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Changes in the Novel Orphan, C5a Receptor (C5L2), during Experimental Sepsis and Sepsis in Humans

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Sepsis is associated with extensive complement activation, compromising innate immune defenses, especially in neutrophils (PMN). Recently, a second C5a receptor (C5L2) was detected on PMN without evidence of intracellular signaling. The current study was designed to determine changes in C5L2 in blood PMN during sepsis. In vitro exposure of PMN to C5a, but not to fMLP, led to reduced content of C5L2. Following cecal ligation and puncture-induced sepsis in rats, PMN demonstrated a time-dependent decrease in C5L2. In vivo blockade of C5a during experimental sepsis resulted in preservation of C5L2. Similarly, PMN from patients with progressive sepsis showed significantly reduced C5L2 expression (n = 26), which was virtually abolished in patients who developed multiorgan failure (n = 10). In contrast, sepsis survivors exhibited retention of C5L2 (n = 12/13). The data suggest that C5L2 on PMN diminishes during sepsis due to systemic generation of C5a, which is associated with a poor prognosis. The Journal of Immunology, 2005, 174: 1104–1110.

In response to tissue trauma or pathogen-associated molecular patterns (1), the immune system initiates and pursues a harmonically orchestrated local inflammatory response that is finely tuned by humoral (2, 3), cellular (3, 4), and neural networks (5). If the intensity of trauma is excessive or if the bacterial load is too high, there may be an overwhelming demand on both the innate and adaptive immune systems (4), resulting in a chaotic, dysregulated systemic inflammatory response, as occurs in sepsis (6, 7). A hallmark of sepsis seems to be uncontrolled activation of the complement system, resulting in excessive generation of anaphylatoxins, especially C3a and C5a (8, 9), which is accompanied by the ensuing dysfunction of neutrophils (PMN)4 (8, 10, 11). Generation of C5a in the pathogenesis of sepsis has been described as too much of a good thing (12). Increases in plasma levels of C5a and decreased binding of C5a to PMN during sepsis and the adult respiratory distress syndrome were reported (10, 13). Such changes were associated with a poor outcome. During experimental sepsis, blood PMN have diminished the ability to bind C5a (14) by down-regulation of C5aR (15). In contrast, in lungs, kidneys, liver, and heart, vascular C5aR is up-regulated in an IL-6-dependent manner (16). Furthermore, blockade of either C5a or C5aR improves survival rates of animals with sepsis (17, 18). In an LPS-induced sepsis model in mice, C3aR expression has been described as being increased on both epithelial and smooth muscle cells (19), suggesting an important role of complement receptors during the systemic inflammatory response of sepsis.

Recently, a putative chemoattractant receptor, C5L2, belonging to a subfamily of C3a, C5a, and fMLP receptors, has been described, and abundant expression has been found on granulocytes and immature dendritic cells (20). This orphan receptor showed high affinity binding for both native C5a and C5aDesArg (21). Whether the C5L2 protein can bind C3a or C4a is still a matter of controversy (22, 23). Unlike C3aR (24, 25) or C5aR (24, 26), C5L2 is uncoupled from G proteins due to an amino acid replacement of arginine by leucine in the so-called DRY region at the end of the third intracellular transmembrane domain (22, 23). Following C5a binding, C5L2 seems neither to induce classical signaling nor to cause biological responses (20–23). When C5L2 was transduced into several cell types, C5a failed to induce chemotaxis, degranulation, or intracellular calcium mobilization (22, 23), which was in striking contrast to C5aR-transfected cells. In addition, slow ligand on and off rates have been reported for C5L2 (23), suggesting that C5L2 may function as a decoy or scavenger receptor for C5a (23). Another interpretation is that C5L2 interaction with C5a prolongs the inflammatory response beyond the transient effects of C5a due to retention of the C5a-C5L2 complex on the cell surface in the absence of internalization of this complex (21). However, the function of C5L2 still remains unclear. Its role in the systemic inflammatory response is not known.

The current studies determine changes in C5L2 during experimental and clinical sepsis. We describe for the first time that C5L2 expression on PMN is down-regulated during sepsis, being associated with a poor clinical outcome.

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4 Abbreviations used in this paper: PMN, neutrophil; CLP, cecal ligation and puncture; MOF, multiorgan failure.

