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SHORT COMMUNICATION

The interaction between C5a and both C5aR and C5L2 receptors is required for production of G-CSF during acute inflammation

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The complement activation product, C5a, is a key factor for regulation of inflammatory responses. C5a and $C5a_{desArg}$ bind to their receptors, C5aR and C5L2, but the functional roles of C5L2 remain controversial. We screened the patterns of 23 inflammatory mediators in cultures of LPS-activated mouse peritoneal elicited macrophages (PEMs) in the presence or absence of recombinant mouse C5a. Production of most mediators studied was suppressed by C5a, whereas G-CSF production was enhanced. G-CSF gene expression and secretion from PEMs was amplified two- to threefold by C5a in a dose- and time-dependent fashion. The degradation product $C5a_{desArg}$ promoted lower levels of G-CSF. The effects of C5a on G-CSF were associated with activation of PI3K/Akt and MEK1/2 signaling pathways. C5a did not enhance G-CSF production in cultures of PEMs from either C5aR- or C5L2-deficient mice, indicating that both C5a receptors are indispensable for mediating the effects of C5a in the production of G-CSF. Finally, G-CSF levels in plasma during polymicrobial sepsis after cecal ligation and puncture were substantially lower in C5aR- or C5L2-deficient mice as compared with that in C57BL/6J WT mice. These findings elucidate the functional characteristics of the C5L2 receptor during the acute inflammatory response.

Keywords: Akt · Cecal ligation and puncture · Macrophages · MEK1/2 · Sepsis

Introduction

Proteolytic cleavage of complement proteins following activation of the classical, alternative, and lectin pathways can generate substantial quantities of the anaphylatoxin, complement activation fragment (C5a) [1]. Rapid inactivation by carboxypeptidase removes the C-terminal arginine, converting C5a to C5a lacking the C-terminal arginine ($C5a_{desArg}$). Both C5a and $C5a_{desArg}$ (with much lower affinity) are ligands for the G-protein coupled C5aR receptor (CD88) [2, 3]. C5aR is abundantly expressed on innate immune cells of the myeloid lineage, lymphocytes, and in lower numbers on epithelial and endothelial cells [4–6]. In polymorphonuclear leukocytes (PMNs) and macrophages, ligation of C5a with the C5aR receptor leads to rapid buildup of cytosolic Ca_2^+, activation of MAPK signaling pathways, chemotaxis, respiratory burst, release of toxic granules, and regulation of cytokine expression [2, 3, 7].

A second C5a receptor, C5L2 (GPR77), has been identified [8]. Initially, C5a was thought to be a nonsignaling "decoy" receptor [8]. Indeed, binding of C5a or $C5a_{desArg}$ to C5L2 does not induce rapid Ca_2^+ currents [9]. However, accumulating evidence suggests distinct functional roles of C5L2 in disease. For example, both C5aR and C5L2 receptors are critical factors during

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polymicrobial sepsis after cecal ligation and puncture (CLP) [10]. The expression of C5L2 in PMNs is downregulated during severe sepsis, which is a marker of poor prognosis [11]. C5L2 determines the outcome of experimental allergic asthma [12]. Polymorphisms of the C5L2 gene may be associated with a higher risk for cardiovascular diseases in some populations [13]. Despite these findings, many aspects of the role of C5L2 in acute and chronic inflammation remain enigmatic.

In this report, we describe that C5a promotes the release of G-CSF. These effects required the presence of both C5aR and C5L2 in cultures of macrophages and during polymicrobial sepsis after CLP.

