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J Immunol 2012; 188:5086-5093; Prepublished online 9 April 2012; doi: 10.4049/jimmunol.1102914
http://www.jimmunol.org/content/188/10/5086

Supplementary Material  http://www.jimmunol.org/content/suppl/2012/04/09/jimmunol.1102914.DC1

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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

Markus Bosmann,* Mikel D. Haggadone,* Mark R. Hemmila,† Firas S. Zetoune,* J. Vidya Sarma,* and Peter A. Ward*

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 (polynosinic-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: 5086–5093.
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 C5L2 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 TLR4−/− mice (B6.10ScN-Tlr4−/−JthJ) were purchased from The Jackson Laboratory (Bar Harbor, ME). C5aR (clone 20/70; BioLegend), and PE Akt (Thr308) (clone J1-223.371; eFluor 450 F4/80 (clone BM8; eBioscience), allophycocyanin C5aR and PE IL-27(p28) (clone MM27-7B1; eBioscience), allophycocyanin C5aR and PE C5aR (clone 20/70; BioLegend), and PE Akt (Thr308) (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 (Verity 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 ODN1826 and ODN 1585 control), wortmannin, and Bay 11-7082 (all from InvivoGen); LPS (Escherichia coli, 0111:B4), monensin, and FMLP (all from Sigma-Aldrich); recombinant mouse C5a (<1.0 EU endotoxin/l μg; R&D Systems); and recombinant mouse C5a△(8-11) (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(p28) production when FMLP was used alone or in combination with LPS (Fig. 2E).

**FIGURE 1.** Characterization of IL-27(p28) release from macrophages after activation of TLR3 or TLR4. (A) PEM from C57BL/6J (WT) mice were incubated with agonists for various TLR-agonists (all 1 μg/ml), and secreted IL-27(p28) was detected by ELISA after 10 h. (B) Macrophages from WT mice, LBP−/− mice, and TLR4−/− mice were incubated with LPS (50 ng/ml) for 10 h before detection of IL-27(p28). (C) Dose-response studies of IL-27 (p28) release by PEM after TLR4 activation by LPS (10 μg/ml–10 ng/ml) for 10 h. (D) Dose-response curve of IL-27(p28) production after TLR3 activation by poly (I:C) (10 μg/ml–50 ng/ml) for 10 h. (E) Time course of IL-27 (p28) release after LPS (1 μg/ml). (F) Time course of IL-27(p28) production after treatment with poly (I:C) (10 μg/ml). All experiments shown were done with PEM from C57BL/6J (WT) mice. Data are representative of at least three independent experiments. Error bars represent SEM. *p < 0.05.

**FIGURE 2.** C5a mediates inhibition of IL-27(p28) from TLR4-activated macrophages. (A) ELISA of IL-27(p28) levels in supernatants from macrophages after incubation with LPS alone, the combination of LPS and different concentrations of recombinant mouse C5a (100–10 nM) or C5a alone (100 nM) for 10 h. (B) Suppression of IL-27(p28) production from LPS-activated macrophages by coincubation with C5a (100 nM) at different time points. (C) RT-PCR of mRNA levels for IL-27(p28) in macrophages after treatment with LPS alone or in combination with C5a (100 nM). (D) Effects of recombinant mouse C5a desArg (100 nM) on IL-27(p28) secretion by LPS-activated macrophages for 10 h. (E) IL-27(p28) release from macrophages after costimulation with LPS and FMLP (4 μM) compared with LPS alone. All experiments shown were done with PEM from C57BL/6J (WT) mice, and LPS was used at 1 μg/ml. Data are representative of at least three independent experiments. Error bars represent SEM. *p < 0.05.

*Figures 3A, left panel.* On macrophages from C5aR−/− mice, there was virtually no detectable C5aR (Fig. 3A, right panel), thereby confirming both the phenotype of the C5aR−/− 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 + macrophages (Fig. 3B). In preparations of resting cells, the frequency of IL-27(p28)+C5aR+F4/80+ 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+ 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−/− mice, and C5L2−/− mice were isolated and incubated with LPS alone or in combination with either 10 or 100 nM C5a (Fig. 3C). In PEM from C5L2−/− mice, C5a was fully active to

Resting peritoneal macrophages from WT mice abundantly expressed the receptor C5aR (CD88), as evaluated by flow cytometry

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

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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 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).
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).