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Materials and Methods

Reagents

Unless otherwise indicated, all reagents were purchased from Sigma-Aldrich.

Generation and characterization of Abs against mouse C5L2 and human C5L2

Abs against human C5L2 were raised in rabbits using the extracellular N-terminal sequence of human C5L2 (MGN DSV SYE YGD YSD LSD RPV DC) coupled to keyhole limpet hemocyanin, as described previously (22). The serum recognized RBL cells transfected with human C5L2 (but not untransfected control cells) at dilutions as low as 1/10,000. Binding to C5L2 was totally inhibited by preincubation of serum with immunizing peptide at 100 μg/ml. The Abs were then affinity purified and used in the present study.

The mouse sequence from the N terminus (MMN HTT SEY YED HEH YSD LPD VPV DCP AGT CFT SD) was synthesized by Lampire Biologicals and used to immunize rabbits. The antiserum was characterized by ELISA, flow cytometry, and Western blots.

Experimental sepsis induced by cecal ligation and puncture (CLP)

Male Long-Evans-specific pathogen-free rats (275–300 g; Harlan) were anesthetized by i.p. administration of ketamine (20 mg/100 g body weight). After a midline incision, the cecum was tigated below the ileocecal valve, carefully avoiding bowel obstruction. The cecum was then punctured through and through with a 21-gauge needle. After repositioning the bowel, the abdominal incision was closed in layers (4-0 silk suture and skin clips; Ethicon). Where indicated, animals received immediately after CLP 400 μl of peritoneal rinse or 2 ml of 1% FACS lysing solution (BD Pharmingen) for 10 min.

There are no reported data on changes in C5L2 on PMN during sepsis. Therefore, blood PMN lysates from either sham-treated rats or rats with progressive sepsis (24 h after CLP) were evaluated by Western blot analysis using anti-C5L2 Ab (directed toward the N-peptide sequence of mouse C5L2). As shown in Fig. 1, PMN

PMN isolation

Human or rat PMN were isolated from whole blood by the traditional technique of Ficoll-Hypaque gradient centrifugation (Pharmacia Biotech) and dextran sedimentation, followed by hypotonic lysis of residual RBC.

Measurement of the serum concentration of C5a

The content of human C5a in serum from healthy volunteers and patients with severe sepsis or septic shock was determined by a C5a enzyme immunoassay kit (IBL, Immuno-Biological Laboratories). Studies were performed according to the manufacturer’s protocol.

Immunoblotting for C5L2

After isolation, PMN were lysed with a hypotonic buffer containing a protease-inhibitory mixture (Roche Diagnostic). Equal amounts of protein were determined by bicinchoninic acid protein assay (Pierce) from lysates (each containing 12 μg of protein) were electrophoretically separated under reducing conditions in 10% SDS-PAGE and transferred onto polyvinylidene fluoride membrane. The membrane was blocked in 5% milk for 1 h and then incubated with Abs against mouse C5L2 or human C5L2, using a concentration of 1 or 1.2 μg/ml, respectively. As a secondary Ab, either HRP-conjugated donkey anti-rabbit IgG (1:5000; Amersham) or alkaline phosphatase-conjugated goat anti-rabbit IgG (1:5000; Jackson ImmunoResearch Laboratories), respectively, was added, and the blot was developed using ECL procedure (ECL Plus; Amersham) or alkaline phosphatase color development system (Bio-Rad).

Analysis of C5L2 and the C5aR on PMN

Flow cytometric analysis was conducted immediately after whole blood collection in an EDTA-containing syringe. A total of 7.5 μl of nonlabeled anti-human C5L2 (1.2 mg/ml) and/or 10 μl of FITC-labeled anti-CD88 (clone P12/1; Serotec) was incubated with 100 μl of human whole blood for 20 min at room temperature. For control purposes, nonlabeled isotype-matched IgG (Serotec) or FITC-labeled isotype-matched IgG (Serotec), respectively, was added to control samples in equal amounts. After washing, cells were suspended in PE-labeled anti-rabbit IgG (Serotec) diluted 1/50 in staining buffer (PBS with 0.1% sodium azide and 1% FBS) and incubated at room temperature for 20 min. Erythrocytes were lysed by addition of 2 ml of 1× FACS lysing solution (BD Pharmingen) for 10 min. After twice washing with PBS, the leukocytes were resuspended in a fixing solution (1% paraformaldehyde prepared in PBS plus 0.1% sodium azide) and analyzed on a flow cytometer (BD Pharmingen). The granulocytes and monocytes were separated by the typical forward and side light scatter profiles, using additional staining with anti-CD45/anti-CD14 (Leukogate; Beckman Coulter).

