Inflammatory Responses Induced by Lipopolysaccharide Are Amplified in Primary Human Monocytes but Suppressed in Macrophages by Complement Protein C5a

Vernon Seow, Junxian Lim, Abishek Iyer, Jacky Y. Suen, Juliana K. Ariffin, Daniel M. Hohenhaus, Matthew J. Sweet and David P. Fairlie

*J Immunol* 2013; 191:4308-4316; Prepublished online 16 September 2013; doi: 10.4049/jimmunol.1301355
http://www.jimmunol.org/content/191/8/4308

---

**Supplementary Material**
http://www.jimmunol.org/content/suppl/2013/09/16/jimmunol.1301355.DC1

**References**
This article cites 49 articles, 24 of which you can access for free at:
http://www.jimmunol.org/content/191/8/4308.full#ref-list-1

**Subscription**
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Inflammatory Responses Induced by Lipopolysaccharide Are Amplified in Primary Human Monocytes but Suppressed in Macrophages by Complement Protein C5a

Vernon Seow, Junxian Lim, Abishek Iyer, Jacky Y. Suen, Juliana K. Ariffin, Daniel M. Hohenhaus, Matthew J. Sweet, and David P. Fairlie

Monocytes and macrophages are important innate immune cells equipped with danger-sensing receptors, including complement and Toll-like receptors. Complement protein C5a, acting via C5aR, is shown in this study to differentially modulate LPS-induced inflammatory responses in primary human monocytes versus macrophages. Whereas C5a enhanced secretion of LPS-induced IL-6 and TNF from primary human monocytes, C5a inhibited these responses while increasing IL-10 secretion in donor-matched human monocyte-derived macrophages differentiated by GM-CSF or M-CSF. G\(_{\alpha_i}\)/c-Raf/MEK/ERK signaling induced by C5a was amplified in macrophages but not in monocytes by LPS. Accordingly, the G\(_{\alpha_i}\) inhibitor pertussis toxin and MEK inhibitor U0126 blocked C5a inhibition of LPS-induced IL-6 and TNF production from macrophages. This synergy was independent of IL-10, PI3K, p38, JNK, and the differentiating agent. Furthermore, C5a did not inhibit IL-6 production from macrophages induced by other TLR agonists that are selective for Toll/IL-1R domain–containing adapter inducing IFN-\(\beta\) (polyinosinic-polycytidylic acid) or MyD88 (imiquimod), demonstrating selectivity for C5a regulation of LPS responses. Finally, suppression of proinflammatory cytokines IL-6 and TNF in macrophages did not compromise antimicrobial activity; instead, C5a enhanced clearance of the Gram-negative bacterial pathogen \textit{Salmonella enterica} serovar Typhimurium from macrophages. C5aR is thus a regulatory switch that modulates TLR4 signaling via the G\(_{\alpha_i}/c-Raf/MEK/ERK\) signaling axis in human macrophages but not monocytes. The differential effects of C5a are consistent with amplifying monocyte proinflammatory responses to systemic danger signals, but attenuating macrophage cytokine responses (without compromising microbialidal activity), thereby restraining inflammatory responses to localized infections. \textit{The Journal of Immunology}, 2013, 191: 4308–4316.

Complement is an important cascading network of >30 soluble plasma and insoluble membrane-bound proteins that cooperate in innate immunity in the recognition, opsonization, destruction, and removal of pathogens and infected or damaged cells. Complement activation by classical, alternative, and lectin pathways and other routes is tightly controlled, producing the lytic membrane attack complex, as well as opsonins such as C3b and proinflammatory anaphylotoxins C3a, C4a, and C5a. Elevated plasma levels of C5a or its receptor are associated with inflammatory diseases such as rheumatoid arthritis, inflammatory bowel diseases, respiratory distress syndrome, ischemia-reperfusion injury, and sepsis (1). C5a binds to and signals through the receptor C5aR (CD88), a class A G protein–coupled rhodopsin-like receptor (2), and to a second receptor C5L2, which does not couple G proteins and may be nonsignaling, but may also modulate C5aR (3, 4). C5aR couples to the pertussis toxin (PTX)–sensitive G\(_{\alpha_i}\) and in some cells such as monocytes to the PTX-insensitive G\(_{\alpha_{16}}\), and it recruits \(\beta\)-arrestins-1,2 to the plasma membrane (1, 5). C5a activates several downstream signaling pathways, including Akt, MEK, PI3K, phosphokinase A, phospholipase C (PLC), and NF-\(\kappa\)B (1).

