results in the degradation of nuclear DNA and key cytostructural proteins, and ultimately in the controlled elimination of the cell [40]. DNA fragmentation during apoptosis is readily evident as reduced uptake of the nuclear dye, propidium iodide, by flow cytometric analysis, or through the characteristic nuclear changes seen by light microscopy. Changes at the cell surface include decreased expression of CD16, and exteriorization of phosphatidyl serine, detected as increased binding of the dye, Annexin V. Augmented expression of phosphatidyl serine permits apoptotic neutrophils to be recognized by fixed tissue macrophages of the reticuloendothelial system, and removed from the circulation without evoking an inflammatory response [41].

Apoptosis proceeds more slowly in neutrophils studied *in vitro*, however by eighteen hours of culture, between 30 and 40% of cells will manifest reduced nuclear uptake of propidium iodide, a hallmark of apoptosis. The relative ease with which these changes can be detected, either by light microscopy or by flow cytometry, suggests that neutrophil survival *in vitro* may serve as a marker of cellular activation state, and of the kinetics of the resolution of inflammation.

The mechanism through which neutrophils are programmed to undergo spontaneous cell death is not well understood. Since neutrophils are known to express both a cellular receptor capable of triggering apoptosis (Fas), and its agonistic ligand, Fas ligand, on the cell surface, it has been suggested that autocrine interactions between these two receptors may result in the expression of the apoptotic program [42]. Such a model would explain why apoptosis is accelerated in neutrophils that have phagocytosed bacteria [43]. In contrast, a wide variety of activational stimuli including bacterial products such as endotoxin [44], host-derived inflammatory mediators such as GM-CSF and IL- 1β [45], and the process of transmigration into an inflammatory focus or the engagement of adhesion molecules of the β2 integrin family [46], can result in inhibition of the expression of apoptosis, resulting in prolonged neutrophil survival.

Clinical studies have demonstrated prolonged neutrophil survival, consequent to inhibition of apoptosis in neutrophils harvested from patients with thermal injury [47], following major surgical trauma, or in patients meeting clinical criteria for the systemic inflammatory esponse syndrome (SIRS) [48] (Figure 3). Delayed apoptosis is associated with evidence of increased cellular activation, and can be induced by circulating factors in patient plasma, including the cytokine GM-CSF. In vitro studies show that inhibition of apoptosis following exposure to either LPS or GM-CSF is an active process, dependent on the synthesis and processing of IL-1β by the neutrophil, through the activity of the interleukin 1b converting enzyme [45]. Thus delayed neutrophil

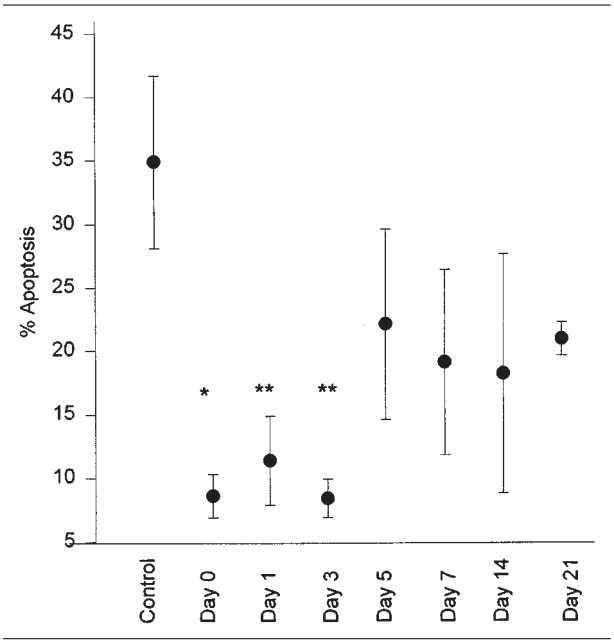

Fig. 3. *Critically ill patients meeting clinical criteria for SIRS show significant inhibition of constitutive neutrophil apoptosis, a state that is persistent, and associated with evidence of enhanced activation, reflected in the generation of reactive oxygen intermediates. From [48]; used with permission.*

apoptosis is both a marker of cellular activation, and a reflection of a biologic effect of the cytokine, IL-1β, acting in an autocrine or paracrine manner. The persistence of activated neutrophils in the circulation and at inflammatory foci may contribute to microvascular and parenchymal cell injury.

Conclusion: Let the Neutrophil Speak

Neutrophil chemiluminescence measured in whole blood can be used to detect bacterial lipopolysaccharide, and hence to identify infection or translocation from the gastrointestinal tract. By examining unstimulated (native) and stimulated (PMA or zymosan) neutrophil oxidant burst activity and opsonin receptor reserve it is possible to gauge the extent of neutrophil activation by proinflammatory mediators such as C5a, PAF, TNF, IL-8 and LTB4, a potential reflection of the pro-inflammatory or cytokinemic phase of immune cell activation. By measuring responsiveness as an index of immune effector cell competence it may be possible to determine whether the patient is progressing to an anergic phenotype. Whole blood neutrophil dependent chemiluminescence may therefore serve as a rapid and informative tool for the staging of immune effector cell status and may be useful as an intermediate biological marker to monitor the efficacy of therapeutic intervention. Conversely monitoring the kinetics of neutrophil apoptosis provides a marker of the persistence or resolution of an activated inflammatory response.

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