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Regulatory Role of C5a on Macrophage Migration Inhibitory Factor Release from Neutrophils

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There is evidence that C5a and macrophage migration inhibitory factor (MIF) both play important roles in experimental sepsis. Humans with sepsis also show elevated levels of both mediators in the blood. Regulation of MIF during sepsis is poorly understood. We now demonstrate that neutrophil depletion greatly reduced serum MIF levels in rats and mice during the onset of sepsis after cecal ligation and puncture. In vitro, C5a induced MIF release from rat and mouse neutrophils. In vivo blockade of C5aR or absence of C5aR led to significantly reduced MIF generation during the onset of sepsis. C5a-induced release in vitro of MIF from neutrophils appeared to be due to up-regulation of MIF in cytoplasmic granules of neutrophils via activation of the protein kinase B signaling pathway together with involvement of PI3K. Our data suggest that C5a plays a role in enhancing MIF release from neutrophils in vitro and during sepsis. These findings represent a previously unrecognized function of C5a and neutrophils in the appearance of MIF in sepsis.


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igration inhibitory factor (MIF) was originally described as a T cell product and was later found to also be generated by pituitary cells, macrophages, eosinophils, tubular epithelial cells in kidney, and epithelial cells in lung. In earlier studies MIF was shown to activate T cells and induce proinflammatory cytokine production in macrophages. MIF production by macrophages can be induced by low levels of glucocorticoids; MIF was found to override glucocorticoid-induced inhibition of proinflammatory cytokine production in monocytes. Such findings suggested a counter-regulatory role for MIF in the context of the effects of glucocorticoids, possibly explaining the proinflammatory potential of MIF. In addition, MIF knockout mice showed protection from p53-induced macrophage apoptosis, revealing a macrophage protective effect of MIF. Recent studies in septic rodents demonstrated that blockade of MIF up to 8 h after experimental induction of polymicrobial Gram-negative sepsis significantly improved survival. In the same study MIF was also found in the plasma of patients with severe sepsis and septic shock. The administration of MIF increased lethality after infusion of LPS, whereas MIF knockout mice demonstrated improved survival after LPS challenge and showed reduced serum levels of TNF-α. In human sepsis, high MIF serum concentrations appear to correlate with bad outcomes. However, to date, there is little knowledge about regulation of MIF production during sepsis and the involvement of other cells, such as neutrophils, in MIF generation during sepsis.

There is now accumulating evidence that activation of the complement system occurs early during the onset of sepsis, with generation of the anaphylatoxin, C5a, which results in numerous harmful effects (reviewed in Ref. 12). C5a is a 74-aa protein that exerts various proinflammatory effects, such as chemotactic responses of neutrophils, release of granular enzymes, production by neutrophils of superoxide anion, vasodilatation, increased vascular permeability, and induction of thymocyte apoptosis during sepsis (reviewed in Ref. 12). Blockade of either C5a or C5aR leads to greatly improved survival in septic rodents (13–15). In humans, C5a is a serum marker that correlates with the severity of sepsis (16). During experimental sepsis, C5a compromises crucial innate immune functions of neutrophils, such as phagocytosis, chemotaxis, and generation of reactive oxygen species (13, 17). Recent work from our laboratory suggests that C5a may play a key role in the regulation of cytokine expression during sepsis (18). We now present evidence suggesting that C5a is able to cause release of MIF from neutrophils in vitro and during the onset of sepsis.

Materials and Methods

Reagents

Recombinant human C5a and other reagents were purchased from Sigma-Aldrich (St. Louis, MO).

Peptide synthesis and production of anti-C5aR Abs

A 37-aa peptide spanning the N terminus of the mouse C5aR (MDPIDNSSFEINYDHYGTMVPNAPDGHILFKRPQGD) was synthesized using an Applied Biosystems 430A peptide synthesizer (Foster City, CA). The peptide was then coupled to keyhole limpet hemocyanin by the glutaraldehyde method and used for the immunization of rabbits and the production of immunoreactive antisera. The anti-peptide-specific Ab was purified by affinity chromatography using the synthetic peptide coupled to cyanogen bromide-activated Sepharose 4B (Amersham Pharmacia Biotech, Piscataway, NJ).