Statistical analysis

All values were expressed as mean ± SEM. Data sets were analyzed by Kruskal-Wallis one-way ANOVA on ranks; differences in the mean values among experimental groups were then compared using a multiple comparison procedure (Dunn’s method). The Spearman rank-order correlation coefficient r was calculated for correlation analysis. Results were considered statistically significant where p < 0.05.

Results

Detection and characterization of C5L2 on rat PMN

There are no reported data on changes in C5L2 on PMN during sepsis. Therefore, blood PMN lysates from either sham-treated rats or rats with progressive sepsis (24 h after CLP) were evaluated by Western blot analysis using anti-C5L2 Ab (directed toward the N-peptide sequence of mouse C5L2). As shown in Fig. 1, PMN

FIGURE 1. Western blot analysis for rat C5L2 in PMN lysates from a normal human, rat PMN from a sham-operated animal, and PMN from a CLP rat 24 h after CLP. Second panel. Indicates loading conditions as revealed by a Western blot for GAPDH. Third panel. Densitometry tracing for C5L2 in the top box. When expressed as the ratio of C5L2/GAPDH, the densitometry values were 0.139, 0.555, and 0.276, respectively, for the three bars in Fig. 1. In the lowest box, anti-mouse C5L2 serum was preabsorbed with the N-terminal peptide of C5L2 (see text). Arrows in this and all subsequent figures indicate the position of the C5L2 protein band. Data are representative of three independent and separate experiments.
from sham-treated rats exhibited a distinct single band (arrow) aligning with the 45-kDa marker, consistent in the reported molecular mass of glycosylated human C5L2 (23). To validate identification of this band, anti-C5L2 was preabsorbed with the C5L2 peptide. This resulted in the disappearance of all PMN-related bands (lowest box), indicating specific detection of C5L2 protein at 45 kDa. Whereas anti-mouse C5L2 cross-reacts with rat C5L2, little or no immunoreactivity occurred with C5L2 in lysates from normal human PMN (first lane, top box). When lysates from rats with CLP-induced sepsis (24 h) were examined, the C5L2 band was less intense than the band found in PMN obtained from a sham-operated animal. These data indicate that C5L2 is expressed on rat PMN and that its expression is altered during sepsis.

Attempts have been made to evaluate the basis for the loss of C5L2 in blood PMN. PMN were obtained from rat blood using the techniques described above and then held on ice for 30 min in the absence or presence of a protease inhibitor mixture containing a combination of serine and cysteine protease inhibitors (Roche, catalogue number 11-973-580-001). When no inhibitor mixture was present, there was reduced ability to detect C5L2 in PMN lysates.

Reduction of C5L2 after in vitro exposure of rat PMN to C5a
Recent reports have suggested that incubation of C5L2-transfected cells with C5a for 5–10 min does not result in internalization of C5L2 (21, 23). We assessed the effects of prolonged exposure (up to 4 h) of rat blood PMN to C5a concentrations found in serum during sepsis (8, 9). When rat PMN were incubated for 1 h with 10 nM rat C5a, no significant change of the C5L2 expression was detectable in PMN lysates (Fig. 2). Cells incubated for 3 or 4 h with 10 nM C5a showed virtually total loss of detectable C5L2, which was in striking contrast to PMN incubated for 4 h in the absence of C5a (control (ctrl), 4 h) where the C5L2 band was still very distinct. These data indicate that prolonged exposure of rat PMN to C5a causes loss of detectable C5L2. When supernatant fluids from rat PMN incubated for 3–4 h with 10 nM rat C5a were probed by Western blots for C5L2, no Ag reactivity was found (data not shown).