Results and discussion

Differential regulation of mediator production by C5a

We have recently reported that the production of several cytokines (IL-17, IL-23, and IL-27) is suppressed by C5a when present in cultures of LPS-activated peritoneal elicited macrophages (PEMs) [5, 14, 15]. To investigate how broad the spectrum of C5a-regulated mediators is, the concentrations of 23 inflammatory mediators were analyzed by a multiplexing bead-based assay (Table 1). Incubation of PEMs from C57BL/6J mice with LPS for 10 h generally increased the release of cytokines and chemokines as compared to untreated control PEMs (Table 1). Simultaneous addition of recombinant mouse C5a (100 nM) to LPS-activated PEMs affected the production of all mediators studied (Table 1). Although proinflammatory mediators were suppressed by C5a, the remarkable finding was that only IL-10 and G-CSF were amplified (by 103 and 197%, respectively). No consistent effects on production of the analyzed cytokines was observed, when C5a was used alone in PEMs (data not shown; [5, 15]). It has been reported previously that cytokines of the IL-12 family are antagonized by C5a [16, 17]. Furthermore, we have recently described the role of C5a-induced IL-10 in downmodulation of the IL-17A/IL-23 axis [15]. These findings demonstrate that the strong proinflammatory anaphylatoxin C5a in high concentrations can mediate anti-inflammatory effects in primed macrophages, which may be beneficial to prevent excessive inflammation. Reciprocal effects of C5a (as an inducer of proinflammatory cytokines/chemokines) have been noted in other cell types such as alveolar epithelial cells [18], microvascular endothelial cells [19], or blood PMNs [20].

Table 1. Regulation of macrophage-derived mediators by C5[a)]

Mediator	CTRL (pg/mL)	LPS (pg/mL)	LPS + C5a (% change)
IL-1α	36 ± 19	388 ± 94	−30**
IL-1β	255 ± 166	2800 ± 683	−30*
IL-2	44 ± 13	83 ± 18	−23*
IL-3	5 ± 2	19 ± 5	−29*
IL-4	9 ± 3	29 ± 8	−22*
IL-5	6 ± 3	15 ± 3	−27*
IL-6	89 ± 46	5516 ± 1455	−64**
IL-9	57 ± 17	350 ± 74	−38**
IL-10	62 ± 21	223 ± 41	+103*
IL-12(p40)	157 ± 66	12 983 ± 4366	−81**
IL-12(p70)	73 ± 20	1427 ± 344	−68*
IL-13	109 ± 60	979 ± 268	−35*
IL-17	43 ± 26	1036 ± 375	−44*
Eotaxin	461 ± 185	2653 ± 613	−30***
G-CSF	38 ± 22	375 ± 59	+197**
GM-CSF	43 ± 12	161 ± 31	−21*
IFN-γ	31 ± 9	279 ± 39	−47*
KC	1148 ± 791	26 097 ± 2087	−28*
MCP-1	1124 ± 663	19 722 ± 4038	−55**
MIP-1α	457 ± 256	21 528 ± 6056	−69**
MIP-1β	2323 ± 1485	130 564 ± 37 936	−60**
RANTES	550 ± 276	22 212 ± 3888	−60**
TNF-α	187 ± 55	59 126 ± 18 211	−77**

[a)] Peritoneal elicited macrophages (PEMs) from C57BL/6J mice were incubated for 10 h with LPS (1 μg/mL) ± rmC5a (100 nM). CTRL denotes supernatants from resting cells. Multiplexing bead-based assay. Data are from four independent experiments. Values represent means ± SEM and values after LPS alone were used as 100% to calculate % change. Student's two-tailed *t*-test, LPS versus LPS+C5a, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

C5a promotes release of G-CSF via Akt and MEK1/2

The current studies were focused on G-CSF, which was the mediator most potently enhanced by C5a in PEMs (Table 1). C5a acted dose dependently to increase G-CSF levels by two to threefold in cultures of PEMs (Fig. 1A). Higher concentrations of G-CSF were observed at all time points (3–24 h) studied (Fig. 1B). Long-lasting modulation of mediator release by C5a has been also reported for other cytokines [5, 15]. Recombinant $C5a_{desArg}$ in combination with LPS also displayed somewhat diminished ability to amplify G-CSF compared to C5a (Fig. 1C). C5a enhanced G-CSF production on the mRNA level was detected by real-time PCR (Fig. 1D). No effects on G-CSF levels were seen when C5a was used alone in the absence of the co-stimulus, LPS (Fig. 1C and D).

