MyD88-dependent production of IL-17F is modulated by the anaphylatoxin C5a *via* the Akt signaling pathway

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ABSTRACT **The interleukin-17 (IL-17) family of cytokines plays important roles in innate immune defenses against bacterial and fungal pathogens. While much is known about IL-17A, much less information is available about the IL-17F isoform. Here, we investigated gene expression and release of IL-17F and its regulation by the complement system. IL-17F was produced in mouse peritoneal elicited macrophages after TLR4 activation by LPS, peaking after 12 h. This effect was completely dependent on the presence of the adaptor protein MyD88. The copresence of the complement activation product, C5a (EC_{50}=10 nM), amplified IL-17F production *via* the receptor C5aR. *In vitro* signaling studies indicated that LPS or C5a, or the combination, caused phosphorylation of Akt occurring at threonine 308 but not at serine 473. Treatment of macrophages with pharmacologic inhibitors of PI3K-Akt greatly reduced production of IL-17F as well as mRNA for IL-17F. In endotoxemia, C5a levels peaked at 6 h, while IL-17F levels peaked between 6–12 h. Full *in vivo* production of IL-17F during endotoxemia required C5a. A similar result was found in the cecal ligation and puncture sepsis model. These data suggest that maximal production of IL-17F requires complement activation and presence of C5a.—Bosmann, M., Patel, V. R., Russkamp, N. F., Pache, F., Zetoune, F. S., Sarma, J. V., Ward, P. A. MyD88-dependent production of IL-17F is modulated by the anaphylatoxin C5a *via* the Akt signaling pathway. *FASEB J.* 25, 4222–4232 (2011). www.fasebj.org**

Key Words: macrophages · endotoxemia · endotoxic shock · cecal ligation and puncture · polymicrobial sepsis

THE COMPLEMENT SYSTEM evolved early during the development of life. It can be found in all vertebrates and all major invertebrate deuterostome groups but is not found in protostome genomes such as *Caenorhabditis elegans* or *Drosophila melanogaster* (1). The function of this arm of innate immune defenses is not only to provide rapid clearance of extracellular pathogens but also to provide immune surveillance and homeostasis (2, 3). Activation of the classic, alternative, and MB-lectin pathways all lead to formation of the C3-convertase followed by the C5-convertase. The terminal complement components C5–C9 interact to form the membrane-attack complex (C5b-9), while C3b opsonizes pathogens directly to facilitate phagocytosis. The cleavage products C3a and the more potent C5a act as powerful anaphylatoxins to recruit phagocytic cells to the site of complement activation and further promote inflammation (4). C5a is rapidly converted to $C5a_{desArg}$ by the serum enzyme carboxypeptidase N, resulting in a half-life for C5a in the circulation of <5 min (5). C5a and the less active $C5a_{desArg}$ both bind with high affinity to the two receptors C5aR (CD88) and C5L2 (GPR77), which are expressed abundantly on myeloid and to a lesser extent on nonmyeloid cells (6). The C5aR receptor is a traditional G-protein-coupled receptor that, when ligated to C5a, results in responses from neutrophils and macrophages that leads to rapid Ca^{2+} currents, activation of MAPK signaling, chemotaxis, respiratory burst, and release of toxic granules (7, 8). C5a and the C5a receptors are critically involved in the pathogenesis and outcome of acute inflammatory diseases, namely acute lung injury/ARDS and sepsis (9–11). Antibody blockade of C5a is protective in experimental sepsis (12). Similarly, blockade or genetic absence of C5aR or C5L2 improve survival during polymicrobial sepsis induced by cecal ligation and puncture (CLP; ref. 13). Furthermore, C5a directly acts on cardiomyocytes to induce mediator release and is involved in the development of cardiac dysfunction during sepsis (14, 15).

The interleukin-17 (IL-17) family of cytokines includes IL-17A-E and IL-17F, of which the founding member, IL-17A, is the best described isoform (16, 17). IL-17F and IL-17A share 50–55% sequence homology, with their genes located close together on chromosome 1 (mouse) and chromosome 6 (human). Both isoforms can exist in disulfide-linked homo- or heterodimers (18). IL-17F binds to the IL-17RA/IL-17RC receptors

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(19). The cellular sources of IL-17F are not only $CD4^+$ Th17 cells but a wide range of cells, including $CD8^+$ T cells, NKT cells, and LTi-like cells (16, 20). Even colon epithelial cells have been reported to express mRNA for IL-17F (21). Target cells of IL-17F are primarily nonmyeloid cells, such as fibroblast, keratinocytes, synoviocytes, and endothelial cells. From these cells, IL-17F induces mediator production of TNF-α, IL-1, IL-6, G-CSF, and CXC chemokines (16, 22, 23). In addition, IL-17F enhances gene expression of antimicrobial peptides (24). Taken together, these effects amplify protective immune responses against microbial infections. Recent studies have suggested that IL-17F is required for clearance of *Klebsiella pneumonia* infection of the lung and *Staphylococcus aureus* from the mucoepithelium (21, 25). In humans, genetic defects for IL-17F or IL-17RA have been associated with chronic mucocutaneous candidiasis disease, further underscoring the role of IL-17F in mucosal immune defense (26). On the other hand, IL-17 family members have also been implicated in the pathogenesis of autoimmune disease. We have recently reported that IL-17A is detrimental during experimental sepsis (27). Here, we report on the regulation of the expression of IL-17F by the complement component, C5a, and the signaling mechanisms involved.

MATERIALS AND METHODS

Animals

All procedures were performed 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. Male mice of the strains C57BL/6J and $MyD88^{-/-}$ (B6.129P2(SJL)-*Myd88*$^{tm1.1Defr}$/J) were purchased from the Jackson Laboratories (Bar Harbor, ME, USA) at an age of 8–10 wk. Mice of the strain $C5aR^{-/-}$ (on a C57BL/6J background) were bred and genotyped at the University of Michigan. All animals were housed under specific pathogen-free conditions.

Isolation and incubation of macrophages

Mice were injected i.p. with 1.5 ml thioglycollate (2.4% w/v; Becton Dickinson, Franklin Lakes, NJ, USA), and peritoneal elicited macrophages (PEMs) were harvested by peritoneal lavage with HBSS 4 d later. After centrifugation (650 *g*, 5 min, 4°C), cells were resuspended in medium [RPMI 1640 with 25 mM HEPES (Life Technologies, Inc., Carlsbad, CA, USA), 100 U/ml penicillin-streptomycin (Life Technologies), and 0.1% BSA (Sigma-Aldrich, St. Louis, MO, USA)]. Purity of macrophage preparations was confirmed by flow cytometry; typically, 85–90% of cells stained positive for F4/80. Cells were plated at a density of 2×10^6 cells/ml in polystyrene culture plates and incubated at 37°C, 5% CO_2. At the end of all experiments, supernatants were cleared of nonadherent cells by centrifugation and stored at −80°C until further analysis.

In vivo experiments

Endotoxemia

Mice were injected i.p. with LPS (*Escherichia coli* 0111:B4, Sigma-Aldrich) in a dose of 10 mg/kg body weight. Body weight of each individual animal was measured directly before injection. Blood was collected from the retro-orbital sinus under deep anesthesia using EDTA (5–10 mM) as an anticoagulant. Plasma was separated by centrifugation (2000 *g*, 10 min, 4°C) and stored at −80°C until further analysis.

