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Complement Activation Product C5a Is a Selective Suppressor of TLR4-Induced, but Not TLR3-Induced, Production of IL-27(p28) from Macrophages

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There is accumulating evidence that the complement activation product, C5a, can orchestrate cellular immune functions. IL-27 (p28/EBI3) is an emerging key player essential for regulating inflammatory responses and T cells. In this article, we report that C5a robustly suppressed IL-27(p28) gene expression and release in peritoneal macrophages. These cells from C57BL/6J mice abundantly produced IL-27(p28) after engagement of either the TLR3 (polyinosinic-polycytidylic acid) or TLR4 (LPS) receptor. Genetic deficiency of either TLR4 or LBP completely incapacitated the ability of macrophages to secrete IL-27(p28) in response to LPS. IL-27(p28)-producing macrophages also expressed the C5aR receptor, thus displaying an IL-27(p28)+F4/80+C5aR+ phenotype. C5a suppressed IL-27(p28) in LPS-stimulated macrophages via interactions with the C5aR receptor rather than the C5L2 receptor. After endotoxemia, C5aR−/− mice displayed higher plasma levels of IL-27(p28) compared with C57BL/6J mice. C5a did not affect the release of IL-27(p28) or the frequency of IL-27(p28)/F4/80+ macrophages after engagement of TLR3. Mechanistically, LPS activated both the NF-κB and the PI3K/Akt pathways, whereas C5a activated only the PI3K/Akt pathway. Engagement of PI3K/Akt was inhibitory for IL-27(p28) production, because PI3K/Akt pharmacologic blockade resulted in increased amounts of IL-27(p28) and reversed the suppressive effects of C5a. Blockade of PI3K/Akt in endotoxemic C57BL/6J mice resulted in higher generation of IL-27(p28). In contrast, the PI3K/Akt pathway was not involved in TLR3-mediated release of IL-27(p28). These data provide new evidence about how complement activation may selectively interfere with production of T cell regulatory cytokines by APCs in the varying contexts of either bacterial (TLR4 pathway) or viral (TLR3 pathway) infection. The Journal of Immunology, 2012, 188: 000–000.
elitis model (35, 36). One hallmark of IL-27 to constrain adaptive immune responses is the induction of IL-10 from T cells (e.g., involving STAT3- and STAT1-dependent mechanisms) (37, 38). In contrast, IL-27 can promote inflammation under at least some experimental circumstances, such as inflammatory bowel disease and autoimmune kidney disease (39, 40). Although defining the biological functions of IL-27 is an ongoing process, it seems that IL-27 may emerge as a novel key player of immunity (41).

In this article, we describe the negative regulation of IL-27(p28) production by the complement component C5a via the C5aR receptor (but not the CSLR2 receptor) in macrophages. Interestingly, such suppression occurred with preference to the nature of IL-27 (p28) transcriptional activation and release. Although C5a substantially inhibited IL-27(p28) after TLR4 activation; there were no such effects seen when IL-27(p28) was induced through TLR3, indicating a degree of specificity for the regulatory effects of C5a. These data provide further evidence about how C5a may selectively influence immune functions by regulating the cytokine milieu in the varying context of either bacterial or viral infection.

Materials and Methods

Animals

All procedures were performed in accordance with the National Institutes of Health guidelines and the University Committee on Use and Care of Animals, University of Michigan. All mice were housed under specific pathogen-free conditions. Ten- to twelve-week-old, male C57BL/6J mice and C5Rα−/− mice (B6.10ScN-Tlr6−/− mice) were purchased from The Jackson Laboratory (Bar Harbor, ME). C5AR−/− mice, C5L2−/− mice, and LBP−/− mice were bred and genotyped at the facilities of the University of Michigan.