The TLRs are an important family of type I transmembrane proteins that respond to infectious organisms and danger signals by initiating inflammatory responses, activating microbial killing and clearance mechanisms, and priming the adaptive immune response (6). Of 10 known human TLRs, TLR4 responds to LPS from Gram-negative bacterial cell walls. TLR4 can signal through two different pathways dependent on, and modulated by, separate adaptor proteins MyD88 or Toll/IL-1R domain–containing adapter inducing IFN-\(\beta\) (TRIF) (7). MyD88 activates IL-1R–associated kinases (IRAKs) and TNFR-associated factor 6 (TRAF6), as well as transcription factors NF-\(\kappa\)B, AP-1, and IFN regulatory factor 5 (IRF5) further downstream. TRIF signals the induction of type I IFNs by recruiting TRAF3 and receptor interacting protein 1 (RIP1) to activate transcription factor IFN regulatory factor 3 (IRF3), NF-\(\kappa\)B, and AP-1. Both MyD88 and TRIF activate AP-1 via downstream MAPK activation (8), although NF-\(\kappa\)B and MAPK activation by TRIF occurs later than for MyD88 (9).

In the present study, crosstalk between C5aR and TLR4 was investigated in human monocytes and human monocyte–derived macrophages (HMDMs) using the respective ligands C5a and LPS. Crosstalk between different signaling pathways in mam-
malian cells can result in unexpected and unique functional outcomes (10). Some pathogens have evolved to evade or exploit host microbiocidal killing by targeting C5aR and TLRs specifically. For example, virulence proteins secreted by *Staphylococcus aureus* bind to C5aR with potent antagonist activity, preventing recruitment of phagocytes to sites of infection (11). *Porphyromonas gingivalis* exploits the C5aR/TLR2 crosstalk by increasing cAMP production in macrophages, which suppresses macrophage immune function and enhances pathogen survival (12). Clearly, these pathogens have evolutionarily adapted to exploit complement/TLR crosstalk to their advantage. Although C5aR and TLR4 signaling have been separately investigated in many studies, there is comparatively little reported about their interplay (13). Among recent reports on C5aR/TLR4 crosstalk, C5a has been found to downregulate TLR4-induced IL-12 production via PI3K-dependent (14) and PI3K-independent (15) pathways in mouse macrophages; to signal via the PI3K-Akt pathway to enhance LPS-induced IL-17F cytokine production (16); and to suppress LPS-induced IL-17A and IL-23 cytokine production in mouse macrophages (17). C5a-induced suppression of the LPS-inducible IL-17A/IL-23 axis was reported to occur via enhanced IL-10 production and ERK1/2 phosphorylation (18). However, most studies reporting C5a modulation of TLR4 signaling had been performed with murine rather than human cells (19, 19). Some interpretations of human disease mechanisms have been made based on extrapolation from observations of mouse studies. However, there are important differences in LPS-induced TLR4 signaling between humans and mice (20–22), necessitating careful assessment of C5aR/TLR4 crosstalk in human cells.

Monocytes and macrophages are important cellular mediators of innate immunity and are targets for C5a and LPS. Some features of LPS signaling are also distinctly different between human monocytes and macrophages (23). Monocytes act as danger sensors in the circulation and typically generate a more rapid, heightened inflammatory response than do macrophages to danger signals. For example, LPS alone can trigger inflammasome activation in monocytes, whereas an additional activation signal such as ATP is required in macrophages (24). The present study compares effects of C5a on LPS responses in primary human monocytes versus donor-matched macrophages derived through differentiating monocytes with either GM-CSF or M-CSF. Surprisingly, we found that C5a differentially modulated LPS responses depending upon the cell type, that C5a activated the G_{i/o}/c-Raf/MEK/ERK axis to selectively downregulate proinflammatory cytokine production from macrophages but not monocytes, and that effects on macrophages do not compromise but instead stimulate pathogen killing. Thus, it is proposed that C5a has distinctly different regulatory effects on myeloid cell functions during localized versus systemic inflammatory responses.