C5a is known to activate PI3K/Akt and MEK/ERK kinases in innate immune cells [5, 14, 16, 21–23]. Using bead-based assays with phosphorylation-specific antibodies, we confirmed that C5a activated MEK1 and Akt (Fig. 1E and F). These signaling pathways appeared to be relevant for mediating C5a-enhanced G-CSF production. Blockade of PI3K/Akt with the small molecule inhibitor, LY294002, and blockade of MEK1/2 with PD98059 or the highly selective inhibitor, U0126, greatly reduced the release of G-CSF from PEMs activated with LPS or the combination of LPS plus C5a (Fig. 1G and H).

G-CSF production is resistant to the inhibitory effects of C5a-induced IL-10

To further elucidate the mechanism by which C5a differentially suppressed proinflammatory cytokines/chemokines, while upregulating G-CSF, we studied the role of IL-10. C5a selectively

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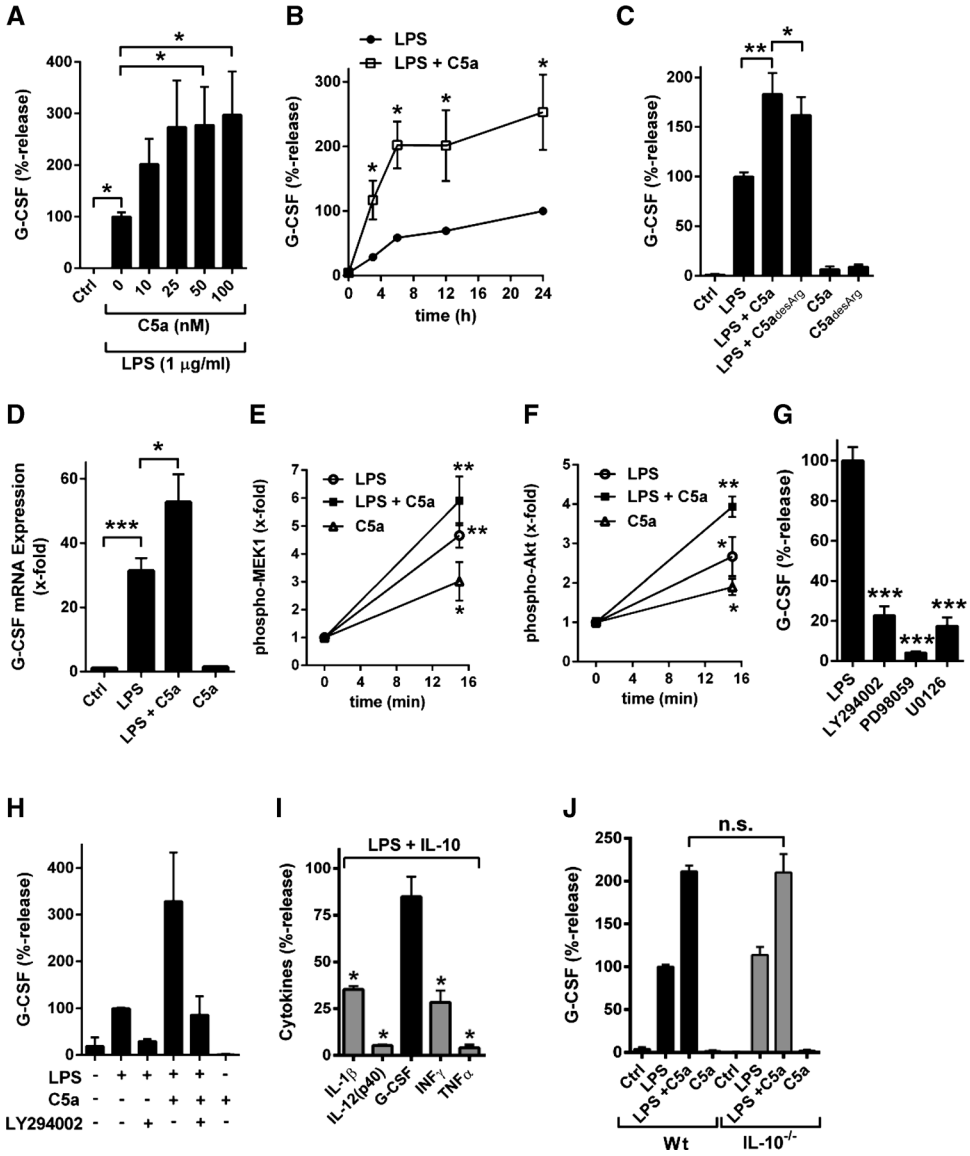