Peritoneal-elicited macrophages

Mice were injected i.p. with 1.5 ml thiglycollate 2.4% (w/v); 4 d later, the peritoneal cavity was lavaged with 8 ml sterile HBSS (without calcium and magnesium). After centrifugation (2000 rpm, 5 min, 4°C), the cells were counted, transferred to polystyrene plates (2 × 106 cells/ml), and incubated at 37°C, 5% CO2. The purity of cell preparations was evaluated by flow cytometry (>85% F4/80+CD11b+). The media used were RPMI 1640 supplemented with 1% Glutamine, 25 mM HEPES, 0.1% BSA, and 100 U/ml Penicillin/Streptomycin. For detection of intracellular IL-27(p28), cells were incubated in the presence of monensin (2 μM) for >12 h. FcRs were blocked with anti-CD16/32 (Fc Block; BD Biosciences). Cells were stained for surface markers before fixation and permeabilization (Cytofix/Cytoperm Plus Kit; BD Biosciences). For detection of phosphorylated Akt, the cells were immediately fixed and permeabilized with the harsh alcohol method (Perm Buffer III; BD Biosciences) before Ab staining. Abs (anti-mouse) were used, together with matched fluorochrome-labeled isotype controls: PE IL-27(p28) (clone MM27-7B1; eBioscience), allophycocyanin F4/80 and PE C5AR (clone 20/70; BioLegend), and PE Act (Thr−89/92) (clone J1-223.371; BD Biosciences). A minimum of 50,000 events was acquired on a BD LSR II flow cytometer with a high-throughput sampler (BD Biosciences) and further analyzed with WinList for Win32 3.0 Software (VeVerity Software) or FlowJo 7.6.4 software. Dot plots in the figures show ≥10,000 events.

Reagents

The following reagents were used: TLR2 (zymosan from Saccharomyces cerevisiae), TLR3 [poly (I:C), high m.w.], TLR5 (flagellin from Bacillus subtilis), TLR9 (type A CpG oligonucleotide ODN1518 and ODN 1585 control), wortmannin, and Bay11-7082 (all from InvivoGen); LPS (Escherichia coli, 0111:B4), monensin, and FMLP (all from Sigma-Aldrich); recombinant mouse C5a (<1.0 EU endotoxin/1 μg; R&D Systems); and recombinant mouse C5aΔkost (Hyclute Biotech).

Statistical analysis

We used GraphPad Prism version 5.04 software for statistical analysis. All values are expressed as mean, and error bars represent SEM. Data sets were analyzed by the two-tailed Student t test, and survival curves were analyzed by the log-rank (Mantel–Cox) test. We considered differences significant when p < 0.05.

Results

Release of IL-27(p28) from macrophages after TLR3 and TLR4 activation

Thiglycollate-elicited peritoneal macrophages (PEM) from C57BL/6J (wild-type [WT]) mice were incubated for 10 h with agonists for several TLRs (Fig. 1A). After activation of TLR3 by poly (I:C) or TLR4 by LPS, IL-27(p28) became abundantly detectable. In contrast, activation of TLR2, TLR5, and TLR9 pathways did not induce IL-27(p28) under the conditions used, despite an earlier report that activation of the TLR9 pathway can induce low levels of mRNA for IL-27(p28) in dendritic cells (43). All TLR agonists were fully biologically active, as indicated by induction of IL-6 and/or KC (Supplemental Fig. 1A, 1B). When macrophages were isolated from TLR4−/− or LBP−/− mice, cells from both mouse strains, as expected, completely failed to secrete IL-27(p28) after LPS treatment (Fig 1B). Dose responses of macrophages incubated with either LPS (10 μg/ml–10 ng/ml) or poly (I:C) (10 μg/ml-0.5 μg/ml) are shown in Fig. 1C and 1D, respectively. Time-course studies indicated the abundant presence of IL-27(p28) with incubation periods >4 h (Fig. 1E, 1F). Overall, these data are consistent with previous reports on the production of heterodimeric IL-27 from APCs after LPS or poly (I:C) treatment (30–33).

IL-27(p28) production in vitro is suppressed by C5a

To evaluate the effects of C5a on IL-27(p28), PEM from WT mice were incubated with LPS alone or in combination with increasing
concentrations of recombinant mouse C5a. As shown in Fig. 2A, the copresence of C5a in concentrations between 10 and 100 nM suppressed the release of IL-27(p28) from LPS-activated macrophages. This C5a-related inhibition of secreted IL-27(p28) was observed at all of the time points studied (Fig. 2B) and was accompanied by C5a-dependent profound suppression of mRNA for IL-27(p28) (Fig. 2C). The natural degradation product of C5a, C5adesArg (100 nM), also suppressed IL-27(p28) when added to cultures of LPS-activated PEM (Fig. 2D). In contrast, other chemotactic peptides for phagocytic cells, such as bacterial FMLP, did not appear to negatively regulate macrophage-derived IL-27 production when FMLP was used alone or in combination with LPS (Fig. 2E).