**Materials and Methods**

**Reagents**

Recombinant human GM-CSF, M-CSF, and IL-10 were purchased from PeproTech. LPS from *Salmonella enterica* (serotype Minnesota RE 595), U0126 monothanolate, wortmannin, and PTX were purchased from Sigma-Aldrich. Imiquimod R837 (IMQ) and polyinosinic-polycytidylic acid (poly(I:C)) were purchased from InvivoGen. Human C5a was purchased from Sino Biological. Goat neutralizing Ab against human IL-10 was purchased from Southern Biotechnology Associates. Abs against human IL-10 were also licensed as PMX53 (1, 25) was synthesized and characterized (analytical HPLC, mass spectroscopy, and nuclear magnetic resonance spectroscopy) in-house as described (26). Abs against phosphorylated c-Raf and total c-Raf were purchased from Cell Signaling Technology. All cell culture reagents were purchased from Invitrogen, and all analytical-grade chemical reagents were obtained from Sigma-Aldrich, unless otherwise stated.

**Isolation of human monocytes and macrophages**

PBMCs were isolated from buffy coats of anonymous donors (Australian Red Cross Blood Service, Kelvin Grove, QLD, Australia) by density centrifugation using Ficoll-Paque Plus (GE Healthcare) following the manufacturer’s instructions. Contaminating erythrocytes were removed by repeated ice-cold sterile water. CD14+ MACS MicroBeads (Miltenyi...
Bacteria were plated out inoculum on Luria–Bertani agar plates and performing colony counts were performed twice and lysed with 0.01% Triton X-100 in PBS, after which lysates were cultured in the presence of 5% CO₂. To generate HMDMs, CD14⁺ monocytes were cultured in the presence of either 10 ng/ml GM-CSF (GM-Mφ) or M-CSF (M-Mφ) for at least 6 d.

**Quantitative cytokine ELISA**

Cells were seeded at 1 × 10⁶ cells/ml and serum-deprived overnight in the incubator prior to treatment. LPS and C5a treatments were premixed prior to 24 h stimulation. Cell culture supernatants were collected and cytokine levels were determined using specific ELISA sets from BD Pharmingen, according to the manufacturer’s instructions.

**Isolation of mRNA and RT-PCR**

Total RNA was extracted from cells using an RNasey Mini Plus kit (Qiagen), according to the manufacturer’s instructions. Total cellular RNA (2–10 μg) was reverse transcribed to cDNA using oligo(dT) primer (1 μg) and SuperScript III (Invitrogen), according to the manufacturer’s instructions. cDNA samples were stored at –20°C until further use.

**Gene expression analysis by real-time PCR**

Gene-specific primers were designed using the free Web-based software Primer-BLAST (National Center for Biotechnology Information). Quantitative real-time-PCR was performed using an ABI Prism 7900 real-time PCR system (Applied Biosystems), cDNA (50 ng), SYBR Green PCR Master Mix (Applied Biosystems), and gene-specific primers. Relative gene expression was normalized against 18S rRNA expression and then converted to fold change against control samples. All samples were analyzed in duplicate. Primer sequences used are: CD68, 5'-TTTGGGTGAGGCGGTTCAG-3' and 3'-CCAGTGCTCTCTGCCAGTA-5'; 18S, 5'-ACCACGGTGACCGGGGA-3' and 3'-CCGGGTCGGGAGTGGGTAAT-5'.

**Quantification of MEK1, ERK1/2, p38, and JNK phosphorylation**

Cells were seeded at 4000 cells/well in white ProxiPlate-384 (PerkinElmer) overnight. On the following day, cells were serum-deprived for 2 h prior to stimulation with C5a (30 nM), LPS (5 ng/ml), or both over a stated time course. Alphascreen SureFire assays (PerkinElmer) were performed according to the manufacturer’s instructions.