Reduction of PMN C5L2 during CLP-induced sepsis; preservation of C5L2 in CLP rats by C5a blockade
During CLP-induced sepsis, blockade of C5a has been shown to cause reduction of C5aR on blood PMN associated with an improved PMN function and enhanced survival (14–17). There are no reported data on the behavior of C5L2 during the course of experimental sepsis. As expected, blood PMN from nontreated animals (control (ctrl)) or from sham-treated animals (sham) exhibited distinct bands in the predicted position for C5L2 (Fig. 3, arrow). Twelve hours after CLP, there were no apparent differences in band intensities in sham-operated animals and CLP animals (Fig. 3). However, at 24 and 36 h after CLP, the C5L2 band was greatly reduced in intensity in PMN from CLP animals treated with preimmune IgG. This was in striking contrast to anti-C5a-treated animals (400 μg i.v. immediately after CLP), in which the C5L2 bands remained intense 24 and 36 h after CLP, with no obvious reduction in intensity when compared with bands in sham or control (ctrl) groups. These findings indicate a C5a-dependent reduction of C5L2 content of blood PMN during progression of experimental sepsis.

Loss of C5L2 in human PMN exposed in vitro to human C5a
PMN from healthy human volunteers (n = 14) were exposed to C5a for increasing periods of time at 37°C, and C5L2 content was determined in cell lysates. As shown in Fig. 4A, PMN lysates from healthy volunteers (control (ctrl)) revealed a single band detected by anti-human C5L2 (N-peptide) Ab aligning with the position of the reference 45-kDa marker. When PMN were incubated with 10 nM human C5a up to 4 h, the C5L2 bands (arrow) were increasingly less intense at 3 and 4 h, whereas PMN incubated in HBSS in the absence of C5a for 4 h still demonstrated a distinct C5L2 band (control (ctrl), 4 h). In companion experiments, cell lysates had diminished C5L2 band intensity at 4 h, but no C5L2 reactivity could be detected in corresponding cell supernatant fluids (Fig. 4B). This suggests that C5L2 may be undergoing proteolytic cleavage under these conditions. Cell viability (as determined by trypan blue exclusion test) at the end of the incubation period was consistently >98% in all samples (data not shown). When human PMN were incubated with 1 μM fMLP for 1–4 h, no change in the intensity of the C5L2 band was noted (Fig. 4C).
Reduction of C5L2 content in PMN from human patients with sepsis

PMN were isolated from 18 patients with sepsis, and cell lysates were evaluated for C5L2 content. Equal amounts of protein (12 μg) were subjected to Western blot analysis, using anti-human C5L2. Typical findings are shown in Fig. 5. In PMN lysates from healthy volunteers (first lane, total n = 9) and from nonsurviving (lanes 2–4, total n = 5) and surviving sepsis patients (lanes 5–7, total n = 13), C5L2 protein bands (12 of 13 tested; Fig. 5B). These observations indicate a sepsis-induced reduction of C5L2 content, especially in those patients who did not survive sepsis.

Expression of C5L2 on PMN during severe sepsis or septic shock in humans and relationship to outcome

We evaluated by flow cytometry PMN from healthy volunteers (n = 14) or from patients with severe sepsis or septic shock (n = 26) to determine the surface expression of C5L2. In contrast to a recent study in which C5L2 could not be detected on PMN from normal donors (28), C5L2 was readily detected by flow cytometry using PMN from healthy volunteers (n = 14). Typical flow cytometric patterns are shown in Fig. 6A and are summarized in Fig. 6B. PMN from healthy volunteers exhibited an intense region for C5L2 staining in the left upper sector (as compared with the isotype control; inset). PMN from septic patients showed a markedly reduced staining for C5L2. Whereas PMN C5L2 expression was virtually lost in septic patients who developed multiorgan failure (MOF), a significantly higher level of C5L2 was found on PMN from septic patients who showed no signs of MOF. In addition, a weak, but significant negative correlation of C5L2 expression was found in the sepsis-related organ assessment score (p = 0.047; r_s = −0.40; data not shown). In accordance with the reduced C5L2 content (Fig. 5B), these data show a significantly reduced C5L2 expression in PMN during sepsis (Fig. 6) in association with poor outcome.
Discussion

Despite aggressive management of sepsis and sepsis-related complications, the lethality rate remains at 40–60% for patients in septic shock (29, 30). Randomized clinical trials have been performed with various anti-inflammatory approaches (enrolling >12,000 patients), all of which failed to show improvement in survival (overall lethality of 38% in each group) (31, 32).