Neutrophil isolation from whole blood and in vitro stimulation

Citrate was used as an anticoagulant (Baxter Health Care, Mundelein, IL) for the isolation of human neutrophils from blood, using Ficoll-Paque gradient centrifugation (Pharmacia Biotech, Uppsala, Sweden) followed by dextran sedimentation. Hypotonic RBC lysis was achieved using sterile
H₂O. Neutrophils were resuspended in DMEM (BioWhittaker, Walkersville, MD) containing 10% calf serum. A final concentration of 6 × 10⁶ cells/ml was used for stimulation at 37°C for the times indicated with C5a (10–50 nM) or LPS (20 ng/ml), or both. Supernatant fluids were collected after pelleting the cells and were frozen at −80°C until used for ELISA analysis. For certain experiments neutrophils were preincubated for 30 min with 50 μM PI3K inhibitor LY294002 (Cell Signaling Technology, Beverly, MA), which inhibits phosphorylation of the protein kinase B (AKT) pathway.

Quantitation of MIF by ELISA

MIF levels in lysates of human neutrophils were determined using ELISA kits for human MIF (Chemicon International, Temecula, CA) according to the manufacturer’s instructions. Various dilutions for supernatant fluids from LPS-stimulated and LPS- plus C5a-stimulated neutrophils were analyzed. Also, companion ELISA tests were used for mouse and rat MIF in serum from animals that underwent cecal ligation and puncture (CLP).

Western blot analysis

Neutrophils were isolated from human blood and stimulated at 37°C in vitro with recombinant human C5a (10–50 nM), LPS (20 ng/ml), or both. Approximately 2 × 10⁶ cells/condition were used for whole cell lysis using Laemmli buffer containing 5% ME. Lysates were transferred to a polyvinylidene difluoride membrane. Membranes were incubated overnight with Abs to phosphorylated and nonphosphorylated human/rat AKT (Cell Signaling Technology) or to MIF (Novus Biologicals, Littleton, CO). For detection of the protein, ECL Plus was used (Amersham Pharmacia Biotech, Piscataway, NJ) according to the manufacturer’s instructions.

MIF production in rats and in C5aR−/− mice

Specific, pathogen-free, 300-g, male Long-Evans rats or C57BL/6 mice (The Jackson Laboratory, Bar Harbor, ME) were used for all CLP studies. Anesthesia was achieved by i.p. injection of ketamine. In the CLP model (19), approximately one-third of the cecum in rats or two-thirds of the cecum in mice was ligated through a 3-cm abdominal midline incision. The ligated part of the cecum was punctured through and through with a 21-gauge needle. After repositioning of the bowel, the abdomen was closed in layers, using a 4.0 surgical suture (Ethicon, Somerville, NJ) and metallic clips. C5aR−/− mice and backcrossed littermate control mice (on the background of C57BL/6) were used. Where indicated, mice received 40 μg of anti-C5aR Ab i.v. in 200 μl of Dulbecco’s PBS solution immediately after CLP. Control animals received similar amounts of IgG Ab.

Neutrophil depletion in rats and mice

Neutrophil depletion was achieved using i.p. injection of rabbit anti-mouse neutrophil IgG or anti- rat neutrophil IgG (Accurate Chemicals & Scientific, Westbury, NY) 18 h before induction of CLP according to the manufacturer’s instructions. Control groups received equal amounts of normal rabbit serum by i.p. injection.

Collection of serum samples from septic animals

After induction of CLP, animals were killed at the indicated time points, and blood was drawn from the inferior caval vein. Blood samples were allowed to clot at 5°C for 6 h before centrifugation at 4000 rpm for 15 min at 4°C. Serum was collected and immediately frozen at −80°C until used for ELISA.