**Western blot analysis**

Equal amounts of total cell lysates (10 μg) were subjected to SDS-PAGE, transferred to polyvinylidene difluoride membranes, and proteins were detected using appropriate Abs according to the manufacturer’s instructions.

**Bacterial clearance assay**

Intracellular survival of **Salmonella enterica** serovar Typhimurium (S. Typhimurium) strain SL1344 within HMDMs was monitored in gentamicin exclusion assays (27). M-Mφ were seeded at 200,000 cells/well in penicillin/streptomycin-free media containing M-CSF (10 ng/ml). On the following day, cells were infected with S. Typhimurium at a multiplicity of infection (MOI) of 100. One hour after infection, cells were washed with gentamicin (200 μg/ml) in penicillin/streptomycin-free media and further incubated (1 h) to kill extracellular bacteria in the same media. Additional incubations beyond this 2 h time point were carried out in media containing 20 μg/ml gentamicin. At specific time points, cells were washed twice and lysed with 0.01% Triton X-100 in PBS, after which lysates were cultured overnight on Luria–Bertani agar. Colony counts were performed to assess intracellular bacterial loads. The MOI was also confirmed by plating out inoculum on Luria–Bertani agar plates and performing colony counts. Treatments with C5a (30 nM) were conducted by coinfection with bacteria upon infection. For antagonism assays, cells were pretreated with the C5a inhibitor 3D53 (1 μM) 30 min prior to infection. 3D53 was also added back following the washing with gentamicin-containing media.

**Statistical analysis**

Data were plotted and analyzed using GraphPad Prism version 5.0c for Mac OS X (GraphPad Software). Statistically significant differences were assessed using a Student t test for paired comparison. All values of independent parameters are shown as mean ± SEM of at least three independent experiments, unless otherwise stated. A p value < 0.05 was considered statistically significant.
the biological roles of CSL2 in inflammation are highly controversial. Our group has previously developed 3D53, a potent, irreversible antagonist of C5aR that does not bind CSL2 (1, 29, 30). 3D53 blocked the immunomodulatory effects of C5a on TLR4 signaling in both macrophages and monocytes (Fig. 1D–F), thus demonstrating that the observed responses of C5a were mediated via C5aR rather than CSL2.

Temporal study of C5aR/TLR4 crosstalk in HMDMs

To further characterize the phenotypic switch in C5a responsiveness during monocyte to macrophage differentiation, time course experiments were performed. As expected, mRNA levels of the macrophage marker CD68 were increased after 4 d of differentiation in the presence of either GM-CSF or M-CSF (Fig. 2A). The suppressive effect of C5a on LPS-induced cytokine production was apparent by day 4 for TNF, and by day 6 for IL-6 (Fig. 2B, 2C). Increased IL-10 production was also observed by day 4 (Fig. 2D). The suppressive effects of C5a on LPS-induced IL-6 and TNF production in macrophages correlated with increased C5AR and TLR4 expression during monocyte to macrophage differentiation (Supplemental Fig. 1A, 1B). Levels of C5AR and TLR4 mRNA were not affected by C5a treatment, but LPS decreased C5AR expression by ∼30% (Supplemental Fig. 1C, 1D), consistent with a previous study (31).

C5a suppresses LPS-induced IL-6 and TNF via Gαi- and MEK-dependent signaling

C5a-dependent C5aR signaling involves coupling to Gαi proteins (5, 32). We used PTX, which specifically interferes with Gαi protein signaling, to investigate whether the blockade of Gαi signaling would reverse inhibition by C5a of TLR4 responses. As expected, all modulatory effects of C5a on LPS-induced cytokine production from monocytes and macrophages was sensitive to PTX (Fig. 3). These results further confirmed involvement of C5aR and not CSL2 signaling in this crosstalk, as C5aR, but not CSL2, employs Gαi signaling (1, 3). We also pharmacologically targeted downstream signaling of C5aR signaling and found that the MEK inhibitor U0126, a selective inhibitor of the ERK pathway (33), prevented immunomodulation by C5a of LPS-induced IL-6 and TNF production in macrophages, but not monocytes (Fig. 3A, 3B). In monocytes, the enhancement of LPS-induced IL-6 production by C5a was altered by the presence of MAPK p38 and PLCβ inhibitors SB203580 and U73122, respectively, whereas the enhancement of LPS-induced TNF production was affected by phosphokinase A inhibitor PKI 14-22 (Supplemental Fig. 2A, 2D). Collectively, these data suggest that C5a-dependent ERK1/2 phosphorylation selectively suppresses LPS-induced IL-6 and TNF production in macrophages. Interestingly, although the literature suggests that ERK1/2 is involved in TLR4-induced IL-10 production from human macrophages (15, 34), we found in the present study that C5a enhanced LPS-mediated IL-10 production in an ERK1/2-independent, but Gαi-dependent, manner (Fig. 3C). Similarly, LPS-induced IL-10 production from monocytes, as well as from GM-Mφ and M-Mφ, was ERK-independent (Fig. 3C).

