Regulatory role of C5a in LPS-induced IL-6 production by neutrophils during sepsis[1]

NIELS C. RIEDEMANN,*,[2] REN-FENG GUO,*,[2] TRAVIS J. HOLLMANN,† HONGWEI GAO,* THOMAS A. NEFF,* JAYNE S. REUBEN,* CECILIA L. SPEYER,* J. VIDYA SARMA,* RICK A. WETSEL,† FIRAS S. ZETOUNE,* AND PETER A. WARD*,[3]

*Department of Pathology, University of Michigan Medical School, Ann Arbor, Michigan, USA, and †Institute of Molecular Medicine for the Prevention of Human Disease, University of Texas-Houston, Houston, Texas, USA

SPECIFIC AIMS

IL-6 has recently been demonstrated to be an important inducer of the G-protein coupled C5a receptor (C5aR) in various organs and tissues in rodents during the early onset of cecal/ligation and puncture (CLP)-induced sepsis. In the current report we sought to investigate the effects of C5a on LPS-induced IL-6 generation in neutrophils in vitro and on in vivo IL-6 production in septic rodents and to describe underlying intracellular signaling mechanisms.

PRINCIPAL FINDINGS

1. Effects of C5a on LPS-induced IL-6 production and gene transcription

We investigated the effects of C5a on LPS-induced IL-6 production in blood neutrophils from healthy rats. ELISA experiments with cell supernatants revealed that 10 nM C5a significantly enhanced LPS (20 ng/mL)-induced IL-6 production in neutrophils at 2 and 4 h of in vitro stimulation in an additive manner. This effect of C5a was reflected at the transcriptional level.

2. Effects of blockade of C5a/C5aR activation on serum IL-6 levels during sepsis

We sought to investigate the contribution of C5a/C5aR activation on IL-6 serum levels during CLP-induced sepsis in rodents. IL-6 serum levels in CLP rats peaked 6 h after CLP (**Fig. 1*A***). To investigate the contribution of neutrophils to IL-6 serum levels, experiments were conducted in neutrophil-depleted animals, in which serum IL-6 levels 6 h after CLP were greatly reduced compared with neutrophil-intact group, suggesting neutrophils as an important source of IL-6 during the onset of sepsis (Fig. 1*B*). We further investigated the effects of C5a on IL-6 serum levels in animals using either control IgG or blocking antibody to C5a (anti-C5a) given intravenously at the start of CLP. The anti-C5a treated group showed significantly lower serum IL-6 levels at 6 h CLP compared with control IgG treated animals (Fig. 1*C*). CLP experiments in C5aR deficient mice (C5aR−/−) showed sharply reduced IL-6 serum levels at 6 h CLP compared with littermate controls (Fig. 1*D*).

3. NF-κB dependency of IL-6 production in neutrophils

To address the question of whether IL-6 production in neutrophils was NF-κB dependent, we conducted experiments in which neutrophils were preincubated with an NF-κB inhibitor (which blocks IκBα phosphorylation) before stimulation with LPS and C5a. IL-6 production in rat neutrophils was completely suppressed with the NF-κB inhibitor (data not shown). In neutrophils from mice deficient in the NF-κB subunit p50, similar levels of IL-6 were produced after LPS stimulation compared with littermate controls, suggesting that IL-6 production in neutrophils was not dependent on the NF-κB subunit p50 even though dependent on NF-κB activation in general.

4. Effects of C5a and LPS on activation of MAPK pathways in neutrophils

To investigate the underlying mechanisms for the observed effects of C5a in LPS-induced IL-6 production in neutrophils, we conducted Western blot experiments with whole cell lysates from neutrophils that had been stimulated in vitro with LPS, C5a or the combination of LPS and C5a for various periods of time. C5a caused rapid (within 5 min) phosphorylation of p38 mitogen-activated protein kinase (p38 MAPK), p44/p42 MAPK (ERK1/2), and p46 c-Jun N-terminal kinases (JNK1/2) (**Fig. 2**). In the case of ERK1/2 and JNK, phosphorylation was transient and observed after 5 min of in vitro stimulation but not at 15 min, while p38 MAPK phosphorylation was still detected 30 min after in vitro stimulation. LPS-induced phosphorylation of p38 could

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[2] These authors contributed equally to this study.