Cecal ligation and puncture (CLP)

The CLP procedure was performed as described previously, with the surgeon blinded to the studied groups of mice (28, 29). Briefly, mice were anesthetized by i.p. injection of ketamine (100 mg/kg body weight; Fort Dodge Animal Health, Fort Dodge, IA, USA) and xylazine (8 mg/kg body weight, Akorn Inc., Lake Forest, IL, USA). After a small midline abdominal incision (1 cm), the peritoneum was opened, and the cecum was exteriorized and ligated with 4-0 braided silk (Henry Schein Inc., Melville, NY, USA) directly below the ileocecal valve. The cecum was punctured through and through with an 18-gauge needle, and a small amount of feces was extruded to assure patency. After wound closure, the animals received 1 ml 0.9% sterile NaCl s.c. for fluid resuscitation. This form of CLP ("high grade") usually causes severe sepsis and results in lethality of most animals within 4–5 d. At the end of experiments, the plasma was processed as described above, and spleens were snap-frozen in liquid nitrogen and stored at −80°C until further analysis.

Measurement of mediator concentrations by ELISA

For detection of mouse IL-17F, an ELISA kit (R&D Systems, Minneapolis, MN, USA) with no cross-reactivity for the other isoforms of IL-17 (IL-17A–IL-17E) was used. Plasma and supernatants were diluted in PBS with 1% BSA to fit in the range of standards. ELISAs were performed according to the instructions of the manufacturer. The ELISA for mouse C5a was performed under optimized conditions with antibodies and recombinant C5a standard purchased separately from BD Biosciences (San Jose, CA, USA).

Isolation of mRNA and real time PCR

Total RNA from macrophages was obtained by the Trizol method. The cDNA was generated with TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster City, CA, USA) in a GeneAmp PCR System 9700 (Applied Biosystems; 25°C for 10 min, 48°C for 30 min, 95°C for 5 min). Amplification was performed with SYBR Green Mastermix in the 7500 real-time PCR System (Applied Biosystems; 50°C for 2 min; 95°C for 10 min; 40 cycles: 95°C for 10 s, 60°C for 60 s, 72°C for 10 s). Results were analyzed by the $2^{-\Delta\Delta Ct}$ relative quantification method and normalized to GAPDH. Primers for mouse IL-17F (QT00144347) were purchased from Qiagen (Valencia, CA, USA). Forward and reverse primer sequences for mouse GAPDH were5′-TACCCCCAATGTGTCCGTCGTG-3′ and 5′-CCTTCAGTGGGCCCTCAGATGC-3′, respectively (Invivogen, San Diego, CA, USA).

Flow cytometry

For intracellular cytokine staining, cells were incubated with Brefeldin A. Samples were processed using the Cytofix/

Cytoperm Plus Fixation/Permeabilization Kit (BD Biosciences), following the instructions of the manufacturer, and blockade of Fc receptors (BD Fc Block). For flow cytometry of phosphoproteins, cells were fixed with fixation buffer (BD Cytofix; BD Biosciences) and permeabilized with the harsh alcohol method (Perm Buffer III, BD Biosciences). A minimum of 50,000 events was acquired on a BD LSR II flow cytometer (BD Biosciences); dot-plot images depict ≥10,000 events. All antibodies used were anti-mouse together with matched fluorochrome labeled isotype controls. From BD Pharmingen (San Diego, CA, USA): AF488 anti-Akt (pS473; clone M89–61), PE anti-Akt (pT308; clone J1-223.371). From eBioscience (San Diego, CA, USA): APC F4/80 (clone BM8), eFluor450 F4/80 (clone BM8), PE-Cy7 CD11b (clone M1/70), PE Mac-3 (clone M3/84), AF488 IL-17F (clone eBio18F10). From BioLegend (San Diego, CA, USA): PE C5aR (clone 20/70).

Antibodies and reagents

Toll-like receptor (TLR) agonists were obtained from Invivogen: TLR2 (zymosan from *Saccharomyces cerevisiae*), TLR3 [poly(I:C)], TLR5 (flagellin from *Bacillus subtilis*), TLR9 (type A CpG oligonucleotide ODN1518 and ODN 1585 control). LPS was from *E. coli* (0111:B4, Sigma-Aldrich), LY294002 and Wortmannin were from Invivogen. Recombinant mouse C5a was from R&D systems (endotoxin level <1.0 EU/μg protein). Normal goat serum was from Equitech-Bio Inc. (Kerrville, TX, USA).

Neutralizing polyclonal goat anti-C5a antiserum was produced as described before and characterized to effectively block rat and mouse C5a (30, 31). Briefly, the carboxyl-terminal peptide of the rat C5a sequence (CTIADKIRKESH HKGMLLGR; amino acid residues 58–77) was synthesized; the peptide was coupled to keyhole limpet hemocyanin by the glutaraldehyde method and used for the immunization of goats and production of sera.

Statistical analysis

GraphPad Prism 5.03 software (GraphPad, San Diego, CA, USA) was used for figure preparation and statistical analysis. All values are expressed as means ± SE. Data sets were analyzed by 1-way ANOVA or Student's *t* test. We considered differences significant at values of $P < 0.05$. *In vitro* experiments were performed independently at least 2 or 3 times, and representative experiments are shown. For *in vivo* experiments, the numbers of animals per group were as indicated in the figure legends ($n \geq 3$).

RESULTS

Release of IL-17F from PEMs after TLR4-activation by LPS

To study the production and release of IL-17F, we used cell cultures of PEMs from C57BL/6J wild-type mice. Macrophages were incubated with agonists for the different TLRs. After 10 h the cell culture supernatants were analyzed for levels of IL-17F by ELISA (**Fig. 1*A***). In