Suppression of IL-27(p28) by C5a is mediated via the C5aR receptor rather than the C5L2 receptor

Resting peritoneal macrophages from WT mice abundantly expressed the receptor C5aR (CD88), as evaluated by flow cytometry (Fig. 3A, left panel). On macrophages from C5aR<sup>−/−</sup> mice, there was virtually no detectable C5aR (Fig. 3A, right panel), thereby confirming both the phenotype of the C5aR<sup>−/−</sup> mice and specificity of the anti-mouse C5aR Ab. Next, PEM from WT mice were left as untreated controls or stimulated with LPS, both in the presence of the protein transport inhibitor monensin. After 12 h, cells were analyzed by flow cytometry for intracellular IL-27(p28) and surface C5aR, with dot-plots gated on F4/80<sup>+</sup> macrophages (Fig. 3B). In preparations of resting cells, the frequency of IL-27(p28)+C5aR+F4/80<sup>+</sup> cells was 2.5%. After LPS treatment, intracellular IL-27(p28) became abundantly detectable, as indicated by the presence of 43.1% IL-27(p28)+C5aR+F4/80<sup>+</sup> cells. All macrophages that produced IL-27(p28) also expressed C5aR, indicating that IL-27(p28)-producing cells respond to C5a via this receptor. Staining results with isotype controls for anti-C5aR and anti–IL-27(p28) are shown in Supplemental Fig. 1C–E. There was also some evidence that macrophages can express the C5L2 receptor, as detected by RT-PCR (data not shown). To further investigate the relative contribution of C5aR and C5L2 in regulating IL-27(p28) production, peritoneal macrophages from WT mice, C5aR<sup>−/−</sup> mice, and C5L2<sup>−/−</sup> mice were isolated and incubated with LPS alone or in combination with either 10 or 100 nM C5a (Fig. 3C). In PEM from C5L2<sup>−/−</sup> mice, C5a was fully active to
suppress IL-27(p28) production. However, in PEM with a deficiency of C5aR, we observed a nearly complete loss of the ability of C5a to inhibit IL-27(p28). Analysis of data from several independent experiments showed that LPS-induced production of IL-27(p28) was reduced by C5a to 40% in WT macrophages and 30% in C5L2−/− macrophages, whereas production remained at 83% in C5aR−/− macrophages (Fig. 3D). These data suggest that C5aR is the predominant receptor facilitating the ability of C5a to suppress IL-27(p28) release in LPS-stimulated PEM.

C5a inhibits TLR4-dependent, but not TLR3-dependent, production of IL-27(p28)

PEM (WT) were stimulated with LPS or poly (I:C) both in the absence or copresence of recombinant mouse C5a. Surprisingly, C5a did not seem to suppress IL-27(p28) production when IL-27(p28) was induced via the TLR3 pathway (Fig. 4A). Similar results were observed by flow cytometry studies (Fig. 4B). In resting macrophages, the frequency of IL-27(p28)+F4/80+ cells was 3.1%, with regions set according to staining with isotype controls (data not shown). C5a inhibited TLR4-dependent, but not TLR3-dependent, production of IL-27(p28) by macrophages (PEM), as assessed by ELISA, after activation by either LPS or poly (I:C) for 20 h. Levels of IL-27(p28) were set to 100% for LPS and poly (I:C) alone. (B) Flow cytometry analysis of intracellular IL-27 (p28) and surface F4/80 in PEM after 12 h of incubation with either LPS or poly (I:C). Data are representative of at least three independent experiments. Error bars represent SEM. *p < 0.05.

C5a inhibits TLR4-dependent, but not TLR3-dependent, production of IL-27(p28) in vivo

PEM (WT) were stimulated with LPS or poly (I:C) both in the absence or copresence of recombinant mouse C5a. Surprisingly, C5a did not seem to suppress IL-27(p28) production when IL-27(p28) was induced via the TLR3 pathway (Fig. 4A). Similar results were observed by flow cytometry studies (Fig. 4B). In resting macrophages, the frequency of IL-27(p28)+F4/80+ cells was 3.1%, with regions set according to staining with isotype controls (data not shown). C5a inhibits TLR4-dependent, but not TLR3-dependent, production of IL-27(p28) in vivo. (A) Survival curves of C57BL/6J (WT) mice (n = 8–12/group) after i.p. injection with different doses of LPS from E. coli (O111:B4). (B) RT-PCR of IL-27(p28) gene expression in spleen homogenates from WT mice 6 h after i.p. administration of either LPS (10 mg/kg BW, n = 6) or poly (I:C) (10 mg/kg BW, n = 5). Sham mice (n = 3) received an i.p. injection of PBS. (C) Levels of circulating plasma IL-27(p28), as detected by ELISA, after 6 h from the same experiment as in (B). Error bars represent SEM. *p < 0.05.

