The Phosphatidylinositol 3-Kinase Pathway Selectively Controls sIL-1RA Not Interleukin-1β Production in the Septic Leukocytes*

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Microbial components such as bacterial endotoxin lipopolysaccharide (LPS) can trigger highly lethal septic shock. The cardinal features of septic leukocytes include the repressed production of inflammatory cytokines, such as interleukin-1 beta (IL-1β), and elevated production of anti-inflammatory cytokines, such as secretory interleukin-1 receptor antagonist (sIL-1RA). Pro- and anti-inflammatory cytokine gene transcriptions are equally repressed in septic leukocytes due to disruption of the LPS signaling pathway at the level of interleukin-1 receptor-associated kinase. The selective elevation of sIL-1RA protein in septic blood is caused by efficient translation of residual sIL-1RA message. In this study, we report that the LPS-inducible phosphatidylinositol 3-kinase (PI3-kinase)-dependent signaling pathway contributes to the elevated translation of sIL-1RA in septic/LPS-adapted leukocytes. We also observe that this pathway is gene specific and does not affect the production of proinflammatory IL-1β protein.

Sepsis occurs when inflammation induced by microbial infection spreads throughout the intravascular space of humans and animals causing failure of multiple organ-systems (reviewed in Ref. 1). Sepsis has a mortality rate of 40–80% and is the major cause of death in critical care units in this country. LPS1 plays a major role in inducing sepsis during infection caused by Gram-negative bacteria (2). Little improvement in the treatment of human sepsis has occurred since the syndrome was defined (3). The lack of knowledge of the altered innate immunity during sepsis may have contributed to the failures of treating sepsis (3).

Blood leukocytes including monocytes, macrophages, and neutrophils are exquisitely responsive to microbial infection and play important roles in the induction of sepsis (4). The pathogenesis of sepsis during infection depends on induction of an autotoxic and apparently dysregulated inflammatory response from blood leukocytes including the activation of a number of genes with both pro- and anti-inflammatory effects (reviewed in 4). During the initial phase of bacterial infection, a transient induction of proinflammatory cytokines including IL-1β and tumor necrosis factor-α (TNF-α) occurs in blood leukocytes from septic patients. This initial phase is followed by a state of imbalance in which leukocytes have decreased production of proinflammatory cytokines and enhanced production of anti-inflammatory cytokines (5). In addition, ex vivo treatment with LPS does not stimulate IL-1β production in leukocytes from septic patients (6). However, anti-inflammatory proteins, such as sIL-1RA (7), are continually induced upon additional challenge with LPS in septic leukocytes. Such imbalance may lead to an adapted state of immunosuppression, thus increasing the mortality risk from subsequent super infection with other microorganisms. Recent reports in both clinical patient cases and animal models support this concept (5, 8).

We refer to this adapted state as the septic leukocyte phenotype. The septic leukocyte phenotype can develop within hours following the onset of Gram-positive and Gram-negative infections and within 3 h when LPS is experimentally administered to humans or animals (9). The septic leukocyte phenotype appears highly reproducible and displays consistent features that are sustained during the clinical course of septic shock in humans and animals (6).

The septic leukocyte phenotype has also been well documented in experimentally induced septic cell lines including human promonocytic THP-1 cells (10) as well as the murine RAW264.7 and HeNC2 macrophage cells (11). Upon initial LPS treatment, proinflammatory cytokines are rapidly induced within these cells. Mimicking human blood leukocytes from septic patients, prolonged treatment of THP-1 cells with LPS induces an adapted state as reflected by the suppression of proinflammatory proteins such as IL-1α and TNF-α. Upon further LPS treatment, LPS-adapted THP-1 cells exhibit continued production of anti-inflammatory proteins such as sIL-1RA (7).