Immunocytochemistry and confocal microscopy

Human blood neutrophils were isolated as described above and plated on glass coverslips (no. 1 thickness, Fisher Scientific, Pittsburgh, PA) fastened to the bottom of punched-out wells on 12-well plates (diagram, 22.6 mm). Coverslips were sequentially coated with poly-γ-lysine and calf skin collagen to promote cell adhesion. Neutrophils were incubated with LPS (20 ng/ml) and C5a (10 nM) for 4 h at 37°C, fixed in paraformaldehyde, and permeabilized with methanol. Intracellular MIF was visualized with a mouse mAb to human MIF (1/10,000 dilution; R&D Systems, Minneapolis, MN) and goat anti-mouse Alexa 568 (Molecular Probes, Eugene, OR) secondary Ab (1/10,000 dilution) in the lissamine-rhodamine channel. Cells were imaged on an LSM 510 laser scanning confocal microscope (Zeiss, Oberkochen, Germany) with a ×63 water immersion lens. In none of the original images in either channel were the intensities at the level of the saturation plateau.

Statistical analysis

All values were expressed as the mean ± SEM. Significance was assigned where p < 0.05. Datasets were analyzed using Student’s t test or one-way ANOVA, with individual group means being compared with the Student-Newman-Keuls multiple comparison test.

Results

Effects of C5a and LPS on MIF production in neutrophils

To investigate the ability of C5a and LPS to induce MIF release, human neutrophils were isolated from blood. The neutrophil content in the preparations was >95% of all cells present based on differential staining with Wright’s stain. The cells were incubated for 6 h in vitro with C5a (10 nM), LPS (20 ng/ml), or both. ELISA measurements on neutrophil supernatant fluids were then conducted. The results in Fig. 1A demonstrate that incubation of human neutrophils for 4 h with human C5a or LPS resulted in significant MIF release. When neutrophils were costimulated with both C5a and LPS, incremental MIF release was observed. Western blot experiments with neutrophils using whole cell lysates from similarly treated cells revealed that incubation of neutrophils with C5a, LPS, or both in all cases resulted in increases in cellular MIF (Fig. 1B). Similar results were obtained with experiments using rat neutrophils (data not shown).

MIF expression in isolated neutrophils was also determined by immunostaining and confocal microscopy. Neutrophils were treated with the combination of LPS and C5a for 4 h under the conditions described above. Cells were fixed and permeabilized, and immunofluorescence was used to visualize intracellular MIF. As shown in Fig. 2, MIF was faintly present in a granular pattern in the cytoplasm (red staining; Fig. 2B). In striking contrast, cells treated with LPS and C5a showed much stronger staining in virtually all cells, exhibiting a granular pattern in the cytoplasm (Fig. 2D). Given the fact that the purity of neutrophils in the isolated cells was >95%, and because of the polymorphonuclear pattern in phase contrast microscopy of the positively staining cells (Fig. 1, A and C), we conclude that neutrophils are the major source of MIF in supernatant fluids of cell cultures and that the source of MIF cannot be related to the small (<5%) contamination by monocytes or eosinophils. In addition, the robust increase in MIF in cells

![FIGURE 1. C5a- and LPS-induced in vitro release of MIF from human neutrophils. A, ELISA measurements of MIF in neutrophil supernatant fluids. Neutrophils were isolated from blood and incubated in vitro with C5a (50 nM), LPS (20 ng/ml), or their combination for 6 h at 37°C at a concentration of 6 × 10⁶ cells/ml. Ctrl, culture medium alone. The asterisks indicate statistical significant difference from the reference group (•) or from all other groups (***). Data are representative of four independent and separate experiments. Incubation and analysis were conducted in separate, quadruplicate samples. B, Western blot measurements of MIF content in whole cell lysates from human neutrophils incubated similar to cells in A. Equal loading conditions were suggested by GAPDH content. Data are representative of three independent experiments.](http://www.jimmunol.org/content/135/6/3158/F1.large.jpg)
treated with C5a and LPS suggests transcriptional up-regulation of MIF (Figs. 1B and 2D).