Figure 1. C5a-induced amplification of G-CSF production from macrophages. (A) PEMs from C57BL/6J mice were stimulated for 8 h with LPS (1 μg/mL) alone or in combination with different doses of recombinant mouse C5a (10–100 nM) followed by detection of G-CSF by ELISA in supernatant fluids. (B) Time course of G-CSF secretion from PEMs after LPS (1 μg/mL) alone or together with rmC5a (100 nM), ELISA. (C) Comparison of activity of rmC5a (100 nM) and rmC5a$_{desArg}$ (100 nM) to enhance G-CSF in supernatants from LPS-activated PEMs, 8 h, ELISA. (D) Real-time PCR of mRNA for G-CSF from PEMs left as untreated controls (Ctrl), after LPS (1 μg/mL), LPS plus C5a (100 nM), or C5a alone, 6 h. (E) Phospho-MEK1 (serine 217/serine 221) in PEMs after incubation with C5a (100 nM) and LPS (1 μg/mL) alone or in combination. Phosphorylation levels in untreated cells were used as onefold, bead-based assay. (F) Phospho-Akt (threonine 308) after stimulation with C5a, LPS, or LPS plus C5a, bead-based assay. (G) Relative release of G-CSF from LPS-activated PEMs after blockade of PI3K/Akt with LY294002 (25 μM) or MEK1/2 with PD98059 (50 μM) or U0126 (1 μM), 8 h, ELISA. All experiments were done with cells from C57BL/6J mice. (H) PEMs were treated with combinations of LPS, C5a, and LY294002 as indicated followed by quantitation of G-CSF, 8 h, ELISA. (I) PEMs (C57BL/6J) were incubated for 8 h with LPS alone or LPS plus rmIL-10 (10 ng/mL) followed by detection in supernatants of IL-1β, IL-12(p40), G-CSF, IFN-γ, and TNF-α. Amounts of cytokines after LPS alone were used as 100% values, bead-based assays. (J) Relative release of G-CSF in PEMs from C57BL/6J mice compared to PEMs from IL-10$^{-/-}$ mice after LPS ± C5a, 8 h, ELISA. All data are shown as mean ± SEM of three to six samples pooled from three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, Student's two-tailed *t*-test.

promoted the production of IL-10 from LPS-activated PEMs (Table 1). We have recently reported that C5a-induced IL-10 release is essential for suppression of proinflammatory cytokines such as IL-17A and IL-23 by C5a [15]. Interestingly, the release of G-CSF was resistant to the inhibitory effects of recombinant IL-10, even when used in high concentrations (Fig. 1I). At the same time, proinflammatory cytokines (IL-1β, IL-12, IFN-γ, TNF-α) were greatly reduced by IL-10 (Fig. 1I). When the production of endogenous IL-10 was interrupted using PEMs from IL-10$^{-/-}$ mice, no differences were seen in G-CSF production after LPS alone or in combination with C5a (Fig. 1J). This is in clear contrast to mediators such as IL-12, IL-17A, or IL-23 for which the