Biochemical studies indicate that LPS-induced gene transcription accounts for the elevated cytokine production in both human blood leukocytes and model cell lines (12). Cytokine gene transcription is repressed and no longer induced by LPS in septic blood leukocytes or LPS-adapted cell lines (12). Studies in our laboratory as well as in others indicate that the differential regulation of molecular signaling leads to an altered state of innate immunity in septic leukocytes. Toll-like receptors (TLRs) have been shown in part to mediate LPS and other microbial-induced cytokine gene expression (reviewed in 14). We observed that the interleukin-1 receptor-associated

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1 The abbreviations used are: LPS, lipopolysaccharide; IL, interleukin; sIL-1RA, secretory interleukin-1 receptor antagonist; TNF-α, tumor necrosis factor-α; TLR, toll-like-receptor; IRAK, interleukin-1 receptor-associated kinase; PI3, phosphatidylinositol 3-kinase; ELISA, enzyme-linked immunosorbent assay; PAGE, polyacrylamide gel electrophoresis; TBS, Tris-buffered saline.
kinase (IRAK), which lies proximal in the LPS-PLC signaling pathway, is inactivated and reduced in quantity within 3 h of LPS stimulation. The alteration of IRAK is sustained for at least 17 h in the THP-1 cell line model of the septic leukocyte phenotype (15). Furthermore, a constitutive disruption of IRAK occurs in blood leukocytes of patients with sepsis.2 It is likely that a disruption in IRAK signaling contributes to the repressed transcription of a set of LPS responsive genes, including proinflammatory IL-1β and TNF-α. Repression of transcription and rapid degradation of proinflammatory cytokine mRNAs contribute to decreased proinflammatory cytokine transcription and rapid degradation of proinflammatory cytokine translation in adapted leukocytes.2 This indicates that the pathway controlling sIL-1RA translation is not disrupted and is responsible for further LPS challenge.

In this investigation, we sought to identify the LPS signaling pathway(s) that are not interrupted and control translation of sIL-1RA in septic leukocytes. Using human blood from healthy donors and septic patients as well as the model THP-1 cell line, we observed that the PI3-kinase-dependent signaling pathway is still responsive to LPS in the septic leukocyte and selectively mediates LPS-induced translation of sIL-1RA but not IL-1β.

MATERIALS AND METHODS

Selection of Septic Patients—Informed consent was obtained using a consent form endorsed by the Institutional Review Board of Wake Forest University Baptist Medical Center. The following was used to identify septic patients with five points or greater signifying certain diagnosis (95% confidence) and four points signifying probable diagnosis (>90% confidence). Major criteria (2 points each): positive blood cultures (excluding Staphylococcus epidermidis) and the failure of at least one of the following organ systems: cardiovascular (systolic blood pressure <90 or low systemic vascular resistance), pulmonary (on ventilator for respiratory failure with diffuse pulmonary infiltrates), renal (creatinine >2.5), and microvasculature (lactic acidosis with pH <7.30 and lactate >3). Minor criteria (1 point each): presence of local infection, fever (>101°F) or hypothermia (<97°F), tachycardia (heart rate >100), leukocytosis (white cell count >15,000) or leukopenia (white cell count <4,000), disseminated intravascular coagulation (increase in fibrin degradation products), and pressor agents (dopamine, dobutamine, norepinephrine, phenylephrine, or large quantities of fluids).

Whole Blood Analysis—To study protein production by whole blood leukocytes, 1 ml of whole blood was stimulated with 500 ng/ml Escherichia coli 0111:B4 LPS (Sigma) for 20 h at 37 °C in 5% CO2 and plasma was separated by centrifugation for 5 min at 1500 g and frozen at -20 °C until ELISA analysis. When required, blood samples were incubated with specific PI3K inhibitors 200 nM wortmannin (Calbiochem) or 30 µM LY294002 (Calbiochem) for 30 min at 37 °C prior to stimulation with LPS for the times indicated. All of the results are typical of at least three independent experiments.

THP-1 Cell Culture and Induction of LPS-adaptation—THP-1 cells were maintained in RPMI 1640 medium (Life Technologies, Inc.) supplemented with 10 units/ml penicillin G, 10 µg/ml streptomycin, 2 mM L-glutamine, and 10% fetal bovine serum (HyClone Laboratories, Logan, UT) at 37 °C and 5% CO2 in a humidified incubator as described previously. Low passage number and log-phase cells were used for all experiments. LPS-adapted THP-1 cells were prepared by treating with LPS (1 µg/ml E. coli LPS 0111:B4, Sigma) for 16 h. The cells were centrifuged, washed once in serum-free RPMI medium, resuspended in normal RPMI medium at 1 x 10^6 cells/ml, and stimulated as described in the figure legends. Cell viability for whole blood samples was determined by trypan blue dye exclusion before centrifuging the cells at 5,000 x g for 4 min and isolating the supernatant. For all assays, normal control cells were treated similarly but were not exposed to LPS during the initial incubation period. When required, cells were incubated with specific PI3K inhibitors 100 nM wortmannin (Calbiochem) or 20 µM LY294002 (Calbiochem) for 30 min at 37 °C prior to stimulation with LPS for the times indicated. All of the results are typical of at least three independent experiments.

