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Evidence for a functional role of the second C5a receptor C5L2

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SPECIFIC AIMS

Recent research demonstrates that C5a/C5aR play an important role for the development of sepsis and harmful impairment of crucial innate-immune functions. The aim of current report was to investigate the presence and regulation of the second C5a receptor, C5L2, the putative "default" or nonsignaling receptor for C5a on neutrophils and various organs during sepsis (after cecal ligation and puncture) and to evaluate role for C5L2 in balancing the biological responses to C5a.

PRINCIPAL FINDINGS

1. 5′-RACE PCR, cDNA cloning and sequence comparison of the rat C5L2 with the sequence of other C5L2s

Using the mouse C5L2 sequence, we PCR-amplified an 865 bp cDNA fragment from rat lung. To obtain the full-length coding region of rat C5L2, we performed a BLAST search of the rat EST database using the partial rat C5L2 as the query sequence. One EST clone (dbEST 14223745) was obtained and contained an incomplete sequence of rat C5L2, terminating at the 3′-end with a poly (A) tail. To isolate sequences extending further upstream, we employed a 5′-RACE approach using a ligation-anchored rat lung cDNA template. A nested pair of primers was designed and two sequential rounds of PCR were carried out using two rat C5L2-specific nested primers in conjunction with adaptor primers. When the PCR products and the EST sequence were assembled together, we obtained the composite-deduced amino acid sequence for C5L2. Comparison of the translation of the cDNA with other known C5L2 sequences showed that rat C5L2 is 59.8% identical to human C5L2 and 86.4% identical to mouse C5L2. We further analyzed the expression of rat C5L2 mRNA in rat tissues using Northern blot analysis. Rat C5L2 was expressed in all tissues examined with highest expression in liver and thyroid. To demonstrate cell surface expression of rat C5L2, we transfected a mammalian expression plasmid-encoding rat C5L2 into HEK-293 cells and performed confocal fluorescence microscopy analysis. Cells transfected with the rat C5L2 demonstrated cell surface expression of C5L2 when αC5L2 was used.

2. Comparison of C5L2 expression to C5aR expression in neutrophils during CLP-induced sepsis

We recently found that compromised neutrophil functions during sepsis correlate with the reduction in C5aR content on neutrophil surfaces. To determine whether the expression of C5L2 on blood neutrophils was also changed during sepsis, both C5L2 and C5aR content on rat blood neutrophils were quantitatively evaluated by flow cytometric analysis 0, 12, 24, and 36 h after the onset of CLP. As shown in **Fig. 1*A***, blood neutrophils from control animals (time 0) showed positive staining for both C5L2 and C5aR. During experimental sepsis, C5L2 content on blood neutrophils was significantly increased 24 and 36 h after onset of CLP. In contrast, C5aR content on neutrophils significantly dropped (2.5-fold) 24 h after CLP and steadily increased thereafter, suggesting that surface C5L2 and C5aR may be independently regulated in blood neutrophils during sepsis. To determine the mechanism by which C5L2 content on neutrophils is regulated during sepsis, we evaluated C5L2 mRNA levels in purified neutrophils 0, 24, and 36 h after CLP. As shown in Fig. 1*B*, the C5L2 mRNA level dramatically increased 24 and 36 h after the onset of CLP, suggesting a transcriptional mechanism was involved in increased expression of C5L2. In contrast, C5aR mRNA levels showed no differences in blood neutrophils isolated at various time points after CLP (Fig. 1*B*). To visualize C5L2 changes in neutrophils during sepsis, we conducted confocal fluores-

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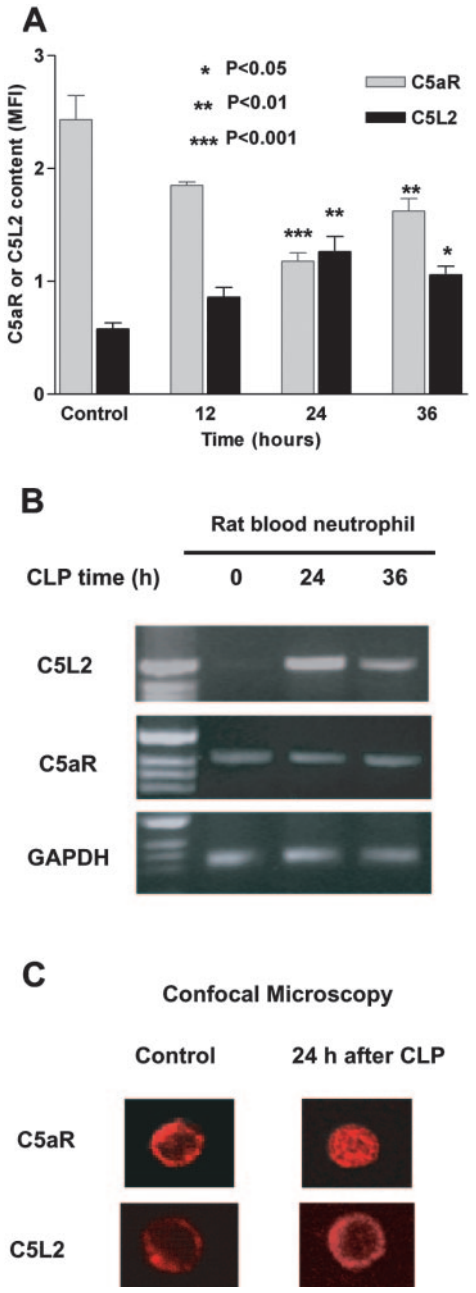