[3] Correspondence: Department of Pathology, University of Michigan Medical School, 1301 Catherine Road, Ann Arbor, MI 48109-0602, USA. E-mail: pward@umich.edu

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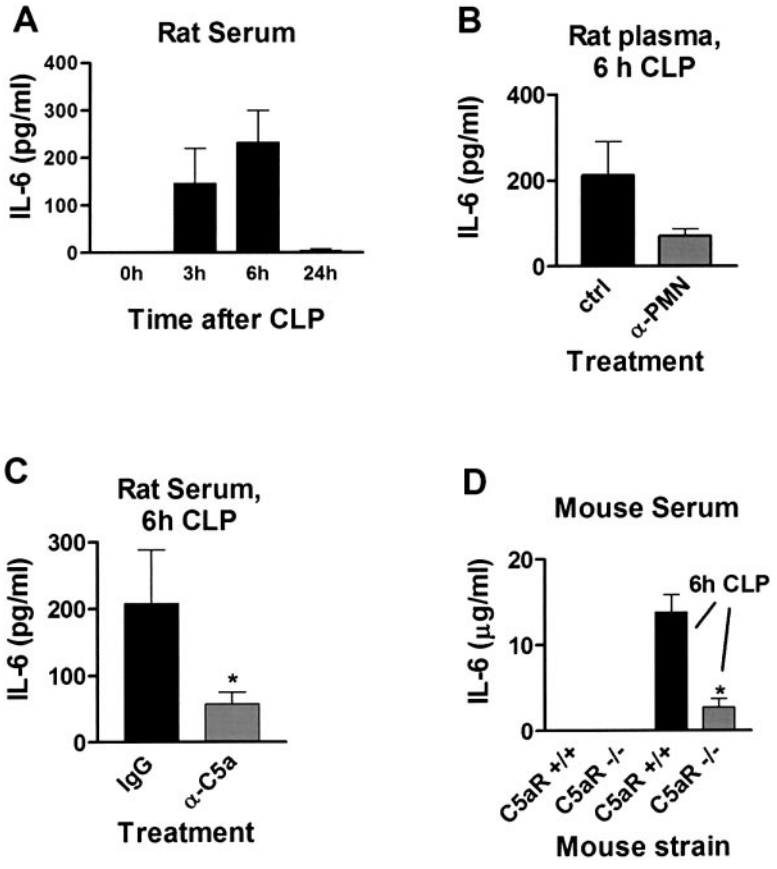

Figure 1. Effects of blockade of C5a/C5aR activation on serum IL-6 levels during sepsis. *A*) ELISA analysis of rat serum samples at various time points after CLP. Data are representative of 4–6 different serum samples per group. *B*) ELISA analysis of plasma samples for IL-6 from neutrophil-depleted rats 6 h after CLP. Neutrophil-depletion was achieved by intraperitoneal injection of anti-neutrophil antibody 18 h prior to CLP. Data are representative of 4–5 animals per group. *C*) IL-6 ELISA analysis of rat serum samples at 6 h after CLP in animals treated intravenously with anti-C5a IgG (500μg/rat) or equal amounts of irrelevant IgG at the start of CLP. Data are representative of 6 different serum samples per group. *D*) IL-6 ELISA analysis of serum samples from $C5aR^{-/-}$ and $C5aR^{+/+}$ mice 6 h after CLP. Data are representative of 4 different animals per group.