During experimental and clinical sepsis, impairment of innate immune functions of PMN seems to be associated with decreased C5a binding (10, 14) and reduced C5aR expression (15, 27, 33) on PMN. The variety, complexity, and differences in the biological effects of C5a and C3a suggested possible existence of additional receptors for these anaphylatoxins. Recently, a second C5a receptor (C5L2) belonging to the seven-transmembrane-spanning receptor subfamily was discovered (20, 34). However, the published data for C5L2 have provided contradictory reports concerning its cellular expression and function (22, 23, 28). C5L2 was described as being abundantly expressed on both PMN (23) and immature dendritic cells (20), although in a recent report C5L2 expression could not be demonstrated on human PMN (28). It is also a matter of debate as to whether C5L2 binds C3a, C3adesArg, and C4a (22, 23). There is emerging evidence that C5L2 binds both C5a and its rapidly desarginated form, C5a_{desArg}, with high affinity (21, 23, 28), in contrast to C5aR, in which the affinity for C5a is as much as 100-fold higher than for C5a_{desArg}. The biological function and role of C5L2 during sepsis are still in the dark.

To elucidate the status of the novel C5L2 protein during sepsis, three approaches have been chosen. Excessive generation of C5a during sepsis (10) was simulated in vitro by exposure of PMN to C5a concentrations found in humans with sepsis (9, 10). Secondly, C5L2 content on PMN was evaluated during CLP-induced sepsis, which is known to closely mimic the pathophysiological conditions of sepsis in humans (35, 36). And, finally, C5L2 expression was determined in human blood PMN in conditions of severe sepsis or septic shock, as defined by consensus criteria for the definition of sepsis (6, 7).

On both rat and human PMN, C5L2 was detected by Western blot analysis as a ~45-kDa protein (Figs. 1 and 5A), consistent with the described molecular mass of the glycosylated form of C5L2 (23). It has been suggested that C5L2 functions as a high affinity scavenger receptor (21) for C5a and C5a_{desArg}, and that such interaction might be effective in clearing or limiting the inflammatory response. In vitro exposure of either rat or human PMN to 10 nM rat C5a or human C5a, respectively, revealed a time-dependent loss of C5L2 in PMN (Figs. 2 and 4). The C5a concentration of 10 nM was chosen based on C5a levels reported in sera of patients with severe trauma or sepsis (8–10) and our findings of significantly increased serum levels of C5a from 1.8 ±...
0.2 nM (n = 14 healthy volunteers) to 11.3 ± 2.6 nM (n = 26 patients with sepsis). In simultaneously performed flow cytometry studies, C5a exposure of human PMN (up to 4 h) also exhibited a time-dependent decrease of C5L2 on PMN surfaces (data not shown). By Western blot analysis, C5L2 content was diminished in cell lysates and was not detected in supernatant fluids after incubation of PMN with C5a (Fig. 4B), suggesting a loss of C5L2 on PMN after exposure of PMN to C5a for 2–4 h. Similar results have been described for the heptahelical CC-chemokine decoy receptor, D6, which is capable of ligand internalization and degradation in the absence of the classical G protein-dependent signaling properties (37, 38). Other groups have demonstrated that monocytes exposed to bacterial LPS show reduction in RNA expression of the IL-1 decoy receptor (IL-1RII), suggesting that under certain conditions, a down-regulation of the decoy receptor can occur, perhaps causing monocytes to be more responsive to IL-1β (39).