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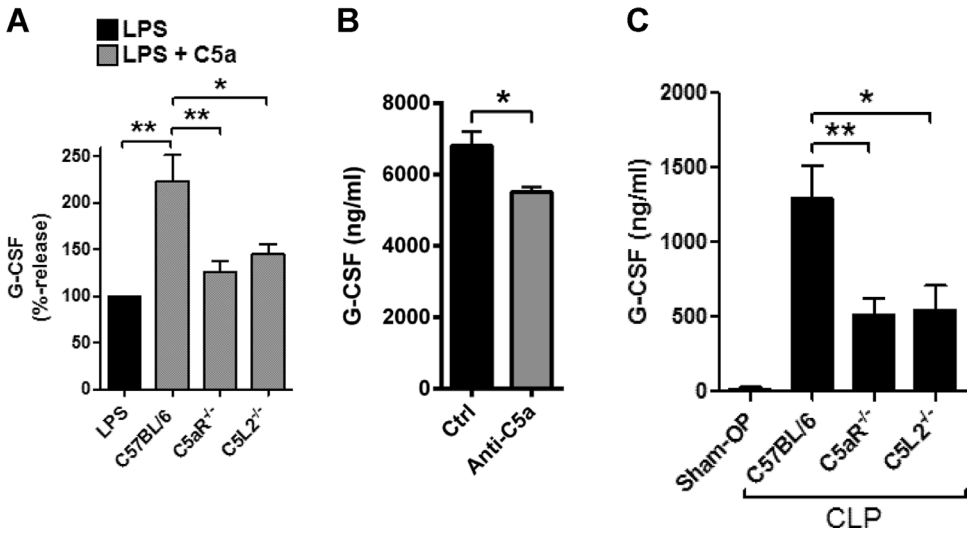

Figure 2. Studies on the production of G-CSF with genetic absence of the C5aR receptor or C5L2 receptor. (A) Relative release of G-CSF from PEMs from C57BL/6J (Wt), C5aR$^{-/-}$, or C5L2$^{-/-}$ mice after LPS (1 μg/mL) alone or together with C5a (100 nM), 8 h, ELISA. Results with LPS alone were used as the 100% value for each mouse strain. (B) Detection of G-CSF in plasma of C57BL/6J mice 12 h after endotoxic shock (LPS 10 mg/kg body weight i.p.). Mice were pretreated with neutralizing polyclonal anti-C5a antiserum or control (Ctrl) serum (500 μL i.p., $n \geq 7$ per group), ELISA. (C) Plasma concentrations of G-CSF in C57BL/6J (Wt) mice after sham-OP ($n = 7$) or cecal ligation and puncture (CLP, $n = 15$) compared with concentrations after CLP in C5aR$^{-/-}$ mice ($n = 14$) or C5L2$^{-/-}$ mice ($n = 11$). Sham-OP was performed only with Wt mice, 24 h, ELISA. Data are shown as mean + SEM of six wells pooled from three independent experiments (each done in duplicate, A), and mean + SEM of the indicated number of mice pooled from one experiment (B) or two experiments (C). $^{*}p < 0.05$, $^{**}p < 0.01$, Student's two-tailed *t*-test.

modulatory effects of C5a are mitigated with genetic deficiency or antibody blockade of IL-10 [15, 24, 25].

Dependency of G-CSF release in the presence of C5aR and C5L2

There is an ongoing debate on the precise functional roles of the C5L2 receptor as compared to the C5aR receptor [1]. We used PEMs from C5aR$^{-/-}$ mice and C5L2$^{-/-}$ mice to investigate the roles of both receptors for C5a-mediated amplification of G-CSF. Both C5aR and C5L2 were required for C5a enhancement of G-CSF production in cultures of PEMs from C5aR$^{-/-}$ mice and C5L2$^{-/-}$ mice (Fig. 2A), since the absence of either receptor caused G-CSF levels to fall to baseline levels (with LPS alone).