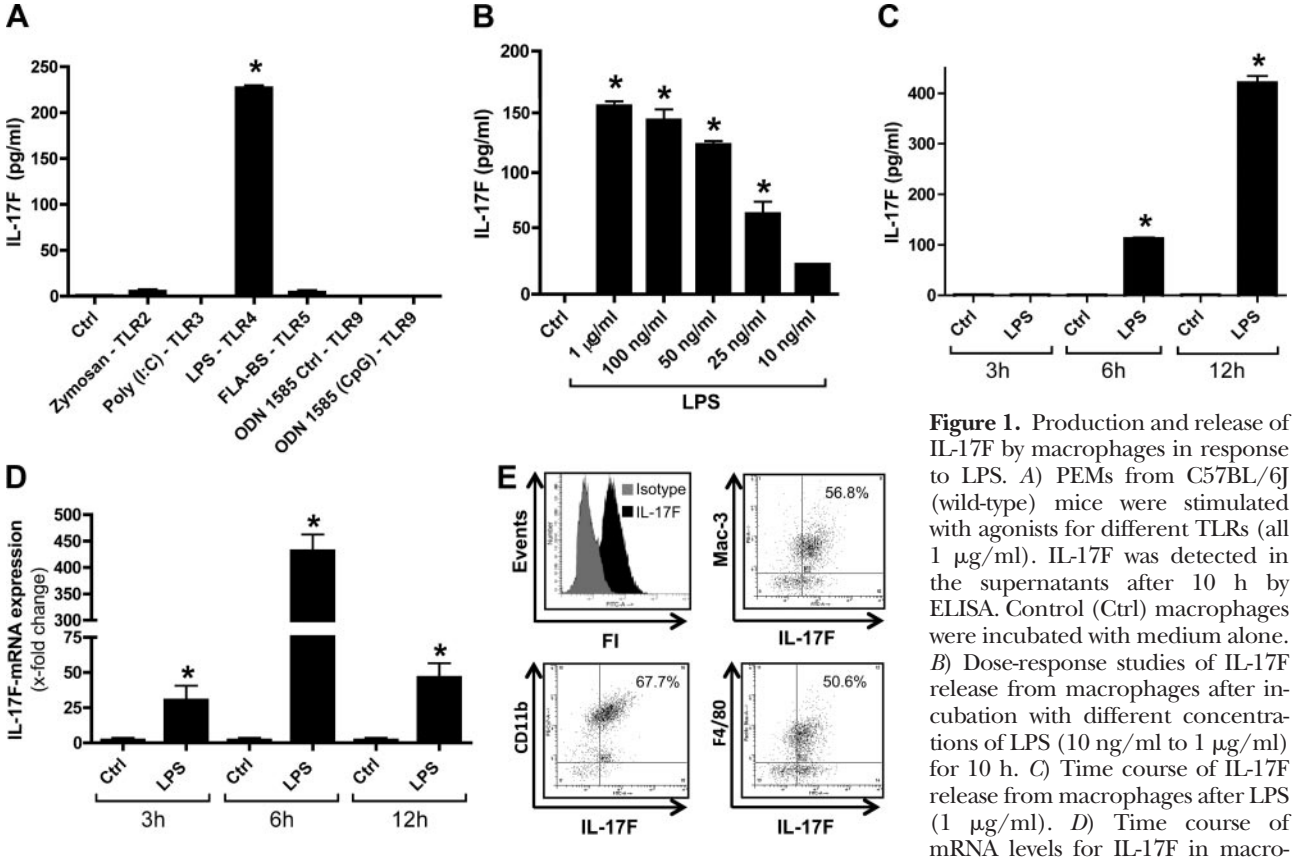

Figure 1. Production and release of IL-17F by macrophages in response to LPS. *A*) PEMs from C57BL/6J (wild-type) mice were stimulated with agonists for different TLRs (all 1 μg/ml). IL-17F was detected in the supernatants after 10 h by ELISA. Control (Ctrl) macrophages were incubated with medium alone. *B*) Dose-response studies of IL-17F release from macrophages after incubation with different concentrations of LPS (10 ng/ml to 1 μg/ml) for 10 h. *C*) Time course of IL-17F release from macrophages after LPS (1 μg/ml). *D*) Time course of mRNA levels for IL-17F in macrophages after LPS (1 μg/ml) as detected by RT-PCR. *E*) Flow cytometry with intracellular staining for IL-17F or isotype control (top left) or costaining for IL-17F and macrophage surface markers (Mac-3, CD11b, and F4/80). Macrophages were stimulated with LPS (1 μg/ml) for 6 h in the presence of Brefeldin A before staining. FI, fluorescence intensity. *$P < 0.05$ *vs.* Ctrl; Student's *t* test.

supernatant fluids from macrophages incubated with medium alone (control), IL-17F was not detectable (detection limit ~20 pg/ml). Macrophages stimulated with LPS (1 μg/ml) for activation of the TLR4 receptor robustly released IL-17F after 10 h. Using agonists for the pattern-recognition-receptors TLR2, TLR3, TLR5, and TLR9, no appearance of IL-17F in supernatants was observed. Next, we performed dose-response studies using different concentrations of LPS and detection of IL-17F levels after 10 h (Fig. 1*B*). LPS induced IL-17F from macrophages with an EC_{50} of 25–50 ng/ml. Concentrations of LPS above 100 ng/ml resulted in robust production of IL-17F. For all further experiments, LPS at a concentration of 1 μg/ml was used to induce maximal IL-17F release, unless otherwise indicated. LPS at 1 μg/ml (*E. coli*, 0111:B4) did not exert cytotoxic effects on PEMs for the incubation period employed in these experiments (data not shown). A time course of IL-17F release after TLR4 activation by LPS is shown in Fig. 1*C*. IL-17F was not detectable at the 3 h time point, but was present after 6 h. Additional release of IL-17F was observed at the 10–12 h time points. Levels did not further increase with longer incubation times, such as 24 h (data not shown). We also analyzed the abundance of mRNA for IL-17F in PEMs in response to LPS (Fig. 1*D*). In untreated macrophages, IL-17F mRNA was barely detectable, but increased 25-fold at 3 h and 400-fold at 6 h when macrophages were stimulated with LPS. Levels of IL-17F mRNA declined at 12 h. Accordingly, the increases of IL-17F on the mRNA level preceded appearance of IL-17F protein in the supernatant fluids (Fig. 1*C*, *D*). Finally, we investigated the presence of intracellular IL-17F in macrophages by flow cytometry (Fig. 1*E*). LPS-stimulated peritoneal cells stained with higher fluorescence intensity using an anti-IL-17F antibody as compared to the isotype control (Fig. 1*E*, top left). Intracellular staining for IL-17F correlated with the presence of macrophage surface markers. Percentages of double-positive cell populations were 56.8% for IL-17F$^+$Mac-3$^+$, 67.7% for IL-17$^+$CD11b$^+$, and 50.6% for IL-17F$^+$F4/80$^+$, respectively. Notably, the markers Mac-3 and CD11b have also been described to be present on cells of the myeloid lineage other than macrophages. CD11b can be expressed on neutrophils and Mac-3 is known to also be expressed to some extent on dendritic cells and endothelial cells. Therefore, we used the surface marker F4/80 for all further experiments as a marker for murine macrophages.

MyD88 is essential for expression and release of IL-17F after TLR4 activation by LPS

The myeloid differentiation primary response gene 88 (MYD88) is critical for the production of inflammatory cytokines in response to a variety of microbial products. On the molecular level, MyD88 serves as universal adaptor protein to all TLRs (except TLR3) and the IL-1R1 receptor. Thus, the inflammatory response is dependent on MyD88, although especially for TLR4 additional MyD88-independent signaling pathways exist. For example, in the genetic absence of MyD88, antigen-presenting cells can still respond to LPS by activation of NFκB with delayed kinetics and release cytokines such as IL-27p28 at reduced levels (32, 33). To investigate the role of MyD88 in the TLR4-induced production of IL-17F, we incubated PEMs from wild-type mice and MyD88$^{-/-}$ mice with LPS. Cell culture fluids were harvested 10 h later (**Fig. 2*A***). Wild-type macrophages released IL-17F robustly after LPS incubation, whereas cells with the genetic absence of MyD88 showed no release of detectable IL-17F. The concentration of LPS used in these experiments was 1 μg/ml, which is a dose ~20 times higher than the EC_{50} concentration of LPS for wild-type macrophages to produce IL-17F (Fig. 1*B*). The data on the absence of IL-17F protein release from LPS-stimulated MyD88$^{-/-}$ macrophages was supported by similar findings, when mRNA levels for IL-17F were measured (Fig. 2*B*). For these experiments macrophages were incubated for 6 h, since in the previous experiments (Fig. 1*D*) the highest levels for IL-17F mRNA after LPS were found at this time point. In unstimulated macrophages from wild-type mice, IL-17F mRNA was again barely detectable but became abundantly present with addition of LPS. However, in LPS-stimulated macrophages from MyD88$^{-/-}$ mice, IL-17F mRNA remained undetectable after 40 cycles of real time PCR (Fig. 2*B*, right).