C5a and LPS synergize for Raf/MEK/ERK phosphorylation in macrophages

The data for PTX and U0126 inhibitors showed that C5a-modulated suppression of LPS-mediated inflammatory responses was dependent on Gαi coupling and MAPK ERK1/2 phosphorylation. Therefore, we further investigated additional checkpoints in the signaling pathway between Gαi signaling and upstream of ERK phosphorylation (Gαi/Raf/MEK/ERK) signaling cascade to further map the crosstalk signaling mechanisms between C5aR and TLR4. Interestingly, C5a phosphorylated c-Raf only in macrophages (both GM-Mφ and M-Mφ) but not in monocytes (Fig. 4A). Co-treatment of GM-Mφ or M-Mφ with LPS and C5a synergistically promoted c-Raf phosphorylation. This synergy was not observed in monocytes. LPS triggered MEK1 phosphorylation in macrophages (Fig. 4B). Although C5a and LPS each induced MEK1 phosphorylation in monocytes and macrophages, the synergistic effect of LPS and C5a cotreatment was only observed in

**FIGURE 3.** C5a suppression of LPS-induced IL-6 and TNF in macrophages is sensitive to PTX and MEKi (U0126) treatment. To inhibit Gαi-dependent interactions, cells were pretreated with PTX (200 ng/ml, overnight) or, for MEK1/2 inhibition, U0126 (1 μM, 30 min) prior to LPS (5 ng/ml) with and without C5a (30 nM) stimulation of monocytes (gray) and HMDMs (GM-Mφ, black; M-Mφ, white). ELISA was used to detect (A) IL-6, (B) TNF, and (C) IL-10 secreted cytokines relative to the LPS response (100%) at 24 h after stimulation. Error bars are means ± SEM of three independent experiments (n = 3). *p < 0.05, **p < 0.01, and ***p < 0.001 by Student t test.
macrophages. Consistent with this, cotreatment of GM-Mφ and M-Mφ with LPS and C5a synergistically promoted ERK1/2 phosphorylation, whereas this synergy was not observed for monocytes (Fig. 4C).

C5a and LPS synergize for phosphorylation of ERK1/2 but not p38 or JNK in macrophages

Because C5a together with LPS potentiates ERK1/2 in macrophages, we next investigated the temporal profile of ERK1/2 activation together with other MAPKs (p38 and JNK) in response to C5a versus C5a plus LPS. C5a induced a transient peak in ERK1/2 phosphorylation within 5 min in monocytes (Fig. 5A) and macrophages, whereas this synergy was not observed for monocytes (Fig. 4C).

C5a-mediated amplification of LPS-induced IL-6 production (Supplemental Fig. 2A), but not TNF production (Supplemental Fig. 2B), from these cells was affected by the p38 inhibitor SB203580. Furthermore, SB203580 did not block the suppression by C5a of LPS-induced IL-6 and TNF production in macrophages (Supplemental Fig. 2B, 2C, 2E, 2F).