**RESULTS**

sIL-1RA and IL-1β Enzyme Immunoassay—sIL-1RA and IL-1β levels in culture supernatants and plasma samples were assayed in duplicate and quantified by enzyme-linked immunosorbent assay with Quantikine Enzyme Immunoassay Kit® (R&D Systems, Minneapolis, MN) against human IL-1 receptor antagonist and IL-1β according to the manufacturer's instructions.

RNA Isolation and Reverse Transcription-Polymerase Chain Reaction Analysis—Normal and adapted THP-1 cells were stimulated with 1 µg/ml LPS for 0 to 24 h. Total RNA was isolated from 1 x 10^6 cells/condition using RNA STAT-60® (Teltest B, Inc., Friendswood, TX) according to the manufacturer's instructions. One microgram of total RNA/concentration was analyzed by radiolabeled reverse transcription-polymerase chain reaction analysis for 40 cycles as previously described.

Western Blot Analysis—Whole blood adapted with 1 µg/ml LPS for the times indicated in the figure legends. Cells (1 x 10^6 cells/ml) were centrifuged and lysed in 100 µl of Nonidet P-40 lysis buffer (100 mM Tris, pH 7.4, 100 mM NaCl, 2 mM EDTA, 1% Nonidet P-40, and 1 mM phenylmethylsulfonyl fluoride). Protein concentrations were determined using BCA Protein Assay Reagent (Pierce). Proteins (100 µg of protein/lane) were separated by SDS-PAGE (7% acrylamide) for 4 h. At the end of the 4-h stimulation, each sample was radiolabeled with 300 µCi of [35S]methionine in 2 ml of methionine-free medium (Life Technologies, Inc.) for 30 min. Following the radiolabel pulse, the radiolabeled cells were chased for 0 and 12 h with 2 ml of nonradiolabeled complete medium. The samples were spun briefly for 4 min and isolating the supernatant. For all assays, radiolabeled supernatants were collected. Supernatants were further cleared of cell debris by centrifugation for 5 min at maximum speed in a microcentrifuge. 20 µg of polyclonal anti-sIL-1RA (R&D Systems, Inc.) were added to 2 ml of the radiolabeled supernatant and incubated at 4 °C for 16 h on a rotator. 50 µl of a 50% slurry of prewashed protein G-agarose beads (Bio-Rad) were then added to each sample followed by an additional 16 h at 4 °C. The samples were spun briefly in a microcentrifuge and washed six times in 1× PBS. Each sample was solubilized with 1× SDS sample buffer (80 mM Tris-HCl, pH 6.8, 2% SDS, 50% glycerol, 0.05% bromphenol blue, and 0.2% diethiothreitol), separated by SDS-PAGE, transferred to polyvinylidene difluoride membrane (Bio-Rad), and visualized by autoradiography.

Statistical Analysis and Data Expression—A mean constitutive activity or fold induction was determined for each experiment. Data are presented as the mean ± S.E. Statistics were performed using either two-tailed paired or non-paired t tests to determine significant changes in activities. Data were analyzed using Microsoft Excel 97 Software (Microsoft, Seattle, WA).

**RESULTS**

sIL-1RA and IL-1β Proteins Are Produced Differentially in Septic Whole Blood and THP-1 Cells—We collected whole blood from patients suffering acute sepsis as well as from healthy donors from Wake Forest University Baptist Medical Center. Serum levels of IL-1β and sIL-1RA were collected and then assayed by ELISA. IL-1β and sIL-1RA protein levels in healthy normal human blood are virtually absent (Fig. 1A). Following LPS stimulation, IL-1β protein level increases ~50-fold while sIL-1RA protein level increases about 5-fold by 24 h. In septic blood, both IL-1β and sIL-1RA proteins are constitutively present at low levels. Unlike normal blood, LPS does not induce...
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Efficient Translation of sIL-1RA Message Contributes to LPS-induced sIL-1RA Protein Production in Adapted THP-1 Cells—We have previously shown that transcription of both IL-1β and sIL-1RA is repressed in septic leukocytes as well as in LPS-adapted THP-1 cells (12). As shown in Fig. 2A, the message level of sIL-1RA is low and not induced by LPS in LPS-adapted THP-1 cells. We also observed that the message as well as protein stability of sIL-1RA was not altered following LPS-adaptation/tolerance of THP-1 cells (data not shown). The fact that sIL-1RA protein level is significantly increased in septic/LPS-adapted leukocytes suggests that LPS induces efficient translation of residual sIL-1RA message in septic/LPS-adapted leukocytes.