Figure 1. Expression of C5L2 and C5aR on neutrophils during experimental sepsis. *A)* Whole blood samples from controls (normal rats) and CLP animals were stained with αC5L2 or αC5aR rabbit serum or preimmune serum, and C5L2 and C5aR content on blood neutrophils was evaluated by flow cytometric analysis at the times indicated. Error bars: mean ± SE (n=4–6). *B)* RT-PCR was performed using total RNA from rat neutrophils isolated 0, 24, and 36 h after CLP. Equal loading of the PCR product was demonstrated by expression of GAPDH mRNA. Data are representative of 2 independent experiments with neutrophils pooled from 3 or 4 rats per time point. *C)* C5aR and C5L2 staining of blood neutrophils, as visualized by confocal microscopy.

cence microscopy analysis. In control cells, both C5aR and C5L2 showed a uniform cortical pattern on the neutrophil periphery, indicative of membrane staining (Fig. 1*C*). In contrast, a diffuse pattern of staining was found in the cytoplasmic compartment in 24 h CLP neutrophils for C5aR, but not for C5L2, indicating that C5L2 did not undergo internalization as did C5aR after CLP.

3. Changed in vivo organ binding of ^{125}I-αC5L2 during CLP-induced sepsis in mice

To examine changes in C5L2 expression during sepsis, ^{125}I-αC5L2 (4 μCi), ^{125}I-αC5aR (2 μCi), or ^{125}I-preimmune IgG (2 μCi) were injected intravenously into mice at various time points (up to 12 h) after CLP. We found that each of the four organs examined (lung, liver, kidney, and heart), with the exception of C5L2 in kidney, showed increases in both C5aR and C5L2 content 12 h after CLP compared with levels at time 0. In all but one case, the peak increases were at 12 h compared with 3 and 6 h after CLP. In each case except the kidney, the increases at 12 h were statistically significant (P<0.05). In kidney, the peak increase, which was statistically significant, in C5L2 occurred at 3 h. In all cases, infusion of ^{125}I-preimmune IgG at 0, 3, 6, and 12 h after CLP showed no evidence of increased tissue localization. These data indicate that sepsis causes increases in both C5aR and C5L2 in organs that are targets of multi-organ failure.

4. Expression of mRNA for C5L2 during CLP-induced sepsis

To extend the results from the in vivo binding experiments, we conducted quantitative real-time PCR experiments using RNA extracts from mouse organs. In each of the four organs studied, there was an increase in the amount of mRNA for C5aR during the CLP- induced sepsis, roughly correlating with increased organ binding of ^{125}I-αC5aR. In the lung and liver, an increased mRNA for C5L2 in the real-time PCR was only observed 12 h after CLP. In kidney and heart, there was no significantly increased level of C5L2 mRNA observed after the onset of CLP. These results suggested that the dynamic pattern of ^{125}I-αC5L2 binding to the various organs during sepsis may not be explained solely by an increased gene expression for C5L2 in these organs.

5. Effects of αC5aR and αC5L2 on in vitro and in vivo production of IL-6

To investigate the role of C5aR and C5L2 on IL-6 production, we conducted in vitro experiments with blood neutrophils obtained from healthy rats. As shown in **Fig. 2*A***, C5a significantly augmented LPS-induced robust production of IL-6 in the presence of normal IgG. When neutrophils were preincubated with αC5aR IgG, the augmentation in IL-6 production related to costimulation with C5a disappeared. In dramatic contrast, neutrophil exposure to αC5L2 IgG dramatically increased production of IL-6 in neutrophils exposed to both LPS and C5a. To extend these results, we measured IL-6 levels in serum samples obtained 6 h after CLP in mice treated intravenously with preimmune