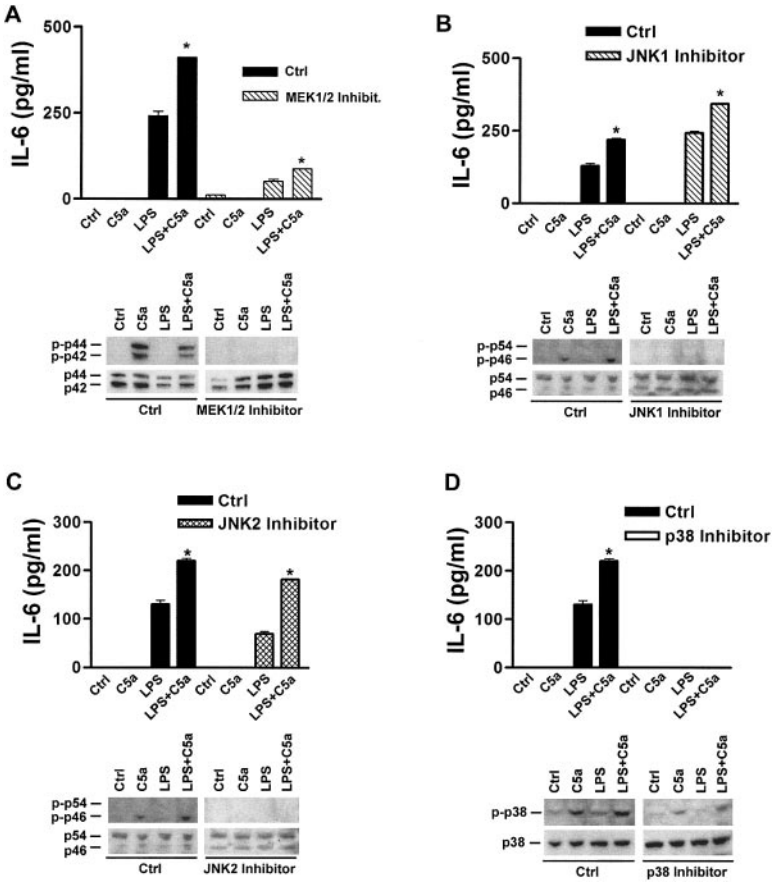

Figure 2. IL-6 ELISA analysis of rat neutrophil supernatant fluids after 4 h stimulation at 37°C with C5a (10 nM), LPS (20ng/mL), or both, and accompanying Western blots from matching whole cell lysates from neutrophils stimulated similarly for 5 min. *A*) Effects of preincubation for 30 min with 20 μM MEK1/2 inhibitor (U0126). *B*) Effects of preincubation for 30 min with 5 μM JNK1 inhibitor (L-form peptide). *C*) Effects of preincubation for 30 min with 20 μM JNK2 inhibitor (SP600125). *D*) Effects of preincubation for 30 min with 20 μM p38 MAPK inhibitor (SB203580). Data are representative of 2–3 independent experiments with neutrophils pooled from 4–6 rats and analyzed in triplicate. *Statistical significant difference from the LPS-only treated group.

be observed at 15–30 min of incubation, peaking at 1 h, but LPS alone did not cause phosphorylation of ERK1/2 or JNK. In the C5a+LPS costimulated neutrophils, an additive effect of p38 phosphorylation was observed at incubation for 1 h (data not shown). These data suggest that C5a is involved in rapid activation of all three kinase pathways, and, in the case of p38, appears to cause sustained (up to at least 1 h) phosphorylation when cells were costimulated with LPS and C5a.

5. Effects of signaling pathway inhibitors on IL-6 production in neutrophils

The experiments in Fig. 2 indicated that C5a can activate all three MAPK pathways, while LPS alone, with time, only activated p38 MAPK in rat neutrophils. Therefore, we investigated the ability of various signaling pathway inhibitors to inhibit the enhancing effects of C5a on LPS-induced IL-6 generation in neutrophils. Neutrophils from normal rats were stimulated in vitro for 4 h with C5a, LPS, or both, after preincubation at 37°C with various inhibitors for 30 min. Inhibition of either MEK1/2 (which is upstream of ERK1/2) or inhibition of p38 resulted in greatly suppressed IL-6 production in neutrophils (Fig. 2*A,D*). In these experiments, C5a-induced phosphorylation of ERK1/2 and p38 was almost completely abolished by inhibitors of MEK1/2 or p38, as demonstrated in the accompanying Western blots. High concentrations of the JNK1 inhibitor or JNK2 inhibitor resulted in no inhibition of IL-6 production, although C5a-induced phosphorylation of JNK was inhibited by both of these compounds (Fig. 2*B,C*). These results suggest that the enhancing effects of C5a on LPS-induced IL-6 production in neutrophils may be elicited by enhanced phosphorylation of ERK1/2 and p38 MAPK, but not JNK.