C5a-mediated suppression of LPS-induced IL-6 and TNF is IL-10 and PI3K independent

C5a has been previously reported to enhance LPS-induced IL-10 cytokine production from macrophages (15), and it has also been reported to suppress IL-17A and IL-23 by enhancing IL-10 production (17). Consistent with such a mechanism, C5a upregulated LPS-induced IL-10 production from macrophages, but it did not alter this response in monocytes (Fig. 1C). However, the ability of the MEK inhibitor to block C5a-mediated suppression of IL-6 and TNF production, without affecting enhancement of IL-10 production in macrophages, implied that the inhibitory effects on IL-6 and TNF were IL-10–independent (Fig. 3C). We therefore examined this hypothesis. LPS-induced IL-6 and TNF production was suppressed with increasing concentrations of IL-10 (Supplemental Fig. 3A, 3B). This is consistent with literature indicating that IL-10 attenuates IL-6 and TNF production from activated macrophages (34, 35). A neutralizing Ab against human IL-10 (10 μg/ml) completely blocked the effect of exogenously added IL-10 (2 ng/ml) in macrophages (Supplemental Fig. 3C, 3D), and its addition prior to LPS stimulation resulted in increased TNF production in GM-Mφ and M-Mφ (Fig. 6B). However, C5a still suppressed IL-6 and TNF production in the presence of the neutralizing Ab against human IL-10 (Fig. 6A, 6B), thus demonstrating that the immunomodulation by C5a was independent of IL-10. Therefore, C5a-enhanced LPS-induced IL-10 production does not account for the inhibition of inducible IL-6 and TNF production by C5a. Apart from IL-10, we also found that the immunomodulation by C5a was also independent of LPS-induced PI3K signaling in GM-Mφ (Fig. 6C, 6D) and in M-Mφ (Fig. 6E, 6F). The presence of the pan-PI3K inhibitor wortmannin at the highest concentration (100 nM) did not abolish C5a-mediated suppression of LPS-inducible IL-6 and TNF release, although wortmannin on its own appears to enhance LPS-induced IL-6 and TNF production from macrophages at 100 nM concentration.

C5a attenuation of LPS-induced IL-6 is selectively TLR4 mediated in macrophages

TLR4 signaling involves two major pathways, the Mal/MyD88- and the TRAM/TRIF-dependent pathways (7). To determine whether C5a affected responses through multiple TLRs in primary human macrophages, poly(I:C) (TLR3 agonist signaling via TRIF) and IMQ (TLR7 agonist signaling via MyD88) were used. In contrast to TLR4 activation by LPS, C5a amplified poly(I:C)-induced (TRIF-dependent) IL-6 production from GM-Mφ (Fig. 7B), whereas it did not induce detectable levels of this cytokine in monocytes and M-Mφ (Fig. 7A, 7C). In the case of IMQ-induced (MyD88-dependent) IL-6 production, C5a enhanced this response in monocytes, as well as in both macrophage populations. C5a also increased poly(I:C)-induced and IMQ-induced IL-10 production in monocytes and M-Mφ (Fig. 7D, 7F), but it did not modulate this response in GM-Mφ (Fig. 7E). These results suggest that suppression of inducible IL-6 production by C5a in GM-Mφ and M-Mφ is selective for TLR4 signaling.

C5a enhances clearance of S. Typhimurium from HMDMs

There is evidence in support of C5aR/TLR crosstalk being exploited by some pathogens for host subversion (12, 35). Therefore, the
impact of C5a on the ability of HMDMs to clear S. Typhimurium was examined. As expected, C5a alone did not have any bactericidal effect on S. Typhimurium (Fig. 8A). Instead, C5a promoted the clearance of this bacterial pathogen from M-Mφ, an effect that was reversed by the C5aR antagonist 3D53 (Fig. 8B). In cytotoxicity assays monitoring lactate dehydrogenase release, infection assay

FIGURE 5. C5a-induced ERK1/2 phosphorylation is amplified in macrophages but not monocytes by LPS. To quantify (A–C) ERK1/2, (D–F) p38, and (G–I) JNK phosphorylation, cells (monocytes, GM-Mφ, and M-Mφ) were treated with LPS (5 ng/ml) with and without C5a (30 nM) for 10 min, after which cells were lysed. Phosphorylated ERK1/2, p38, and JNK were measured by AlphaScreen SureFire assays. Treatments by C5a, LPS, or both are represented by broken lines, dotted lines, and solid lines, respectively. Error bars are means ± SEM of three independent experiments (n = 3). **p < 0.01, ***p < 0.001 by student t test.