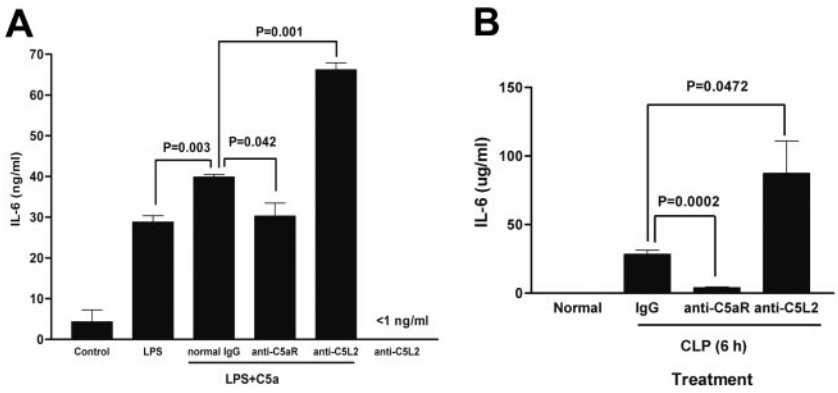

Figure 2. Effects of αC5L2 and αC5aR on in vitro production of IL-6 by blood neutrophils stimulated in vitro with LPS + C5a and serum levels of IL-6 6 h after CLP in rats treated with blocking antibodies to C5aR or C5L2. *A)* IL-6 as measured by ELISA analysis in supernatant fluids from blood neutrophils after in vitro incubation with C5a (10 nM) or LPS (20 ng/mL) in the presence or absence of αC5L2 or αC5aR. Neutrophils were pooled from 4–6 animals per study; incubations were carried out in separate triplicate samples. *B)* ELISA measurements for serum IL-6 levels 6 h after CLP-induced sepsis in animals injected with αC5L2, αC5aR or preimmune IgG. IL-6 could not be detected in sera from control (normal) mice. When used, αC5L2 and αC5aR treatment consisted of 20 μg/mouse at the start of CLP, with a companion group treated to control IgG. Error bars: mean ± SE (n=4).

IgG, αC5aR, or C5L2 IgG (Fig. 2*B*). As expected, serum IL-6 was elevated in mice 6 h after CLP. In αC5aR-treated mice, IL-6 levels were reduced to nearly undetectable levels 6 h after CLP compared with control IgG injected mice. In contrast, αC5L2-treated mice dramatically increased (by nearly 4-fold) serum levels of IL-6, suggesting that there may be competition between C5aR and C5L2 for C5a binding, the balance determining serum levels of IL-6 during sepsis.

CONCLUSIONS AND SIGNIFICANCE

During experimental sepsis in rodents after cecal ligation and puncture (CLP), excessive C5a is generated, leading to interactions with C5aR and loss of innate immune functions of neutrophils and lethality. In the current study, we have analyzed the expression of the second C5a receptor, C5L2, the putative "default" or nonsignaling receptor for C5a. Rat C5L2 was cloned and antibody was developed to C5L2 protein. After CLP, blood neutrophils showed a reduction in C5aR followed by its restoration, while C5L2 levels gradually increased, accompanied by the appearance of mRNA for C5L2 (Fig. 1, **Fig. 3**). We showed that mRNA for C5L2 increased in lung and liver during CLP. Substantially increased C5L2 protein occurred in lung, liver, heart, and kidney after CLP. Using serum IL-6 as a marker for sepsis, infusion of αC5aR dramatically reduced serum IL-6 levels, while αC5L2 caused a nearly 4-fold increase in IL-6 compared with CLP controls treated with normal IgG. When normal blood neutrophils were stimulated in vitro with LPS and C5a, the antibodies had similar effects on release of IL-6. The biological implications of changes in C5a receptors (C5aR and C5L2) during sepsis are not currently known but may be linked to development of multiorgan failure. Whether the regulation of C5L2 expression in the different organs during sepsis is similar to that in neutrophils remains to be defined. But, our data provide the first evidence for a role for C5L2 in balancing the biological responses to C5a and strongly suggest that, depending on the relative numbers of C5aR and C5L2, the generation of C5a may lead to an amplified proinflammatory response or a suppressed proinflammatory response. FJ

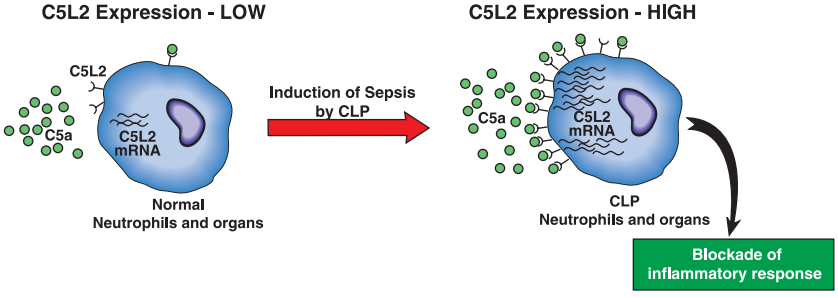

Figure 3. Schematic diagram showing the increased C5L2 expression on neutrophils and various organs during sepsis and the correlated effect on inflammatory response.