C5a modulates IL-17F production through engagement of the C5aR receptor

Cells of the myeloid lineage, including macrophages, are known to abundantly express the C5a receptor, C5aR (CD88). We performed flow cytometry staining of macrophages for C5aR together with staining for the F4/80 marker and intracellular IL-17F (**Fig. 3*A***). We gated on F4/80, so that the histogram shown as Fig. 3*A* only depicts cells that were found positive for F4/80 compared to staining with the isotype control. Thus, flow cytometry analysis revealed that the vast majority of

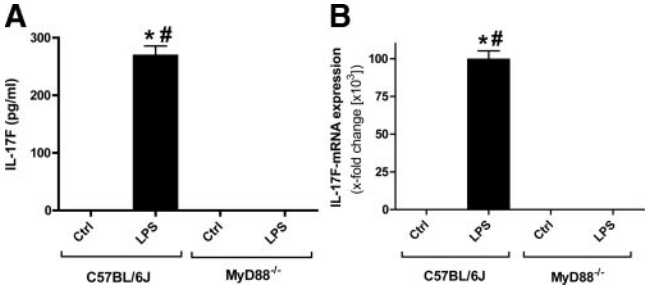

Figure 2. MyD88 is essential for production and release of IL-17F after LPS. *A*) PEMs from C57BL/6J (wild-type) mice and MyD88$^{-/-}$ mice were stimulated in parallel with LPS or left as untreated controls. IL-17F levels in the cell culture supernatants were detected by ELISA after 10 h. *B*) RT-PCR for IL-17F mRNA in wild-type macrophages and MyD88$^{-/-}$ macrophages after LPS, 6 h. LPS was used at 1 μg/ml in all experiments. $^*P < 0.05$ *vs.* Ctrl; $^{\#}P < 0.05$ *vs.* Myd88$^{-/-}$; Student's *t* test.

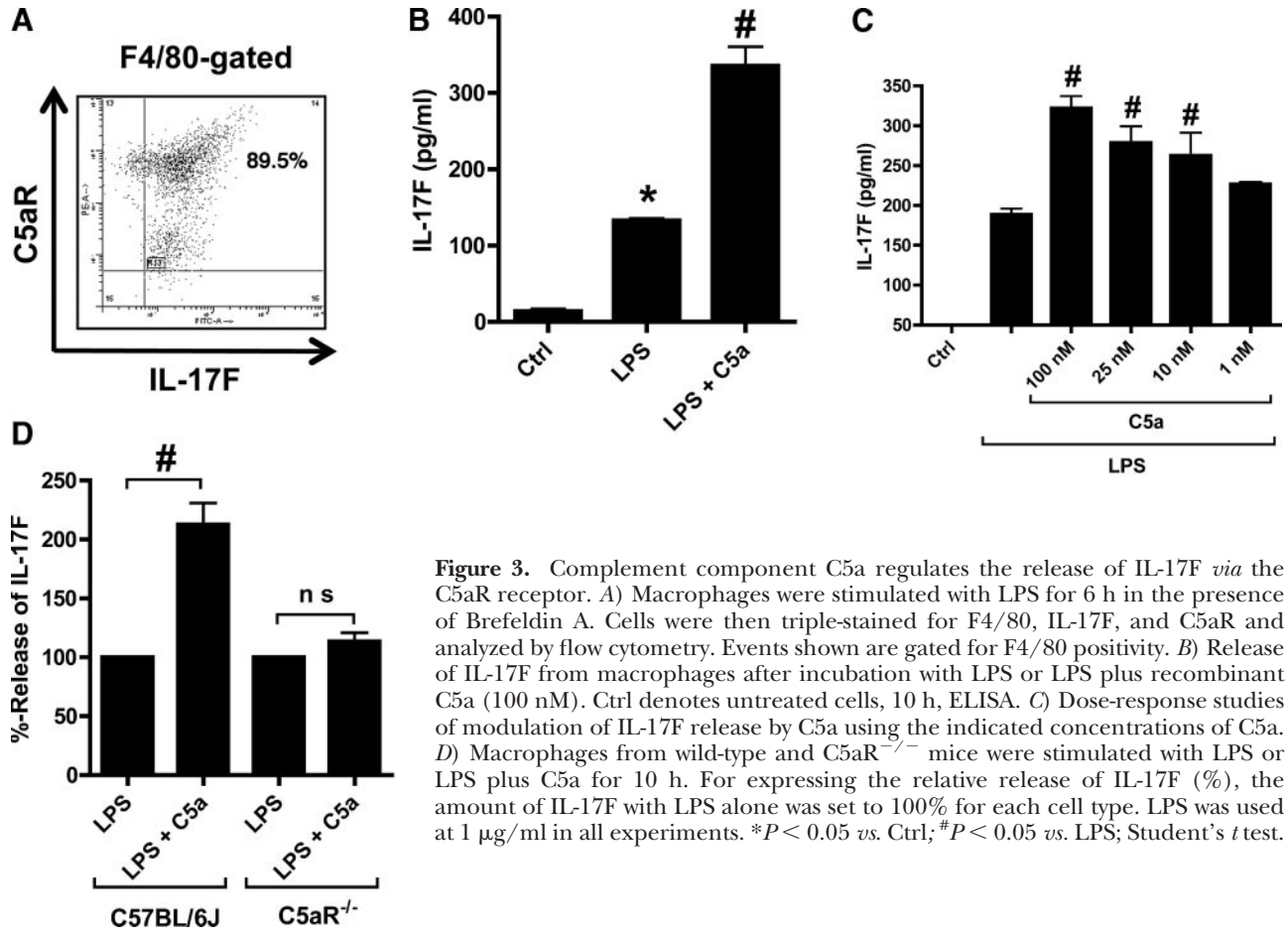

Figure 3. Complement component C5a regulates the release of IL-17F *via* the C5aR receptor. *A*) Macrophages were stimulated with LPS for 6 h in the presence of Brefeldin A. Cells were then triple-stained for F4/80, IL-17F, and C5aR and analyzed by flow cytometry. Events shown are gated for F4/80 positivity. *B*) Release of IL-17F from macrophages after incubation with LPS or LPS plus recombinant C5a (100 nM). Ctrl denotes untreated cells, 10 h, ELISA. *C*) Dose-response studies of modulation of IL-17F release by C5a using the indicated concentrations of C5a. *D*) Macrophages from wild-type and $C5aR^{-/-}$ mice were stimulated with LPS or LPS plus C5a for 10 h. For expressing the relative release of IL-17F (%), the amount of IL-17F with LPS alone was set to 100% for each cell type. LPS was used at 1 μg/ml in all experiments. $^{*}P < 0.05$ *vs.* Ctrl; $^{\#}P < 0.05$ *vs.* LPS; Student's *t* test.

$F4/80^+$ macrophages were not only producing IL-17F after LPS but also abundantly expressed the C5aR receptor on the cell surface ($F4/80^+C5aR^+IL\text{-}17F^+$ cells). Next, we sought to test the hypothesis that C5a may alter the release of IL-17F from $F4/80^+C5aR^+IL\text{-}17F^+$ macrophages, since the expression of C5aR suggests that these cells would be responsive to C5a. Macrophages were left as untreated controls or incubated with LPS alone or with the combination of LPS plus recombinant mouse C5a (100 nM). After 10 h, cell culture fluids were collected, and levels of IL-17F were quantified by ELISA (Fig. 3*B*). Indeed, the addition of C5a resulted in higher levels of IL-17F released from macrophages, which on average were ~200% in the copresence of 100 nM C5a as compared to LPS alone. The effect of C5a in the copresence of LPS was dose-dependent, with an EC_{50} of ~10 nM for C5a (Fig. 3*C*). When cells from $C5aR^{-/-}$ mice were compared to wild-type cells, C5a could not enhance IL-17F levels (Fig. 3*D*). This suggests that ligation of C5a to the C5aR receptor is required to the initiate intracellular signal transduction events for the modulation of IL-17F release by C5a.