To further confirm that LPS induces efficient sIL-1RA translation in adapted leukocytes, we conducted methionine-labeling experiments assaying LPS-induced de novo sIL-1RA protein synthesis in normal and adapted THP-1 cells. Normal and LPS-adapted THP-1 cells were washed with fresh RPMI medium and subsequently incubated in RPMI medium supplemented with [35S]methionine. Cells were stimulated with 1 μg/ml LPS for 12 h at 37 °C. sIL-1RA protein was subsequently immunoprecipitated and analyzed on SDS-PAGE. Newly synthesized sIL-1RA proteins were detected by autoradiography. As shown in Fig. 2B, LPS induced marked increase of [35S]methionine incorporation into sIL-1RA proteins in normal THP-1 cells. Despite low IL-1RA message level in adapted THP-1 cells, we observed that LPS induced similar [35S]methionine incorporation into sIL-1RA protein in adapted THP-1 cells compared with normal THP-1 cells (Fig. 2B). Taken together, our study indicated that the translational efficiency of sIL-1RA message was enhanced in adapted THP-1 cells.

The PI3-Kinase Pathway Contributes to the Enhanced Production of sIL-1RA Protein in Septic Blood and LPS-adapted THP-1 Cells—The PI3-kinase-mediated signaling pathway has been implicated in regulating protein translational efficiency (reviewed in 16). Because PI3-kinase has been shown to be activated by LPS in murine leukocytes as measured by Akt phosphorylation (17), we therefore tested whether LPS-induced sIL-1RA protein production in human septic blood as well as LPS-adapted THP-1 cells is mediated by the PI3-kinase pathway. Wortmannin and LY294002 can specifically inhibit PI3-kinase activation and have been used extensively to study the involvement of PI3-kinase pathway in various biological processes. We therefore employed these inhibitors to study the involvement of PI3-kinase pathway. Whole blood cells collected from healthy and septic patients were treated with either wortmannin or LY294002 and subsequently stimulated with LPS (500 ng/ml) for 24 h at 37 °C. The sIL-1RA and IL-1β protein levels were then assayed by ELISA. As shown in Fig. 3A, pretreatment with wortmannin or LY294002 significantly decreases LPS-induced sIL-1RA protein production in septic human blood cells. Interestingly, we observed no effect of either wortmannin or LY294002 on IL-1β protein production in parallel experiments (Fig. 3A).

We further studied the effect of wortmannin on LPS-induced sIL-1RA protein production in adapted model THP-1 cells. Adapted THP-1 cells were washed and resuspended in LPS-free RPMI medium. Wortmannin was then added to the medium to the final concentration of 100 nM. Adapted THP-1 cells pretreated with wortmannin were subsequently challenged with 1 μg/ml LPS for various amounts of time. Consistent with
the finding from septic blood cells, wortmannin pretreatment dramatically decreased LPS-induced sIL-1RA production in adapted THP-1 cells (Fig. 3B). Similarly, IL-1β production was not affected by wortmannin treatment (Fig. 3B). Experiments using LY294002 gave similar results (data not shown).

In parallel, we also assayed the phosphorylation status of Akt, the downstream target of PI3-kinase. Following LPS and/or wortmannin treatment of the adapted THP-1 cells, total protein extracts were prepared and analyzed by SDS-PAGE. The levels of total Akt and Akt phosphorylated at serine 473 were determined through Western blot using antibodies against total Akt and phosphor-Akt-ser473 (Upstate Biotechnology). As shown in Fig. 3C, we first observed that LPS induced Akt phosphorylation in adapted THP-1 cells. As expected, wortmannin pretreatment abolished LPS-induced Akt phosphorylation (Fig. 3C).