6. Effects of p38 and ERK1/2 inhibition on LPS-induced IL-6 production and accompanying C5a effects in neutrophils

The experiments depicted in Fig. 2 indicated that LPS-induced production of IL-6 was dependent on p38 and ERK1/2 activation. We conducted similar experiments in which neutrophils were preincubated with different concentrations of each inhibitor followed by stimulation with C5a, LPS or both (as described above). Inhibition of either MEK1/2 or p38 activation dose-dependently resulted in progressive inhibition of LPS-induced IL-6 production in neutrophils. Preincubation of neutrophils with the combination of MEK1/2 and p38 inhibitors resulted in additive inhibition of IL-6 generation in neutrophils. Under such conditions, C5a still elicited a significant additive effect on LPS-induced IL-6 production (data not shown). These data suggest that both pathways (p38 and ERK1/2) are involved in LPS-induced IL-6 production in neutrophils in an additive manner.

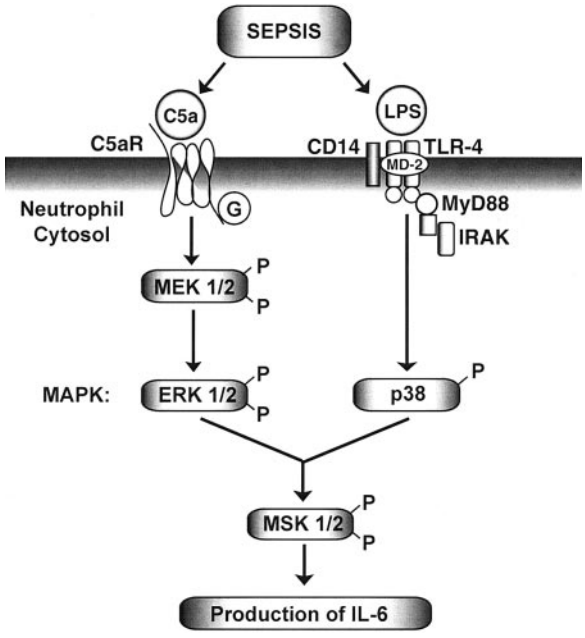

Figure 3. Schematic diagram depicting the employed signaling pathways in neutrophils for IL-6 production during the onset of sepsis in the presence of C5a and LPS.

CONCLUSIONS

Little has been known about mechanisms responsible for the early generation of proinflammatory mediators such as IL-6 during the onset of sepsis. Our data suggest that C5a has an additive effect on LPS-induced generation of IL-6 in neutrophils in vitro and that blockade or genetic loss of C5aR in mice or antibody-blockade of C5a significantly reduced serum levels of IL-6 during sepsis. The availability of neutrophils appears to be important for IL-6 generation during the onset of sepsis. LPS-induced production of IL-6 in neutrophils was NF-κB-dependent (which has also been suggested in other cell types) and dependent on phosphorylation of p38 MAPK as well as p44/p42 MAPK (ERK1/2) but not on phosphorylation of JNK1/2. C5a stimulation of neutrophils elicited a rapid phosphorylation of ERK1/2 and p38 MAPK. Accordingly, we suggest that induction of IL-6 after CLP is neutrophil and C5a/C5aR-dependent, likely due to the ability of C5a to cause activation of ERK1/2 and p38 MAPK signaling pathways with a common distal activation of mitogen and stress activated kinase 1/2 (MSK 1/2) (**Fig. 3**). In the context of earlier findings from our group, the current data suggest positive feedback mechanisms in which early generation of C5a during the onset of sepsis leads to enhanced IL-6 production, resulting in enhanced C5aR expression in various organs and possibly in increased susceptibility to C5a-induced harmful effects on organ function. Understanding the underlying intracellular mechanisms for this regulatory role of C5a may help to develop new therapeutic targets for the treatment of sepsis. FJ