C5a mediates site specific phosphorylation of Akt at amino acid residue threonine 308

We have previously reported that C5a can activate Akt (also known as protein kinase B) in human and rat neutrophils (34, 35). Binding of phosphatidylinositol (3,4,5)-triphosphate (PIP_3) results in translocation of Akt to the plasma membrane and activation of Akt to phospho-Akt by upstream kinases. Specifically, Akt can be phosphorylated at threonine 308 (T308) by the kinase PDPK1 and/or phosphorylation at serine 473 (S473) by the kinase mTORC2. We employed flow cytometry using antibodies specific for the phosphorylation sites T308 and S473 to investigate the effects of C5a and LPS on the Akt signaling pathway in $F4/80^+$ macrophages. A time course of the effects of C5a on the phosphorylation status of Akt at T308 is shown in **Fig. 4*A***. Baseline phosphorylation (0 min) was 2.8% and increased with duration of C5a incubation. After 20 min, the number of phospho-Akt(T308)$^+F4/80^+$ cells was 14.0%, which represented a 5-fold increase. The gate used for data analysis and staining with isotype control compared to the phospho-Akt(T308) antibody is shown in Supplemental Fig. S1. A different pattern was observed when phosphorylation of Akt at S473 was studied in response to C5a (Fig. 4*B*). In fact, no significant increase in the number of phospho-Akt(S473)$^+F4/80^+$ macrophages (1.5% at 0 min *vs.* 2.9% at 20 min) was seen in the presence of C5a (100 nM). Similar results were obtained when phosphorylation of Akt after addition of LPS (1 μg/ml) was studied (Fig. 4*C*). The number of phospho-Akt(T308)$^+F4/80^+$ macrophages shifted from 1.5% at 0 min to 35.2% at 20

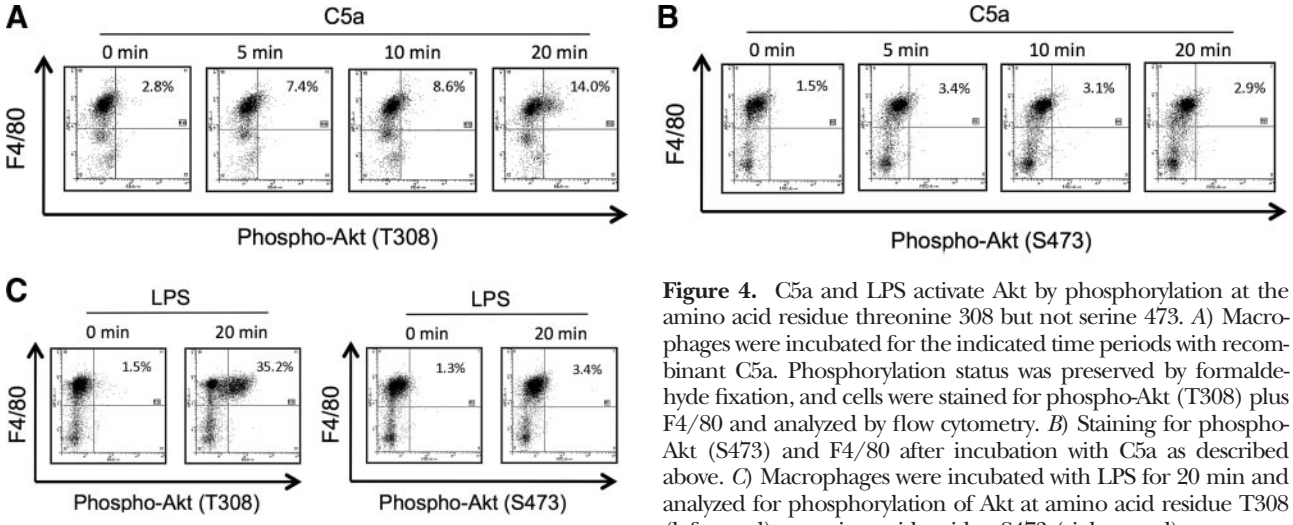

Figure 4. C5a and LPS activate Akt by phosphorylation at the amino acid residue threonine 308 but not serine 473. *A*) Macrophages were incubated for the indicated time periods with recombinant C5a. Phosphorylation status was preserved by formaldehyde fixation, and cells were stained for phospho-Akt (T308) plus F4/80 and analyzed by flow cytometry. *B*) Staining for phospho-Akt (S473) and F4/80 after incubation with C5a as described above. *C*) Macrophages were incubated with LPS for 20 min and analyzed for phosphorylation of Akt at amino acid residue T308 (left panel) or amino acid residue S473 (right panel).

min after incubation with LPS (Fig. 4*C*, left panel). However, LPS had very limited ability to phosphorylate Akt at S473 in F4/80$^+$ macrophages (1.3 *vs.* 3.4%, Fig. 4*C*, right panel).

Synergistic activation of the Akt signaling pathway by C5a and LPS and dependency on MyD88

To further investigate the role of Akt for transmission of the intracellular signals after binding of C5a and LPS to their receptors, we studied the capability of C5a to further enhance the phosphorylation of Akt in the copresence of LPS. According to the results presented above, we focused our further studies on the phosphorylation site T308 of Akt. First, we report the results obtained with PEMs from C57BL/6J wild-type mice: when such F4/80$^+$ macrophages were incubated for different durations of time with LPS alone, the number of phospho-Akt(T308)$^+$F4/80$^+$ double-positive cells increased from 2.3 (0 min) to 30.6% (20 min; **Fig. 5*A***, top panel). When macrophages were stimulated with the combination of LPS and recombinant C5a, the phosphorylation of Akt at site T308 increased more rapidly and to higher absolute cell numbers (Fig. 5*A*, bottom panel). After 5 min with LPS plus C5a, the number of phospho-Akt(T308)$^+$F4/80$^+$ double-positive cells was 13.3.% as compared to 3.6% with LPS alone. At the 20-min time point, LPS plus C5a resulted in 48.2% phospho-Akt(T308)$^+$F4/80$^+$ macrophages, whereas 30.6% double-positive cells were counted when LPS was used alone in this experiment.

In addition to macrophages from wild-type mice, we also studied the time course for Akt phosphorylation after LPS and LPS plus C5a in macrophages from MyD88$^{-/-}$ mice. The experiment and all data in Fig. 5 for wild-type and MyD88$^{-/-}$ macrophages were performed side by side on the same day. One representative of two independent experiments is shown. Not surprisingly, the extent of phosphorylation of Akt in response to LPS was markedly abrogated in macrophages with the genetic absence of MyD88 (Fig. 5*B*, top panel). After 20 min with LPS (1 μg/ml), the number of phospho-Akt(T308)$^+$F4/80$^+$ double-positive macrophages from MyD88$^{-/-}$ mice was only 9.7% as compared to 30.6% in macrophages from wild-type mice (Fig. 5*A*, top panel). On combined incubation with LPS and C5a, macrophages from MyD88$^{-/-}$