The PI3-Kinase Pathway Mediates sIL-1RA Protein Translation without Interfering with Its Transcription—PI3-kinase activation has been reported to regulate diverse biological processes including transcription and translation (reviewed in 18). To determine whether the PI3-kinase pathway regulates sIL-1RA production through interfering with its transcription or translation, we measured the sIL-1RA message level in the adapted THP-1 cells through Northern analyses. As shown in Fig. 3C, we first observed that LPS induced Akt phosphorylation in adapted THP-1 cells. As expected, wortmannin pretreatment abolished LPS-induced Akt phosphorylation (Fig. 3C).

FIG. 3. Wortmannin specifically inhibits LPS-induced sIL-RA not IL-1β protein production in septic blood as well as adapted THP-1 cells. A, sIL-1RA and IL-1β protein levels were determined by ELISA in adapted THP-1 cells treated with either LPS alone, LPS and wortmannin, or LPS plus LY294002; B, sIL-1RA and IL-1β protein levels were assayed by ELISA in adapted THP-1 cells treated with either LPS, wortmannin or LPS plus wortmannin; C, adapted THP-1 cells were washed, resuspended in RPMI medium, and incubated in RPMI medium with or without wortmannin for 1 h. LPS was subsequently added to a final concentration of 500 ng/ml for the indicated amount of time. Protein extracts were isolated and resolved on SDS-PAGE followed by Western transfer. Monoclonal antibodies against unphosphorylated and phosphorylated Akt were used to perform the Western blot. Akt-P, phosphorylated Akt; Akt, unphosphorylated Akt.

FIG. 4. Wortmannin inhibits IL-1RA translation not its transcription. A, wortmannin does not affect sIL-1RA mRNA levels in adapted THP-1 cells. Adapted THP-1 cells were washed with LPS-free RPMI medium, resuspended in fresh RPMI medium, and stimulated with either LPS alone or wortmannin plus LPS for the indicated amount of time. Total RNAs were isolated and analyzed by Northern blot using radiolabeled IL-1RA and glyceraldehyde-3-phosphate dehydrogenase cDNA. B, wortmannin reduces sIL-1RA protein synthesis in LPS-adapted cells. Adapted THP-1 cells were incubated in RPMI medium supplemented with 300 μCi of [35S]methionine and stimulated with LPS (1 μg/ml) or wortmannin plus LPS for 12 h. This figure represents results from two independent experiments.
The effect of wortmannin on sIL-1RA translation in adapted THP-1 cells was directly measured through methionine-labeling experiments using [35S]methionine. LPS-adapted THP-1 cells were washed with fresh RPMI medium and incubated in RPMI medium supplemented with [35S]methionine. Cells with or without 100 nM wortmannin pretreatment were stimulated with 1 μg/ml LPS for 12 h at 37 °C. Total protein extracts were prepared, and the sIL-1RA protein was immunoprecipitated using monoclonal antibody against sIL-1RA and analyzed on SDS-PAGE. The newly synthesized sIL-1RA protein was detected by autoradiography. As shown in Fig. 4A, we observed that pretreatment with wortmannin abolished LPS-induced [35S]methionine incorporation into sIL-1RA protein in adapted THP-1 cells. Collectively, our results indicate that LPS-induced PI3-kinase activation specifically enhances sIL-1RA translation, without interfering with its transcription.

**DISCUSSION**

Two novel observations relevant to innate immunity and sepsis are provided by this study. First, despite repression of cytokine gene transcription due to disrupted TLR-IRAK-mediated signaling (15), a PI3-kinase-dependent pathway responsible for efficient sIL-1RA translation was selectively retained and remained responsive to further LPS challenge in the septic leukocytes. Second, the LPS-responsive PI3-kinase pathway selectively controlled sIL-1RA translation not IL-1β. Our study indicates that differential regulation of LPS-mediated signaling pathways contributes to altered cytokine protein profiles and the septic leukocyte phenotype (Fig. 5).