Absence of the C5aR receptor correlates with increased IL-27(p28) levels in vivo

C57BL/6J (n = 6), C5aR−/− (n = 5), and C5L2−/− (n = 6) mice were injected i.p. with LPS (10 mg/kg BW). Plasma was collected after 12 h, and IL-27(p28) was detected by ELISA. Error bars represent SEM. *p < 0.05. n.s., Not significant compared with C57BL/6J.
not shown). After stimulation with LPS, 38.1% of PEM were IL-27(p28)+F4/80+ cells. This number was reduced to 7.3% with the combination of LPS plus C5a (100 nM). With poly (I:C) alone, the frequency of IL-27(p28)+F4/80+ macrophages increased to 41.5%; this number was affected little when C5a was present together with poly (I:C) (37.2%; Fig. 4B, lower right panel).

Evidence for complement dependency of IL-27(p28) release in vivo

Fig. 5A shows survival curves of male C57BL/6J (WT) mice (12 wk old) after challenge by i.p. injection with different doses of LPS (E. coli, 0111:B4). An LPS dose of 10 mg/kg BW resulted in a survival rate of 17%, and this dose was used for further experiments. As expected, IL-27(p28) mRNA was detectable in spleen homogenates 6 h after injection of mice with LPS or poly (I:C) (Fig. 5B). In plasma samples from the same mice, IL-27 (p28) was present in higher concentrations after injection of LPS compared with poly (I:C) (Fig. 5C). The upregulation of IL-27(p28) after systemic administration of LPS or poly (I:C) in mice was reported by other investigators (44). Given the results indicating that C5a, via C5aR, sharply and specifically reduced IL-27(p28) levels after TLR4 activation, we determined whether the absence of the C5a receptors affected IL-27(p28) levels after endotoxemia. In mice with genetic deficiency of C5aR, plasma levels of IL-27(p28) were significantly increased by an average of 39% compared with responses of C57BL/6J (WT) mice (Fig. 6).

Collectively, the absence of C5aR leading to higher levels of IL-27(p28) in endotoxemic mice fits with the data that C5a interacting with C5aR both in vitro and in vivo results in reduced production of IL-27(p28).
C5a mediates suppression of IL-27(p28) production via activation of the PI3K/Akt pathway

IL-27(p28) gene expression in LPS-stimulated macrophages was reported to involve the NF-κB pathway (31). Indeed, when macrophages were incubated with Bay11-7082 (which prevents proteolysis of IkB and, thereby, NF-κB activation) before the addition of LPS, considerably lower amounts of IL-27(p28) were produced in a dose-dependent manner (Fig. 7A). To determine whether C5a regulates IL-27(p28) production by preventing full NF-κB activation, nuclear extracts from LPS- or LPS plus C5a-treated macrophages were evaluated for DNA-binding activity of NF-κB for its consensus sequence (5′-GGGACTTTCC-3′, Fig. 7B). No evidence was found that C5a blocks (or significantly increases) NF-κB in macrophages from WT mice. In contrast, we recently characterized the synergistic recruitment of the PI3K/Akt pathway by C5a and LPS in such cells (45, 46). In this study, we used a novel bead-based assay with Abs specific for Akt phosphorylation at amino acid residue Thr308 to confirm these effects (Fig. 3C). After poly (I:C) treatment, no phosphorylation of Akt was observed using this assay (data not shown). The selective activation of the PI3K/Akt pathway by LPS, but not by poly (I:C), was also studied by flow cytometry with an Ab specific for Akt phosphorylation at Thr308 (Fig. 7D).