FIGURE 7. C5a suppression of LPS-induced IL-27(p28) is related to differential roles of the NF-κB– and PI3K/Akt–signaling pathways. (A) Effects of different doses of the IκB inhibitor Bay11-7082 on IL-27(p28) production from WT macrophages after LPS treatment for 6 h, as assessed by ELISA. (B) WT macrophages were stimulated for 2 h with LPS alone or the combination of LPS and C5a (100 nM) or were left as untreated controls (Ctrl). Nuclear lysates were prepared and analyzed for NF-κB activity for binding of its DNA consensus sequence (5′-GGGACTTTCC-3′) by immunodetection. (C) Bead-based assay for phospho-Akt (Thr 308). WT macrophages were stimulated for 30 min with LPS and C5a (100 nM) alone or in combination or were left as unstimulated controls. (D) Macrophages were stimulated with either LPS or poly (I:C) or were left as unstimulated controls. Cells were fixed after 60 min, and phosphorylation of Akt (Thr308) was analyzed by flow cytometry. Data in histograms were gated to only show F4/80+ cells. (E) RT-PCR of mRNA levels for IL-27(p28) from macrophages treated with LPS alone or in combination with wortmannin (1 μM) or Bay11-7082 (10 μM) for 6 h. (F) Effects of specific inhibition of the PI3K/Akt pathway by wortmannin (1 μM) on production of IL-27(p28) from macrophages treated with LPS or poly (I:C) for 20 h. (H) PI3K/Akt blockade by wortmannin reverses C5a-mediated suppression of IL-27(p28) release from macrophages after LPS treatment for 8 h, as assessed by ELISA. (I) Plasma levels of IL-27(p28) in C57BL/6J WT mice 8 h after endotoxemia. Mice were injected i.p. with wortmannin (1 mg/kg BW, n = 4) or vehicle (n = 5) 1 h before i.p. LPS treatment (10 mg/kg BW). All data shown, with the exception of those in (I), were collected from PEM from C57BL/6J (WT) mice. Wortmannin and Bay11-7082 were added 1 h before LPS (1 μg/ml), poly (I:C) (1 μg/ml), or C5a (100 nM). Data are representative of three independent experiments or were collected during experiments performed with the numbers of mice indicated. Error bars represent SEM. *p < 0.05.
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 IκB 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 C5L2. This is consistent with other reports suggesting that, for macrophages, the C5L2 receptor plays only a minor role when production of mediators is used as the end point (45–47). In contrast, C5a interactions with C5L2 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-signal-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 absence 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 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

**FIGURE 8.** IL-27(p28) is suppressed by C5a. The proposed simplified scheme summarizes the interactions of C5a and IL-27(p28) in macrophages, highlighting the differential roles of the intracellular signaling pathways involved.
a key factor in this simplified hypothesis. However, the story may actually be more complex. It has been known for decades that complement consumption also occurs during some viral diseases, with the classic example being hemorrhagic dengue fever (54). In principle, any antiviral immune response resulting in production of Abs binding to extracellular viral particles or viral proteins expressed on host cells can trigger the classical pathway of complement activation. However, this may not always be sufficient to ensure complement-dependent virolysis, in part as the result of viral immune-evasive strategies (55).

In summary, the T cell regulatory cytokine IL-27 appears to be crucially involved during several viral, bacterial, and protozoan infections. Our data provide a paradigm of how innate humoral factors, such as C5a, may selectively influence subsequent adaptive immune cell functions.

Acknowledgments

We thank Norman F. Russkamp, Florence Pacheco, Fabien Meta, Vinay R. Patel, and Rachel Voigt for technical assistance and Ron Craig for advice regarding the flow cytometry studies. We cordially thank Beverly Schuermann, Sue Scott, and Robin Kunkel for assistance in the preparation of the manuscript.

Disclosures

The authors have no financial conflicts of interest.

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