FIGURE 6. C5a suppression of LPS-induced IL-6 and TNF in macrophages is IL-10- and PI3K-independent. To determine dependency of IL-10, GM-Mφ (black) and M-Mφ (white) were pretreated with neutralizing Ab against human IL-10 (10 μg/ml, 30 min), or for dependency of PI3K signaling, wortmannin (1–100 nM, pretreated for 1 h) prior to LPS (5 ng/ml) with and without C5a (30 nM) stimulation, after which (A, C, E) IL-6 and (B, D, F) TNF production were monitored at 24 h after stimulation. Error bars are means ± SEM of three independent experiments (n = 3). *p < 0.05, **p < 0.01, ***p < 0.001 by Student t test.
supernatants harvested 8 h after infection showed no differences in macrophage viability irrespective of treatment (DMSO, SLI344, hC5a, 3D53, or combinations), thereby indicating that reduced bacterial loads upon C5a treatment were not a result of increased macrophage death. Thus, C5a enhances human macrophage antimicrobial responses while reducing production of key proinflammatory cytokines from these cells.

**Discussion**

Most pathogens trigger both complement and TLR activation, and crosstalk between these two systems could potentially affect inflammatory and antimicrobial responses in vivo in infectious disease. However, studies on C5aR/TLR crosstalk have typically focused on monocytes or macrophages separately. No previously reported study has directly compared the impact of crosstalk on the functional responses of primary human monocytes versus donor-matched human macrophages. The present study has found that the myeloid differentiation state is an important variable in determining the impact of C5aR/TLR4 crosstalk on inflammatory responses of human monocytes and macrophages. New findings in this study suggest that C5a modulates TLR4 signaling in macrophages to favor pathogen clearance while simultaneously limiting excessive inflammatory responses. This control may be important during localized infections, whereas C5a detection by monocytes likely acts as an alarm signal that triggers or amplifies inflammatory responses during systemic infections.

The mechanisms of C5aR/TLR4 crosstalk are complex and evidently species- and cell type–dependent. In human monocytes, C5aR signaling has been reported to both downregulate (36) and upregulate (37) TLR-induced IL-12 production. Although those studies were performed on IFN-γ–primed human blood monocytes, the second signal used to stimulate the cells was different. C5a suppressed LPS-induced IL-12 production but upregulated *S. aureus* Cowan I–induced IL-12 production. Although not addressed in either study, the variations reported are likely due to different TLRs being activated and differentially affected by C5aR signaling. The present study emphasizes that C5a suppression of IL-6 production is specific to LPS/TLR4 signaling in human

**FIGURE 7.** Suppression by C5a of inducible IL-6 production from macrophages is selective for LPS responses. Human CD14+ monocytes (gray) and HMDMs (GM-MΦ, black; M-MΦ, white) were treated with 1) LPS (TLR4 agonist, 5 ng/ml) with and without C5a (30 nM) stimulation, 2) poly(I:C) (TLR3 agonist, 10 μg/ml) with and without C5a (30 nM) stimulation, or 3) IMQ (TLR7 agonist, 10 μg/ml) with and without C5a (30 nM) stimulation for 24 h in 10% FBS/IMDM. ELISA was used to detect (A–C) IL-6 and (D–F) IL-10 secreted in culture media. Error bars are means ± SEM of three independent experiments (n = 3). *p < 0.05, **p < 0.01, ***p < 0.001 by Student t test.

**FIGURE 8.** C5a decreases *S. Typhimurium* survival in M-MΦ. (A) Growth of *S. Typhimurium* was monitored with and without C5a (100 nM) for 7 h. (B) M-MΦ were infected with *S. Typhimurium* (MOI of 100) with and without C5a (30 nM). For antagonism of C5aR, M-MΦ were pretreated with 3D53 (1 μM) for 30 min prior to C5a stimulation. Intramacrophage survival was assayed at 2 and 8 h after infection. Graph shows a representative of three independent experiments. Error bars are means ± SEM of experimental triplicates. **p < 0.01, ***p < 0.001 by Student t test.
macrophages, because IL-6 production is actually boosted by C5aR crosstalk with other TLRs, such as TLR3 and TLR7. Similarly, in mouse macrophages, C5a was reported to selectively crosstalk with TLR4, but not TLR3, in suppressing IL-27 (p28) production (38). C5a is thus not only a potent chemotactic agent, attracting immune cells to site of infection or injury, but it also differentially modulates host immune responses, possibly tailoring them to match a particular type of infection.