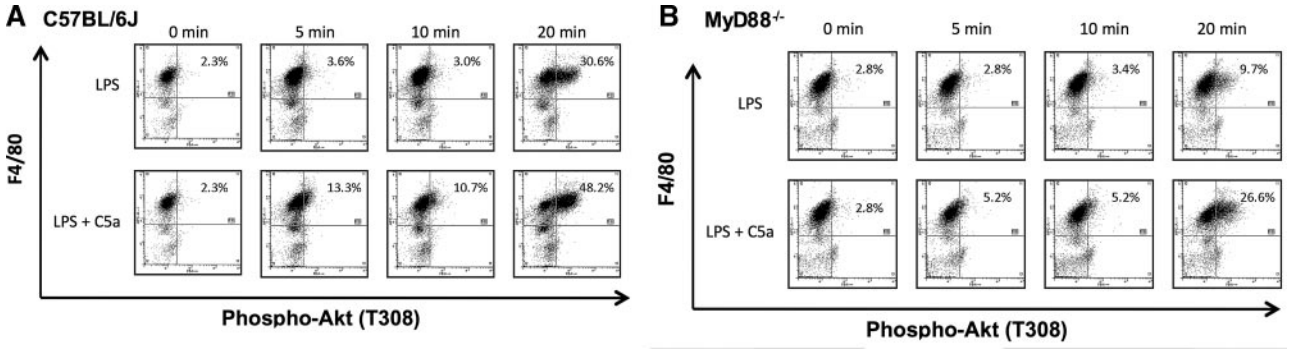

Figure 5. Synergistic activation of Akt by C5a and LPS is reduced in the absence of MyD88. *A*) PEMs from wild-type mice were incubated for the indicated time periods with either LPS alone or the combination of LPS plus C5a. Cells were stained for F4/80 and phospho-Akt (T308) and analyzed by flow cytometry. *B*) Macrophages from MyD88$^{-/-}$ mice were incubated with LPS or LPS plus C5a and stained for F4/80 and phospho-Akt (T308). LPS, 1 μg/ml; C5a, 100 nM.

mice responded with significant but nevertheless less phosphorylation of Akt as compared to wild-type macrophages after 20 min (26.6 *vs.* 48.2%). Incubation of MyD88$^{-/-}$ macrophages with C5a alone resulted in similar levels of Akt phosphorylation at T308 as compared to wild-type macrophages after 20 min (data not shown).

Pharmacologic blockade of the phosphoinositide 3-kinase (PI3K)-Akt signaling pathway abrogates IL-17F production

To address the question of whether the activation of Akt by LPS and C5a is the relevant signal transduction pathway subsequently mediating IL-17F gene expression and release, we studied how IL-17F is affected when Akt inhibition is performed. The pharmacologic small-molecule inhibitors Wortmannin and LY294002 were used for these experiments. Wortmannin irreversibly inactivates PI3K by a covalent binding reaction, which thereby prevents further downstream activation of Akt. PEMs were incubated with different doses of Wortmannin (5 μM–40 nM) for 10 h in the presence of LPS, and IL-17F was detected by ELISA (**Fig. 6*A***). Compared to IL-17F levels after LPS alone, the release of IL-17F from LPS-activated macrophages was profoundly suppressed by Wortmannin at all concentrations employed. In contrast to Wortmannin, LY294002 is a reversible inhibitor of PI3K and displays somewhat less potency. However, IL-17F release from LPS-stimulated macrophages was effectively antagonized even with low concentrations of LY294002 (Fig. 6*B*). Specifically, with the lowest concentration of LY294002 (1 μM) LPS-induced IL-17F levels were reduced by 38%. Further support of our hypothesis that the PI3K-Akt pathway is essential for gene expression of IL-17F after LPS was obtained by RT-PCR. The mRNA levels for IL-17F were substantially lower in macrophages after activation by LPS, when the macrophages had been pretreated with Wortmannin (Fig. 6*C*). Similar suppression of IL-17F mRNA was evident when LY294002 was used to inhibit the PI3K-Akt pathway (Fig. 6*D*). When macrophages were incubated with Wortmannin or LY294002 alone, no effects on IL-17F protein or mRNA was observed (data not shown). In addition, no cytotoxic effects were observed with either Wortmannin or LY294002 under the conditions employed as evaluated by light microscopy at the end of experiments.

C5a regulates the appearance of IL-17F during acute systemic immune responses

According to the data presented above, we have demonstrated that the release of IL-17F is regulated by the anaphylatoxin C5a, when recombinant C5a is added to cell cultures of peritoneal macrophages activated by LPS. To further collect evidence that the complement

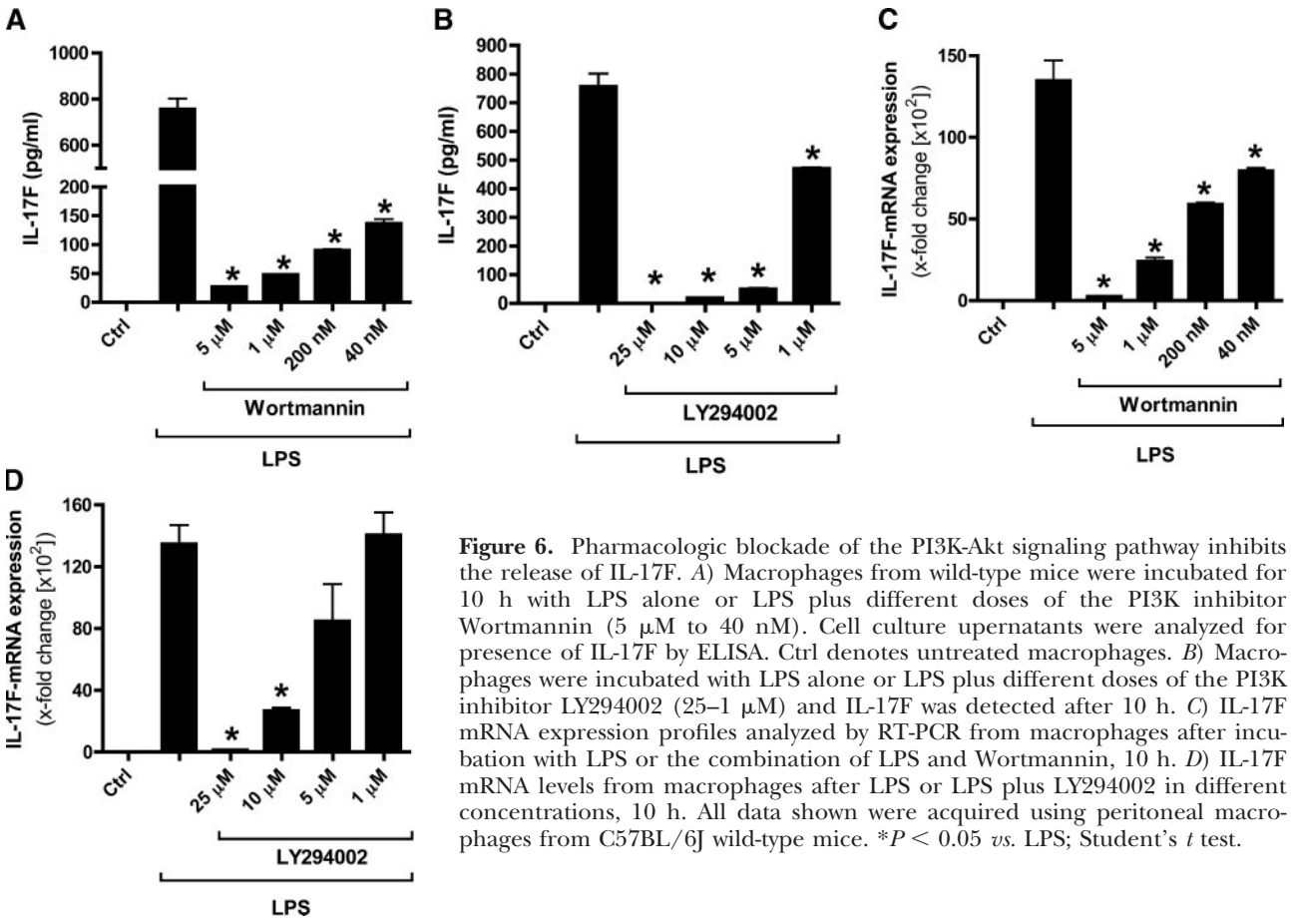

Figure 6. Pharmacologic blockade of the PI3K-Akt signaling pathway inhibits the release of IL-17F. *A*) Macrophages from wild-type mice were incubated for 10 h with LPS alone or LPS plus different doses of the PI3K inhibitor Wortmannin (5 μM to 40 nM). Cell culture upernatants were analyzed for presence of IL-17F by ELISA. Ctrl denotes untreated macrophages. *B*) Macrophages were incubated with LPS alone or LPS plus different doses of the PI3K inhibitor LY294002 (25–1 μM) and IL-17F was detected after 10 h. *C*) IL-17F mRNA expression profiles analyzed by RT-PCR from macrophages after incubation with LPS or the combination of LPS and Wortmannin, 10 h. *D*) IL-17F mRNA levels from macrophages after LPS or LPS plus LY294002 in different concentrations, 10 h. All data shown were acquired using peritoneal macrophages from C57BL/6J wild-type mice. *$P < 0.05$ *vs.* LPS; Student's *t* test.

