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NF-κB-mediated inverse regulation of fractalkine and CX3CR1 during CLP-induced sepsis

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ABSTRACT

Fractalkine is a unique member of the CX3C chemokine family by unfolding its potential through the chemokine (C-X3-C motif) receptor 1 (CX3CR1) with dual function acting both as an adhesion molecule and a soluble chemokine. The regulation of this chemokine is still not clear. Therefore, we were interested in the regulation of fractalkine and of CX3CR1 in experimental sepsis. In addition, we investigated the role of NF-κB for the regulation of fractalkine and of CX3CR1.

Using a mouse model of cecal ligation and puncture (CLP)-induced sepsis, we found elevated fractalkine mRNA levels in the heart, lung, kidney, and liver, as well as increased plasma levels 24 and 48 h after CLP, respectively. In parallel, CLP resulted in a significant downregulation of CX3CR1 mRNA receptor expression in all investigated murine tissues. Septic mice that were pretreated with the selective NF-κB inhibitor pyrrolidine dithiocarbamate (PDTC) were found to have a decreased liberation of proinflammtory cytokines such as TNF-α, IL-1β, IL-6, or IFN-γ. Further PDTC pretreatment attenuated CLP-induced downregulation of CX3CR1 mRNA as well as CLP-induced upregulation of fractalkine mRNA expression in the heart, lung, kidney, liver, and the increase in fractalkine plasma levels of septic mice. In addition, CLP-induced downregulation of renal CX3CR1 protein expression was inhibited by PDTC-pretreatment.

Taken together, our data indicate a CLP-induced inverse regulation of the expression between the relating ligand and the receptor with an upregulation of fractalkine and downregulation of CX3CR1, which seems to be mediated by the transcripting factor NF-κB likely via reduced liberation of proinflammtory cytokines in the whole murine organism.

1. Introduction

Septic syndromes represent a dramatic problem in critically ill patients with a continuously increasing incidence over the last two decades [1]. Despite major advances in supportive therapy, sepsis-related mortality has only modestly decreased with rates still up to 60%, accounting for 135,000 deaths per year in Europe and in the US [2,3]. Septic shock is characterized by major perturbations of the immune homeostasis initially leading to a tremendous systemic inflammatory response followed by an antiinflammatory process [1,4,5]. This compensatory inhibitory response may become deleterious as nearly all immune functions are compromised [4,6–9]. In line, clinical studies have shown that the spontaneous recovery of a functional proinflammatory response after septic shock is associated with survival [6,10,11]. However, much remains to be learned about the pathophysiology of this state of immunosuppression, and the characterization of its mechanistic bases remains a major challenge to improve care for critically ill patients. Recently, a panel of genes was identified, whose peripheral blood expression could efficiently discriminate survivor from nonsurvivor septic shock patients [12]. Among the genes up-regulated in survivors compared with nonsurvivors, the chemokine receptor chemokine (C-X3-C motif) receptor 1 (CX3CR1) showed the highest factor of change (8-fold increase in comparison with nonsurvivors).

CX3CR1 is a G-protein coupled receptor expressed on T lymphocytes [13,14], mast cells [15], natural killer cells [16], dendritic cells [17], platelets [18], neurons, astrocytes, microglial cells [19–21], and monocytes [22,23]. The CX3CR1 ligand chemokine (C-X3-C motif) ligand 1 (CX3CL1), also called fractalkine, is the sole member of the CX3C chemokine subfamily. Compared with classic CC and CXC chemokines, the CX3C chemokine fractalkine backpedals an extraordinary role, because it exists in two forms: membrane anchored or shed chemotactic soluble glycoprotein [24,25]. The soluble fractalkine exerts a potent chemotactic activity [26], whereas the membrane-bound fractalkine is expressed on

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endothelial cells [27–29] and induces leukocyte adhesion and transmigration into injured tissue. A role for CX3CR1-mediated inflammation has been demonstrated in several inflammatory disorders such as rheumatoid arthritis [30,31], atherosclerosis [32,33], and various inflammatory conditions of the kidney, brain, and lung [29,34]. This chemokine–receptor pair has also been implicated in allergic inflammatory airway disease. [35].

Since there is evidence that proinflammatory cytokines can interfere with the expression of CX3CR1 receptor /fractalkine *in vitro* and *in vivo* [29,36–46], one might speculate that as well during sepsis proinflammatory cytokines themselves impair CX3CR1-receptor/fractalkine interaction. In turn, for maximal secretion and for unfolding their full potential, proinflammatory cytokines are all dependent on the transcription factor nuclear factor (NF)-κB, which will be activated by a variety of bacteria and bacterial components [47–49]. Recently, inhibition of NF-κB has been shown to prevent vasoplegia and to improve survival in endotoxemic animals, even though the mechanism remains unclear [50,51]. NF-κB may thus be an important target for new anti-inflammatory approaches for treating sepsis-induced alterations of CX3CR1 receptor and fractalkine expression.

The goal of the current study was to investigate the expression of CX3CR1 receptors and the ligand fractalkine during cecal ligation and puncture-induced experimental septic shock. We examined the expression of CX3CR1 receptor and fractalkine on mRNA and protein level in murine plasma and organs such as the heart, lung, kidney, and liver. Furthermore, the role of NF-κB in the regulation of CX3CR1 receptor and fractalkine expression during experimental sepsis was investigated.