Our findings reveal a novel LPS signaling pathway that is responsive in LPS-adapted leukocytes. In normal leukocytes, LPS triggers TLR4 receptor-mediated signaling and activates a series of kinases including IRAK as well as various mitogen-activated protein kinases (11, 15). Prior exposure to LPS was reported to render leukocytes hyporesponsive to further LPS challenge, a phenomenon also known as endotoxin tolerance, which we refer to here as LPS adaptation. LPS-adapted leukocytes were shown not to respond to further LPS challenge as measured by suppression of IRAK kinase (15) and TLR4 signaling as well as all forms of mitogen-activated protein kinases (11). Disruption of these LPS signaling events may account for the repressed cytokine gene transcription that occurs in septic leukocytes or experimentally LPS-adapted cell lines. However, LPS-adapted leukocytes still express several anti-inflammatory cytokines including IL-10 and sIL-1RA (7). This indicates that endotoxin tolerance is not a total inhibition of cellular activities but rather an adaptation or reprogramming of cellular signaling events. Until now, it is not known which LPS signaling pathway still remains open and is responsible for the continued sIL-1RA production in LPS-adapted leukocytes. We show in this report that LPS induces PI3-kinase pathway activation in LPS-adapted cells (Fig. 3C). The activation of PI3-kinase is commonly measured through the phosphorylation of endogenous Akt protein, a direct downstream target of PI3-kinase (reviewed in Ref. 19). PI3-kinase pathway activation and Akt phosphorylation was observed in LPS-stimulated normal macrophages, neutrophils, as well as monocytes (17, 20, 21). However, there has been no previous biochemical study regarding the PI3-kinase activity in septic or LPS-adapted leukocytes. In this report, we are the first to report that Akt undergoes phosphorylation in LPS-adapted THP-1 cells upon further LPS treatment.

In addition, our work provides a novel link between PI3-kinase pathway activation and sIL-1RA protein translation in septic/adapted leukocytes. The phospholipid products of PI3-kinase, phosphatidylinositol 3,4-bisphosphate and phosphatidylinositol 3,4,5-trisphosphate, can act as second messengers and activate several downstream kinases including Akt, inositol phosphate kinase, and several calcium-dependent protein kinase C forms (18). These downstream kinases can subsequently regulate multiple cellular events including gene transcription (13) as well as protein translation (17). Among the multiple targets of PI3-kinase, the activation of Akt, mTOR, and p70S6 kinases have been well documented to regulate ribosomal assembly and protein translational efficiency (16). We show here that inhibition of PI3-kinase by either wortmannin or LY294002 abrogates sIL-1RA protein production induced by LPS in septic whole blood or LPS-adapted THP-1 cells (Fig. 3, A and B). We observed that LPS-induced sIL-1RA message levels in human septic leukocytes or LPS-adapted THP-1 cells is not affected by PI3-kinase inhibitors (Fig. 4A), suggesting that the PI3-kinase pathway selectively controls sIL-1RA translation not its transcription or message stability. We also observed that rapamycin, an inhibitor of mTOR, inhibits LPS-induced sIL-1RA protein production (data not shown). In addition, pulse-chase experiments show that inhibition of PI3-kinase significantly decreases incorporation of [35S]methionine into newly synthesized sIL-1RA protein (Fig. 4B). Our findings indicate that the PI3-kinase pathway plays a critical role in directly regulating sIL-1RA translation.

Interestingly, we observe that inhibition of PI3-kinase pathway does not interfere with other cytokine production such as IL-1β (Fig. 3, A and B). LPS-induced IL-1β message and protein levels in both septic blood and LPS-adapted THP-1 cells are not affected by PI3-kinase inhibitors. This indicates that the LPS-activated PI3-kinase pathway specifically regulates sIL-1RA translation not its transcription or message stability. The mechanism of such regulation remains to be elucidated.

Taken together, we conclude that septic/LPS-adapted leukocytes can still respond to LPS stimulation and undergo activation of PI3-kinase pathway. LPS-induced PI3-kinase pathway activation selectively contributes to the efficient translation of sIL-1RA not IL-1β. Our work further underscores that the septic leukocyte phenotype is a complex adaptation of cellular signaling events, involving not only repression of TLR-IRAK and several other signaling pathways but also selective activation of the PI3-kinase pathway. This altered phenotype may represent a modification of innate immunity that could either protect the host from further injury or produce an immunocompromised state. Further biochemical examination of the fine interplay of these signaling events is needed for better understanding and treatment of sepsis and other inflammatory diseases.

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