system through C5a may regulate IL-17F-dependent immune functions, we sought to study the effects of C5a on IL-17F during acute systemic inflammation *in vivo*. We used a model of lethal endotoxemia, where a high dose of LPS (*E. coli*, 10 mg/kg body weight) is administered i.p. In this experimental setting, mice begin to develop signs of acute illness (lethargy, hypothermia, periorbital exudates, piloerection) within a few hours, and lethality is 60–80%, with most of the deaths usually occurring between 24–48 h after LPS injection (data not shown). Using this model of endotoxemia, plasma was collected at several time points after challenge with LPS but before mice succumbed to cardiovascular shock. A time-course study of C5a appearance in plasma from endotoxemic wild-type mice revealed that C5a levels reached the maximum 6 h after injection of LPS (**Fig. 7*A***). Levels of C5a were 5- to 6-fold higher as compared to levels detected in the plasma of mice injected with vehicle alone (sham). We cannot rule out the possibility that the C5a levels detected in sham-treated mice represent cross-reactivity of the ELISA assay with C5 rather than true presence of C5a. Next, we investigated the kinetics of circulating IL-17F. As shown in Fig. 7*B*, levels of IL-17F in plasma peaked at 6–12 h in the endotoxemia model. Thereby, the appearance of C5a preceded the release of IL-17F in plasma. To test the hypothesis that levels of IL-17F in endotoxemia are influenced by the presence of C5a, we used a blocking polyclonal goat anti-C5a antiserum to neutralize C5a. When anti-C5a serum was given 2 h before the administration of LPS, the levels of IL-17F were reduced by ~50% as compared to animals that received a normal control serum followed by LPS (Fig. 7*C*). Finally, we expanded our investigation on polymicrobial sepsis after cecal ligation and puncture (CLP). This model of disease has been considered by many to be the gold standard for research on experimental sepsis and similar to the endotoxemia model results in a systemic inflammatory response. In mice 10 h after CLP, we found IL-17F to be present at levels of ~150 pg/ml (Fig. 7*D*), meaning IL-17F release was lower as compared to the findings in the endotoxemia model. The CLP procedure was performed in a design causing severe sepsis and high mortality (high-grade CLP). In control mice undergoing anesthesia and sham surgery, IL-17F in plasma was not detectable. Similar to the findings after endotoxemia, the neutralization of C5a with administration of a blocking anti-C5a antiserum 2 h before CLP resulted in a significant reduction of circulating IL-17F after 12 h (Fig. 7*E*). In summary, the release of IL-17F appeared to be regulated by C5a in the two studied experimental models (endotoxemia and CLP), which are known to be characterized by a

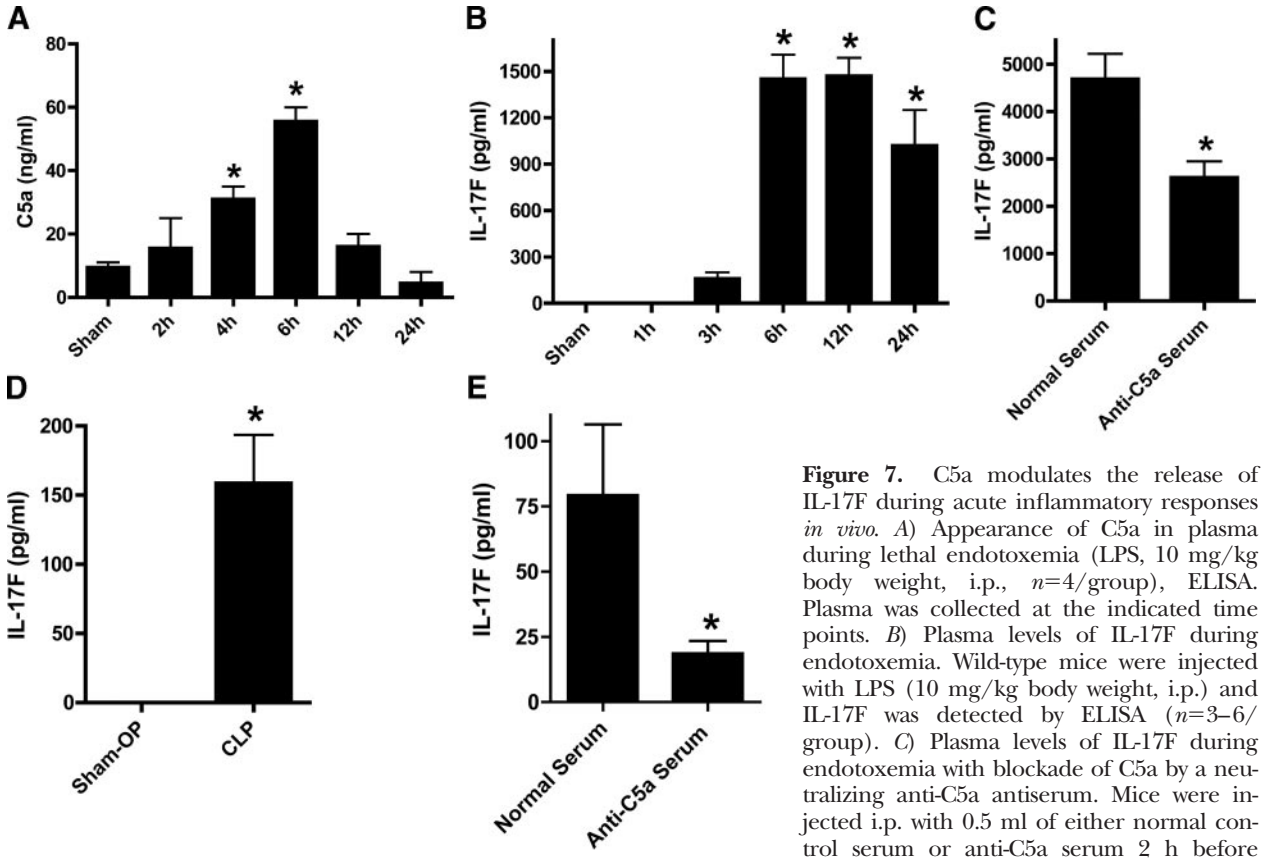

Figure 7. C5a modulates the release of IL-17F during acute inflammatory responses *in vivo*. *A*) Appearance of C5a in plasma during lethal endotoxemia (LPS, 10 mg/kg body weight, i.p., n=4/group), ELISA. Plasma was collected at the indicated time points. *B*) Plasma levels of IL-17F during endotoxemia. Wild-type mice were injected with LPS (10 mg/kg body weight, i.p.) and IL-17F was detected by ELISA (n=3–6/group). *C*) Plasma levels of IL-17F during endotoxemia with blockade of C5a by a neutralizing anti-C5a antiserum. Mice were injected i.p. with 0.5 ml of either normal control serum or anti-C5a serum 2 h before injection of LPS (10 mg/kg body weight, i.p.). Plasma was analyzed for appearance of IL-17F after 12 h. *D*) Levels of IL-17F in plasma during polymicrobial sepsis induced by cecal ligation and puncture (CLP), 12 h. Mice underwent either sham surgery (n=3) or high-grade CLP (n=4). *E*) Levels of IL-17F in plasma after CLP with blockade of C5a. Neutralization of C5a was achieved by anti-C5a antiserum and compared to normal control serum as described above for endotoxemia; $n \geq 6$ for each group. *$P < 0.05$; Student's t test.

severe systemic inflammatory response, including activation of the complement system.