To study the effects of C5a on G-CSF production during the acute inflammatory response in vivo, we blocked endogenous C5a during endotoxic shock, which resulted in moderately decreased G-CSF (Fig. 2B). Next, we used the model of polymicrobial sepsis after CLP to study G-CSF production. Sham-operated C57BL/6J mice received only anesthesia and laparotomy without manipulation of the cecum. The amounts of G-CSF in plasma of sham-OP mice were low but G-CSF was greatly increased in C57BL/6J mice 24 h after CLP (>1000 ng/mL, Fig. 2C). At the same time, plasma concentrations of G-CSF were substantially lower in C5aR$^{-/-}$ mice and C5L2$^{-/-}$ mice after CLP (Fig. 2C).

Collectively, it appears that neither C5a receptor (C5aR and C5L2) by itself is able to compensate for the genetic absence of the other receptor, respectively. These findings are quite surprising because C5a does not require presence of the C5L2 receptor (as studied in C5L2$^{-/-}$ PEMs) for suppression of other cytokines such as IL-17A, IL-23, or IL-27 [5, 14], suggesting that the effects of C5a on G-CSF production from PEMs are distinct from the regulation of other mediators by C5a (Table 1). Interestingly, a recent study has reported C5aR internalization after ligand binding and co-localization with intracellular C5L2 in human neutrophils, which may suggest a potential crosstalk of the two receptors [26].

Complement activation is an essential arm of innate immune responses, capable of providing immediate clearance of pathogens by direct lysis (membrane attack complex) and facilitating opsonization/phagocytosis of pathogens. C5a directs chemotaxis of PMNs to the site of inflammation and activates such cells. In addition, our data demonstrate that C5a signaling amplifies the production of G-CSF in vitro and in vivo. G-CSF is a critical growth factor for the proliferation of PMNs during hematopoiesis in the bone marrow. Recombinant G-CSF is an FDA-approved pharmaceutical drug for the treatment of chemotherapy-related severe neutropenia. In one report, C5-deficient mice displayed an impaired mobilization of hematopoietic stem/progenitor cells [27]. C5a promotes the egress of PMNs from the bone marrow into peripheral blood [28]. Our data suggest that C5a via enhanced production of G-CSF may promote the PMN-dependent acute inflammatory response with dependency on both C5aR and C5L2 receptors. Respiratory burst and granule release from PMNs contributes to tissue injury during inflammation (e.g. in the setting of sepsis). In accordance with our findings, the blockade of complement activation using compstatin analogs (which block the C3 convertase) reduced G-CSF levels in human blood during simulated hemodialysis [29]. Moreover, G-CSF appears

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to upregulate C5aR (CD88) expression on PMNs [28]. In summary, C5a promotes the influx and proliferation of PMNs through multiple mechanisms, whereas different effects are seen with respect to adaptive immunity such as suppression of Th1 and Th17 cytokines.

Concluding remarks

C5a possesses broad-spectrum activities for regulating the release of inflammatory mediators from macrophages. G-CSF is regulated by C5a different than with most other mediators. C5aR and C5L2 are both indispensable for transmission of signaling by C5a in order to amplify G-CSF production in vitro and in vivo.

Materials and methods

Mice

All procedures were in accordance with the U.S. National Institutes of Health guidelines and the University Committee on Use and Care of Animals (UCUCA), University of Michigan. We used male mice of the strains C57BL/6J, IL-$10^{-/-}$ (B6.129P2-Il10tm1Cgn; Jackson Laboratories, Bar Harbor, ME, USA), C5a$R^{-/-}$, and C5L$2^{-/-}$ (both breeding and genotyping were done at the University of Michigan). Mice were housed under specific pathogen-free conditions. The generation of C5a$R^{-/-}$ and C5L$2^{-/-}$ mice by targeted gene disruption has been described previously [30,31]. Animals used were the progeny of at least ten generations of breeding of the knockout mice on the C57BL/6J background.