2. Methods

2.1. Animals

After approval of the Regensburg Animal Protection Committee, all animal experiments were performed according to National Institutes of Health Guide for the Care and Use of Laboratory Animals. Male C57BL/6J mice (20–25 g) were purchased from Charles River (Sulzfeld, Germany). Mice were anesthetized with sevoflurane (Abbott, Chicage, USA), using a Trajan 808 (Dräger, Lübeck, Germany). Sepsis was induced by cecal ligation and puncture (CLP). After mobilization and ligation of the cecum, it was punctured six times with an 18-gauge needle. In sham-treated animals, a similar procedure was performed but without CLP. To ensure adequate fluid resuscitation, animals received a subcutaneous injection of lactated Ringer's solution (50 μl/g) [52] directly after finishing the intervention. Sham and CLP animals were killed 24 or 48 h ($n = 6$ per group) following surgery. CLP and sham treatment were also performed in mice pretreated with the NF-κB inhibitor pyrrolidine dithiocarbamate (PDTC, 100 mg/kg, Sigma, Munich, Germany, $n = 6$ per group; intraperitoneally 2 h before CLP treatment). The dose of PDTC was chosen from data taken from the literature [49,50,53–57].

2.2. mRNA extraction and real-time PCR analysis

Total RNA from tissues and plasma was extracted and reverse-transcribed, and real-time PCR was carried out using the Light-Cycler system (Roche, Basel, Switzerland) as described [58]. Each primer set for CX3CR1 receptor (5′–3′→s: aag ttc cct tcc cat ctg ct; as: caa aat tct cta gat cca gtt cag g) and ligand fractalkine (s: cac ctc ggc atg acg aaa t; as: ttg tcc acc cgc ttc tca a) was checked using a BLAST search to ascertain that the sequences were unique for each gene. β-Actin was used as reference gene.

2.3. Tissue and plasma concentrations of cytokines, NF-κB and fractalkine

Tissue concentrations of TNF-α, IL-1β, IFN-γ, and NF-κB as well as plasma concentration of fractalkine were determined using Enzyme Linked Immunosorbent Assay (ELISA) kits (R&D Systems, Minneapolis, USA) and set into proportion to total protein.

2.4. Protein preparation

Kidneys were homogenized in ice-cold homogenization buffer in the presence of protease inhibitors followed by centrifugation with 500*g* for 15 min at 4 °C. The resultant supernatant was centrifuged at 20,000*g* for 30 min at 4 °C. The resultant supernatant was used for determination of tissue cytokine concentration, and the resultant pellet was reconstituted in blotting buffer and used for Western blotting.

2.5. Western blot

Protein samples (40 μg) were electrophoretically separated on a 10% polyacrylamide gel and transferred to nitrocellulose membrane, which was blocked overnight in 5% nonfat dry milk diluted in Tris-buffered saline with 0.1% Tween-20, and then incubated for 1 h at room temperature with a polyclonal antibody against CX3CR1 (Santa Cruz Biotechnology, Santa Cruz, USA; 1:500; sc-30030). After washing, the membrane was incubated for 2 h with secondary goat-anti rabbit antibodies (Santa Cruz Biotechnology, Santa Cruz, USA; 1:500; β-actin, 1:5000) and subjected to a chemiluminescence detection system (Amersham). Semiquantitative assessment of bands was performed densitometrically.

2.6. Statistical analyses

Data were analyzed by ANOVA with multiple comparisons followed by the *t* test with Bonferroni adjustment. $P \leqslant 0.05$ was considered significant.

3. Results

3.1. Effect of CLP on CX3CR1 and ligand expression

CLP-induced a strong liberation of proinflammatory cytokines, such as TNF-α, IL-1β, IFN-γ, and IL-6 (Table 1). CX3CR1 mRNA was downregulated 4 times of control levels in the kidney, 4–5 times of control levels in the liver, 2–3 times of control levels in the lung, and 2–4 times of control levels in the heart 24 and 48 h after CLP induction, respectively (Fig. 1). Fig. 2 demonstrates protein expression of CX3CR1 as determined by western blot analysis.

Table 1
CLP-induced plasma cytokine levels are reduced by NF-κB inhibition. Effect of pyrrolidine dithiocarbamate pretreatment (PDTC, 100 mg/kg, 2 h before CLP intraperitoneally) on interleukin (IL)-1β, tumor necrosis factor (TNF)-α, interferon (IFN)-γ, and transcripting factor NF-κB expression in murine plasma 24 h after cecal ligation and puncture (CLP) treatment. Plasma concentrations of IL-1β, TNF-α, IFN-γ, and NF-κB protein are given as pg/ml. Mean ± SEM of 6 animals/group.

	TNF-α	IL-1β	IFN-γ	NF-κB
Sham	n.d.	n.d.	0.8 0.1	1.0 ± 0.1
PDTC	n.d.	n.d.	n.d.	0.5 ± 0.1
CLP 24 h	10.2 ± 1.0	142 ± 22	19.8 ± 3.2	68 ± 4.2*
CLP + PDTC 24 h	1.8 ± 0.2#	12.0 ± 3.1#	1.2 ± 0.2#	4.3 ± 1.6*,#

n.d. means not detectable.
* $P \leqslant 0.05$ vs. sham.
# $P \leqslant 0.05$ vs.CLP.