Several experimental sepsis studies have indicated protective effects by blockade of either C5a or C5aR (14, 17, 18). Therefore, we investigated the effects of i.v. infused anti-C5a on C5L2 in PMN during CLP-induced sepsis. As expected and in accordance with previous reports (14, 17, 18, 40), CLP rats treated with anti-C5a showed improved outcomes (75% survival), whereas the survival rate in CLP animals treated with preimmune IgG was <25% 48 h after CLP. In the current studies, blood PMN from CLP animals treated with preimmune IgG revealed a time-dependent loss of C5L2. In the early stages of experimental sepsis (<12 h after CLP), blood PMN from all animals had easily detectable C5L2 regardless of the experimental manipulations. Following the appearance of clinical signs of sepsis (>12 h), C5L2 in blood PMN was barely detectable by Western blot analysis, and by 36 h it had virtually disappeared. It is noteworthy that during the experimental sepsis course there was a ~12- to 18-h delay in C5L2 reduction relative to changes described for C5aR (14). Other mediators of the complement cascade or the chemokine/cytokine network could be involved in C5L2 reduction (e.g., excessively generated C3a or its degradation products) (18, 27). The loss of C5L2 during progressive CLP sepsis was in striking contrast to the condition of anti-C5a-treated CLP animals that had minimal clinical signs of sepsis and exhibited preservation of C5L2 on blood PMN (Fig. 3). These findings indicate that generation of C5a during sepsis or in vitro exposure of PMN to C5a leads to loss of C5L2, whereas blockade of C5a in CLP animals not only maintains expression of C5L2, but greatly improves the clinical outcome. Several studies support the concept that decoy receptors are beneficial during inflammatory responses. In a model of Streptococcus pyogenes-induced arthritis in mice, the presence of osteoprotegerin, a decoy receptor for receptor activator of NF-κB ligand, prevented bone destruction in the infected joints (41). In another inflammation model in which Fas ligand-induced lung inflammatory injury was studied, the presence of the decoy receptor 3 analog reduced PMN recruitment into the alveolar compartment and inhibited the appearance in bronchoalveolar lavage fluids of proinflammatory chemokines (42). Recently, the anti-inflammatory IL-10, 12 h after C5a exposure of human PMN (up to 4 h) also exhibited a time-dependent decrease of C5L2 (Fig. 3). These findings indicate that generation of C5a during sepsis or in vitro exposure of PMN to C5a leads to loss of C5L2, whereas blockade of C5a in CLP animals not only maintains expression of C5L2, but greatly improves the clinical outcome. Several studies support the concept that decoy receptors are beneficial during inflammatory responses. In a model of Streptococcus pyogenes-induced arthritis in mice, the presence of osteoprotegerin, a decoy receptor for receptor activator of NF-κB ligand, prevented bone destruction in the infected joints (41). In another inflammation model in which Fas ligand-induced lung inflammatory injury was studied, the presence of the decoy receptor 3 analog reduced PMN recruitment into the alveolar compartment and inhibited the appearance in bronchoalveolar lavage fluids of proinflammatory chemokines (42). Recently, the anti-inflammatory IL-10, has been found to uncouple proinflammatory chemokine receptors and to generate functional decoy receptors that act as scavengers for inflammatory cytokines (43).

When C5a binds to C5aR, serine and threonine residues in the C-terminal region (cytoplasmic) of the receptor are phosphorylated, resulting in rapid internalization involving ~60% of the receptor and subsequent recycling of C5aR to the membrane in the span of 80–90 min (44–46). During experimental sepsis, blockade of C5a resulted in retention (or fast recovery) of both C5a-binding capacity (14) and C5aR content on PMN (15). In contrast, C5L2 has been described as not undergoing rapid internalization in response to ligand binding (21, 23). This may be due to a different pattern of serine/threonine phosphorylation in the carboxyl tail of C5L2. As recently described, there is only a modest increase in phosphorylation of C5L2 in response to 5-min exposure to C5a, in contrast to robust phosphorylation of C5aR (23) within the first minutes. The in vitro and in vivo findings in the present study demonstrate for the first time that in humans with severe sepsis or septic shock, a significant reduction of the C5L2 expression occurs in PMN (Fig. 6). It is striking that C5L2 content in PMN obtained from patients with sepsis and surviving an observation period of 30 days exhibited higher levels of C5L2 than patients with sepsis who failed to survive (Fig. 5B). Furthermore, reduced C5L2 expression seems to correlate with sepsis-induced MOF (Fig. 6), which experimentally has been shown to be attenuated by anti-C5a (14) and by C5aR antagonist (C5aRa) treatment (47), although C5aRa fails to directly block C5a interaction with C5L2 (28). Based on the current data, it is not possible to determine whether C5L2 functions as a decoy receptor to mitigate against the consequences of interactions between C5a and C5aR. It is likely that C5L2 functions as a C5a/C5aDes/Arg scavenging receptor in PMN during sepsis. As a recent report has suggested, the IL-1β decoy receptor may be a target for potential gene therapy in arthritis (48). Although speculative, up-regulation of C5L2 might be a novel therapeutic approach to block the harmful effects of C5a during sepsis and developing MOF.

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