Effects of neutrophil depletion on serum levels of MIF after CLP
ELISA experiments performed on serum samples from rats at various times after CLP revealed that MIF was released into the serum early during the onset of sepsis, reaching a plateau at 6 h (Fig. 3A). Similar findings were obtained in serum from CLP mice (data not shown). To investigate the potential contribution of neutrophils to MIF release after CLP, we depleted rats of neutrophils with an anti-neutrophil Ab, which was injected 18 h before CLP. Compared with the control group, which received an irrelevant IgG Ab, neutrophil-depleted animals showed substantially lower levels (by 58%; p < 0.05) of MIF in the serum 6 h after CLP (Fig. 3B). Similar experiments were conducted in mice and also showed that neutrophil-depleted animals demonstrated significantly reduced serum levels (by 81%) of MIF 6 h after CLP (Fig. 3C). These results suggest the requirement for neutrophils in MIF generation during the early period of sepsis in rodents, although a contribution from eosinophils cannot be ruled out.

Requirement for C5aR in MIF release during sepsis
To investigate whether the observed effects of C5a on MIF release from neutrophils in vitro had an in vivo parallel, we subjected C5aR<sup>−/−</sup> mice and C5aR<sup>+/+</sup> mice to CLP and conducted ELISA measurements on serum samples obtained 6 h after CLP. The results demonstrated that the genetic absence of C5aR resulted in significantly reduced (by 55%) MIF appearance in serum during the early onset of CLP-induced sepsis in mice (Fig. 4A). To extend these findings, we conducted experiments in which C5aR<sup>+/+</sup> mice were injected i.v. with anti-C5aR Ab or irrelevant IgG at the start of CLP. Six hours after CLP, mice treated with anti-C5aR Ab demonstrated significantly less (65%) MIF than mice treated with irrelevant IgG Ab (Fig. 4B).

Evidence for involvement of AKT and PI3K in release of MIF from activated neutrophils
We investigated the ability of C5a to induce phosphorylation of the AKT signaling pathway in human neutrophils using Western blot techniques applied to whole cell lysates from human neutrophils. C5a caused rapid phosphorylation of AKT, within 5 min of in vitro incubation with 10 nM C5a (Fig. 5A). This activation appeared to be transient, because it was greatly diminished at 15 min and beyond. In contrast, incubation of neutrophils with 20 ng/ml LPS did not result in phosphorylation of AKT after 5 min of incubation, whereas faint phosphorylation of AKT could be detected after 30 min of incubation with LPS. With the combination of LPS and C5a, strong phosphorylation of AKT was noted at 5 min, with loss of phosphorylation at 15 min and faint, but discernible, phosphorylation at 30 min, similar to the pattern found with C5a alone.

To investigate whether AKT activation was involved in C5a- or LPS-induced release of MIF from neutrophils, we conducted in

FIGURE 2. Confocal microscopy of MIF in neutrophils. Neutrophils were isolated from human blood in the usual manner and incubated in vitro with C5a (50 nM), LPS (20 ng/ml), or the combination for 4 h at 37°C. Cellular MIF was visualized with anti-MIF Ab and goat anti-mouse Alexa 568 secondary Ab in the lissamine-rhodamine channel. Control neutrophils and activated cells were viewed under phase microscopy (A and C) and under the rhodamine channel (B and D).

FIGURE 3. Participation of neutrophils in MIF appearance in serum. A, ELISA analysis for MIF in rat serum at various time points after CLP. Data are representative of four to six different serum samples per group. B, ELISA analysis of MIF in serum samples obtained from neutrophil-depleted and neutrophil-intact rats 6 h after CLP. Neutrophil depletion was achieved by i.p. injection of anti-neutrophil Ab 18 h before CLP. Data are representative of five animals per group. C, ELISA analysis of MIF in mouse serum samples 6 h after CLP in neutrophil-depleted mice and mice treated with normal rabbit IgG. Data are representative of five animals per group. *, Statistically significant difference from the reference group.
vitro experiments in which human neutrophils were preincubated with 50 μM PI3K inhibitor for 30 min. This inhibitor blocks the activation of the kinase (PI3K) upstream of AKT. The cells were subsequently stimulated with C5a (10 nM), LPS (20 ng/ml), or both for 6 h at 37°C (Fig. 5B) or for 5 min for Western blot determinations (pAKT; Fig. 5C). The results presented in Fig. 5B demonstrate that preincubation of neutrophils with PI3K inhibitor almost totally inhibited the C5a- or LPS-induced (or their combination) release of MIF. Furthermore, C5a-induced AKT phosphorylation in neutrophils was completely abolished in the presence of the PI3K inhibitor (Fig. 5C). Our data suggest that C5a- and LPS-induced release of MIF from neutrophils is regulated by activation of the AKT (and the PI3K) signaling pathways, which has not previously been described.