DISCUSSION

Our data indicate that the expression of the IL-17F isoform is regulated by the complement activation product C5a. Production and release of IL-17F from F4/80$^+$ macrophages in response to LPS was completely dependent on the TLR4-associated adaptor protein MyD88. In the presence of C5a, the levels of IL-17F increased synergistically with the effects of LPS. Conversely, with blockade of C5a during acute systemic inflammation using the models of endotoxemia and CLP, the amounts of IL-17F detectable in plasma were reduced. The intracellular signaling mechanisms underlying the observation that C5a and LPS interact for the gene expression of IL-17F appear to be linked to Akt. C5a and LPS both induced rapid activation of Akt. Interestingly, phosphorylation of Akt in F4/80$^+$ macrophages was mostly heavily targeted for the amino acid residue threonine 308 with very limited phosphorylation at amino acid residue serine 473. The extent of Akt phosphorylation was higher when LPS and C5a were used in combination as compared to LPS or C5a alone, which is consistent with the finding that LPS plus C5a resulted in higher levels of IL-17F released from macrophages. Blockade of the PI3K-Akt signaling pathways with the specific inhibitors Wortmannin and LY294002 profoundly incapacitated macrophages for producing IL-17F protein and mRNA. Furthermore, the activation of Akt was dependent of MyD88. The proposed molecular mechanisms according to the data presented in this report are shown in **Fig. 8**.

There are accumulating reports in the literature that IL-17 isoforms can be produced by cells of the innate immune system in addition to IL-17 derived from Th17 cells (20, 27, 36–38). Cells of the myeloid lineage, such as neutrophils, have been suggested to be a source of IL-17 after LPS instillation into the lung or after kidney ischemia-reperfusion injury (36, 39). Substantial levels of IL-17A were still released in the genetic absence of αβ T cells after CLP, whereas γδ T cells contributed to IL-17A production in this model (27). Levels of IL-17A after CLP were reduced but not completely absent in Rag-1$^{-/-}$ mice after CLP (29). Interestingly, in endotoxic shock the genetic absence of αβ T cells, γδ T cells, or CD4$^+$ cells did not result in diminished plasma IL-17A levels after 12 h, whereas depletion of F4/80$^+$ macrophages did (unpublished results.) Furthermore, IL-17A can be produced by peritoneal macrophages and is suppressed by IL-10 in these cells (40). Genetic absence of IL-10 was accompanied by elevated expression of RORγt in macrophages together with increased IL-17A release on LPS stimulation (40). LPS-stimulated wild-type macrophages that produced lower levels of IL-17A failed to show evidence for expression of RORγt. In a murine model of asthma, the house dust mite induced production of IL-17A was attenuated in

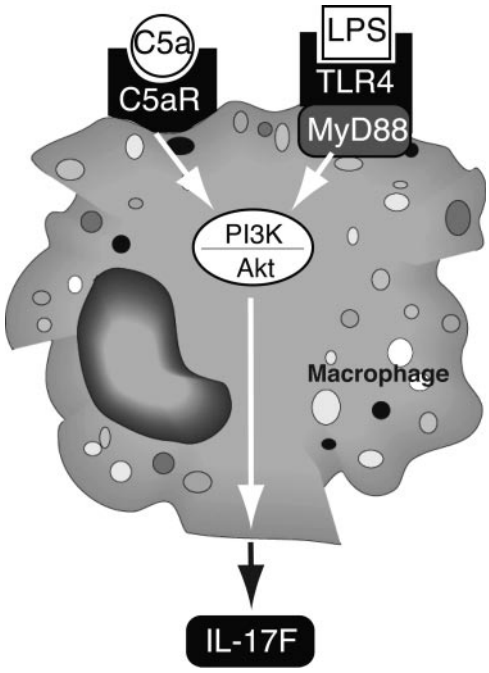

Figure 8. Proposed mechanisms for the regulation of the release of IL-17F based on the data presented herein.

MyD88 deficient mice (41). In addition, macrophages produce IL-17A in response to chitin in a manner that was dependent on both TLR2 and MyD88 (42). However, most studies so far have focused on IL-17A, whereas much less is known about the production and regulation of IL17F from innate immune cells. The production of cytokines in response to LPS is not necessarily entirely dependent on MyD88 since TLR4 can signal using both the MyD88 and TRIF pathways. For example, IL-18 can be cleaved from proIL-18 in Kupffer cells from MyD88 deficient mice and IL-27p28 levels after LPS are only reduced by 50–70% in macrophages from such animals (33, 43). However, according to our findings, MyD88 is essential for the release of IL-17F in response to LPS, since we observed complete absence of IL-17F generation with genetic deficiency of MyD88.

There is growing evidence that the complement system can positively or negatively regulate the responses of components of the innate and adaptive immune system. Specifically, the anaphylatoxin C5a is being generated in large quantities after formation of the C5-convertase. C5a does not only attract and activate neutrophils and other phagocytic cells to the site of complement activation but also interferes with the extent of mediator production by these cells, *via* signal transduction molecules such as Akt. For example, C5a has differential effects on TNF-α release from rat neutrophils and alveolar macrophages (35). Furthermore, C5a enhances the expression of IL-8 from LPS-activated human neutrophils and regulates IL-6 from rat neutrophils after CLP (44, 45). Furthermore, C5a has been demonstrated to interfere with the release of Th1 cytokines such as IL-12 and IL-23 from LPS-stimulated murine macrophages and human mono-

cytes (46, 47). Lymphocytes, namely, T cells are also responsive to C5a, since T cells express the C5aR receptor and are chemoattracted to C5a (48). Clonal expansion of T cells is also enhanced by C5a through limitation of lymphocyte apoptosis and C5a enhances T-cell activation (49, 50). Several reports have recently suggested a role of the complement system and C5a to interfere with Th17 cell responses and expression of IL-17A (51–54). For example, in a model of severe airway hyperresponsiveness, IL-17A apparently was reciprocally regulated by the anaphylatoxins C5a and C3a, when mouse strains deficient of the complement factor C5, C5aR, or C3aR were studied (52). Dendritic cells from C5aR-deficient mice have been found to promote the induction of Th17 cells after activation of TLR2 or with ovalbumin (54). However, the exact influence of complement activation on directing and orchestration of Th1, Th2, and Th17 cell responses may vary depending on the disease model studied. Notably, the time course and magnitude of C5a generation as well as the responsiveness to C5a after binding to the C5aR receptor may differ depending if chronic or acute inflammatory diseases are studied. To our knowledge, the data presented here is the first detailed report specifically addressing the effects of C5a on the IL-17F isoform and characterizing some of the intracellular signaling events involved. It suggests that the extent of IL-17F-dependent immune response can be modulated and fine-tuned by the complement system. In our future work, we plan to further address the question on how the complement system regulates innate and adaptive immune functions and how the understanding of these mechanisms can eventually be employed for new therapeutic strategies for human disease. FJ

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