Cecal ligation and puncture (CLP)

The CLP surgery was performed as described elsewhere [32]. Briefly, mice were anaesthetized with ketamine/xylazine i.p. before laparotomy, ligation of 4–5 mm of the distal cecum with puncture (21-G needle) and postoperative administration of 1 mL 0.9% NaCl subcutaneously. The severity of the procedure was designed to obtain mortality rates of 20–50% after 7 days. Sham mice received anesthetics, laparotomy, and fluid resuscitation without manipulation of the cecum.

Peritoneal elicited macrophages (PEMs)

Thioglycollate (1.5 mL, 2.4%, i.p.) was injected to mice and macrophages (>80% F4/80^{+}C$D11b^{+}$) obtained by peritoneal lavage 4 days later [14]. PEMs were cultured at 2×10^6 cells/mL in RPMI 1640 medium supplemented with 100 U/mL penicillin-streptomycin, 0.1% BSA, and 25 mM HEPES at 37°C and 5% CO_2. At the end of experiments, nonadherent cells were pelleted by centrifugation (650 × *g*, 5 min, 4°C) and supernatants were stored at −80°C until further analysis.

Detection of proteins

Mouse G-CSF was quantified by ELISA (R&D Systems) according to the instructions of the manufacturer. Bead-based assays were used for detection of phosphorylated MEK1 (serine 217/serine 221) (BioRad) or phosphorylated Akt (threonine 308) (Millipore). Cells were lysed in the presence of protease and phosphatase inhibitors (Bio-Plex Cell Lysis Kit, BioRad) and assays performed according to instructions of the manufacturers. A multiplex bead-based assay (BioPlex Pro, 23-plex group I, BioRad) was used for simultaneous quantification of the following cytokines/chemokines: IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12(p40), IL-12(p70), IL-13, IL-17, eotaxin (CCL11), G-CSF, GM-CSF, IFN-γ, KC (CXCL1), MCP-1 (CCL2), MIP-1α (CCL3), MIP-1β (CCL4), RANTES (CCL5), and TNF-α. All samples from bead-based assays were analyzed on a Luminex-200 instrument as described before [32].

Real-time PCR

Total RNA was isolated (RNeasy Mini Kit, Qiagen) and reverse transcribed (TaqMen reagents) followed by real-time amplification (SYBR Green Master Mix, Applied Biosystems) on a 7500 Real-Time PCR instrument (Applied Biosystems). The $2^{-\Delta\Delta CT}$ method with normalization to GAPDH and untreated controls was used for calculation of results. Primers for mouse G-CSF (#QT00105140) were purchased from Qiagen. Primer sequences for mouse GAPDH were: (fo) 5′-TACCCCCAATGTGTCCGTCGTG-3′, (re) 5′-CCTTCAGTGGGCCCTCAGATGC-3′.

Reagents

The following reagents were used for the studies: Recombinant mouse C5a (R&D Systems and Hycult); LPS (*Escherichia coli*, 0111:B4, Sigma-Aldrich); recombinant mouse $C5a_{desArg}$ (Hycult); LY294002 (InvivoGen); PD98059 (InvivoGen); and U0126 (InvivoGen). We used a neutralizing anti-C5a antiserum as described before [14].

Statistical analysis

The GraphPad Prism Version 5.04 software was used for statistical analysis. All values are expressed as mean and error bars represent SEM. Data sets were analyzed by two-tailed Student's *t*-test across independent experiments. In vitro experiments were performed independently at least three times and in vivo experiments were done with numbers of mice as indicated in the figure legends. Differences were considered significant when $p < 0.05$.

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Abbreviations: **C5a**: complement activation fragment C5a · **C5a$_{desArg}$**: C5a lacking the C-terminal arginine · **CLP**: cecal ligation and puncture · **PEM**: peritoneal elicited macrophage · **PMN**: polymorphonuclear leukocyte

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