Discussion

Neutrophils have received little attention as a source of MIF. In a model of gastric ulcer formation in rats, macrophages were thought to be the major source of MIF (20). Our data suggest that neutrophils are one of the important sources for MIF and may play an important role in MIF release during the onset of experimental sepsis. Our findings are especially interesting, because to date there is little information about the source(s) and regulatory mechanism(s) for MIF appearance during the onset of experimental sepsis. It has been suggested that the pituitary gland may be a major source of MIF generation after endotoxin infusion into rodents (2).

The current studies demonstrate that LPS and C5a significantly induce MIF release from neutrophils. As shown in Fig. 2, little MIF was observed in nonstimulated cells. The small amount detectable was found in cytoplasmic granules of neutrophils. In contrast, neutrophils exposed to LPS and C5a evoked very strong immunostaining for MIF (Fig. 2D), implying that the release of MIF from neutrophils was probably due to transcriptional up-regulation. LPS is known to enhance MIF generation in macrophages (3), and MIF may be specifically generated in response to Gram-negative infections. MIF knockout mice showed reduced ability to clear Leishmania major due to impaired macrophage function (21) and had a greater susceptibility to Salmonella typhimurium infection (22). In contrast, Gram-positive exotoxins have been shown to be potent inducers of MIF production in macrophages; also, anti-MIF Abs protect mice from lethal Gram-positive exotoxin challenge (23). MIF has been demonstrated to play a crucial role in LPS-induced lung injury (24) and in noninfectious acute pancreatitis (25). Interestingly, recent findings suggest that MIF may be involved in the regulation of TLR expression (26), which suggests an important feedback mechanism for MIF and LPS.

Recently, we have found in vitro that C5a negatively regulates LPS-induced release of TNF-α by neutrophils, but has the opposite effect on TNF-α release from alveolar macrophages (18). In the same study, treatment of CLP mice with anti-C5a caused enhanced serum levels of TNF-α and protected the signaling pathways in neutrophils from C5a-induced dysfunction. In addition, C5aR−/−
mice or wild-type mice treated with anti-C5aR at the onset of sepsis had decidedly lower levels of MIF in serum and had much lower lethality (Fig. 4). Previous as well as the current data suggest that neutrophils may be an important source of TNF-α and MIF during sepsis. C5a enhances MIF release from neutrophils in vitro (Fig. 1). C5aR is clearly required for sepsis-induced generation of MIF. As shown in Fig. 3, neutrophil-depleted animals demonstrated significantly reduced serum levels of MIF 6 h after CLP. MIF can be strongly induced in neutrophils (Fig. 2), suggesting that neutrophils may be an important source of MIF during sepsis. In addition, it is possible that other neutrophil products (e.g., TNF-α) might promote the release of MIF from other cell types, such as macrophages. The extent to which eosinophils and monocytes contribute to the appearance of MIF in serum during sepsis is not known.

The intracellular mechanisms leading to MIF generation in various cell types are inadequately understood, although activation of the transcription factor NF-κB is presumed to be involved in MIF generation in macrophages. As for the mechanism for C5a-induced MIF generation in neutrophils, our data implicate activation of the AKT pathway (Fig. 5). Activation of the AKT pathway in neutrophils by MIF generation in neutrophils, our data implicate activation of the generation in macrophages. As for the mechanism for C5a-induced the transcription factor NF-

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