Wortmannin is a specific pharmacologic small molecule inhibitor of the PI3K/Akt pathway. Treatment of PEM with wortmannin before the addition of LPS resulted in substantially higher levels of mRNA for IL-27(p28) (Fig. 7E). In contrast, Bay11-7082 (see above) reduced levels of IL-27(p28) mRNA compared with LPS alone (Fig. 7E). Wortmannin increased levels of secreted IL-27(p28) >2-fold, in a dose-dependent manner, if added before LPS (Fig. 7F). In contrast, such effects were not observed when wortmannin was used in combination with poly (I:C) (Fig. 7G). This is consistent with the fact that no activation of Akt occurred with poly (I:C) in macrophages (Fig. 7D). When macrophages were treated with the combination of LPS and C5a in the copresence of wortmannin, C5a was unable to suppress IL-27(p28) production (Fig. 7H). Finally, when WT mice were given wortmannin (1 mg/kg BW i.p.) before the induction of endotoxemia, this resulted in much higher levels of IL-27(p28) in plasma (Fig. 7I). In conclusion, LPS activated both NF-κB and PI3K/Akt; however, although NF-κB appeared to be an activating pathway for IL-27(p28) gene expression, PI3K/Akt was a negative regulator. C5a suppressed IL-27(p28) by amplified upregulation of PI3K/Akt activity.

Discussion

The data presented above characterize the negative regulation of IL-27(p28) gene expression by the complement activation product C5a. The effects of C5a were related to its interactions with C5aR rather than with CS1L2. This is consistent with other reports suggesting that, for macrophages, the CS1L2 receptor plays only a minor role when production of mediators is used as the end point (45–47). In contrast, C5a interactions with CS1L2 are essential during other experimental models, such as sepsis or asthma (17, 19). We and other investigators previously described the activation of the PI3K/Akt pathway by C5a via C5aR in several cell types, such as neutrophils, macrophages, and lymphocytes (21, 45, 46, 48, 49). As shown above, pharmacologic blockade of Akt phosphorylation resulted in enhanced production of IL-27(p28) in LPS-stimulated macrophages. Interestingly, at the same time, LPS appeared to activate both stimulatory- and inhibitory-signalizing pathways for IL-27(p28) transcription (Fig. 8). The net result was abundant release of IL-27(p28). However, when the PI3K/Akt pathway was blocked, hyperproduction of IL-27(p28) occurred both in vitro and in vivo because of the loss of this inhibitory pathway. C5a via C5aR shifts the balance of positive and negative regulatory pathways toward PI3K/Akt without affecting NF-κB activation, thereby antagonizing IL-27(p28) release (Fig. 8). This also explains the specificity of C5a for selective inhibition of IL-27(p28) only after activation of TLR4. TLR4 ligation with the LBP/LPS complex resulted in phosphorylation of Akt, whereas TLR3 activation did not. Blockade of PI3K/Akt did not cause increased IL-27(p28) levels after poly (I:C) treatment. Although C5a may still induce phosphorylation of Akt in the presence of poly (I:C), this may not be sufficient to exceed a necessary activation threshold for this pathway.

There is growing evidence that C5a regulates mediator production from innate immune cells. For example, C5a profoundly suppressed LPS-induced production of IL-12 from human monocytes and murine macrophages (47, 50). For the release of TNF-α, the effects of C5a appear to be cell-type specific, depending on whether neutrophils or macrophages are studied (51). Two earlier reports mentioned regulation of mRNA for IL-27(p28) by C5a in human monocytes and murine macrophages, which is consistent with our findings (47, 52). Furthermore, we recently reported that C5a modulates MyD88-dependent levels of the isoforms IL-17A and IL-17F (45, 46). In addition, C5a can act as an anti-inflammatory mediator by augmentation of early (3–12 h) release of IL-10 from macrophages (46). C5a-dependent effects on immune cell functions may be beneficial for pathogen clearance or in the context of sepsis associated with inflammatory dysregulation and poor outcome (51, 53). In general, the complement system can be viewed as an essential arm of immune defense providing surveillance of the extracellular compartments against bacteria and parasites rather than intracellular viruses. It seems plausible to expect that C5a can interfere with the IL-27 system after sensing of extracellular bacteria (TLR4) but not intracellular viruses (TLR3). Generation of C5a may signal ongoing bacterial infection to macrophages and prevent abundant release of IL-27, because IL-27 helps to terminate and curtail inflammation via IL-10 induction from T cells in the chronic state of inflammation (37, 38). Of note, rIL-27 did not induce early IL-10 production in cultures of macrophages within 24 h (data not shown), consistent with the idea that IL-27 mainly acts on lymphocytes. The termination of chronic inflammation is only desirable after pathogen clearance is achieved, as would be indicated by decreasing complement activation, resulting in decreasing levels of C5a. The short in vivo half-life of C5a compared with other molecules (e.g., LPS) may be
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