The differentiating agents GM-CSF and M-CSF have differential effects on macrophage lineage populations, which contribute to their functional heterogeneity (39). GM-Mφ are often considered as “M1-like” macrophages, whereas M-Mφ are classed as “M2-like” macrophages, according to their cytokine and gene expression profiles (40, 41). Whereas LPS-induced inflammatory cytokine production was clearly enhanced in GM-Mφ compared with M-Mφ in the present study, C5a exerted a similar suppressive effect on inflammatory responses in both human macrophage populations. Thus, the phenotypic switch in C5a responsiveness during monocyte to macrophage differentiation occurred irrespective of the differentiating agent. In the future, it will be of interest to determine whether other macrophage-polarizing factors (e.g., IL-4, IgG) influence the ability of C5a to suppress TLR4-inducible inflammatory mediator production. Although the basal levels of C5AR and TLR4 increased during the course of differentiating monocytes to macrophages, the differences in the expression levels are unlikely to be the cause of the phenotypic switch in the crosstalk. First, C5a treatments did not alter C5AR expression levels. Second, C5a had opposing effects on inducible cytokine production when the macrophages were challenged with poly(I:C) or IMQ, instead of LPS.

The effects of C5a on TLR4 responses were PTX-sensitive in both monocytes and macrophages, suggesting a Gaii-sensitive mechanism. C3a can also bind to a PTX-sensitive receptor, C3aR, that can recruit Gi coupling when activated. Others have shown that C3a acting on IFN-γ–primed macrophages from C5AR−/− mice (14), as well as other Gi-specific ligands acting on IFN-γ–primed human monocytes (42), can negatively regulate LPS-induced IL-12 production. Also, PI3K signaling has been implicated in C5a regulation of LPS signaling. The pan-PI3K inhibitor wortmannin was reported by La Sala et al. (42) to block the suppression by C5a of IL-12p70 production, but this was not the case in the study performed by Hawlisch et al. (14). In our study, wortmannin treatment had no impact on C5a-mediated suppression of LPS-induced IL-6 and TNF production in human macrophages under the conditions examined. This suggests that the crosstalk is not only tightly regulated at multiple checkpoints in the signaling cascade, but that it is also highly dependent on the activation status of the cells.

As shown previously in human neutrophils, C5a can activate the Raf/MEK/ERK signaling cascade either directly via Gi coupling or indirectly via Gi coupled activation of PLCβ/PKC pathways (32, 43). We found that C5a-mediated suppression of LPS-induced IL-6 and TNF was independent of PLCβ signaling (Supplemental Fig. 2). Although C5a quickly phosphorylated ERK1/2 in both monocytes and macrophages, consistent with the literature (14, 15), only in macrophages did C5a increase c-Raf phosphorylation in the presence of LPS, leading to a further downstream increase in phosphorylation of MEK and ERK. Because LPS and C5a did not synergize to alter other MAPKs (p38 and JNK) in monocytes and macrophages, we attribute the differences in monocytes and macrophages to the activation of the Gaii-c-Raf/MEK/ERK pathway by C5a with LPS in macrophages versus monocytes.

Low concentrations of LPS failed to stimulate ERK phosphorylation. However, only high concentrations of LPS triggered delayed ERK1/2 activation (20 min after stimulation) by comparison with the C5a response, which occurs within 5 min. The MAP3K tumor progression locus 2 is essential for activation of ERK1/2 by multiple TLRs, including TLR4, in macrophages (44). Consistent with this, we observed that LPS triggered MEK1 phosphorylation in macrophages independently of activation of another MAP3K, c-Raf. ERK activation can produce different results, depending on the stimulus and cell types, especially when comparing primary cells versus laboratory cultured cell lines (45). C5a enhanced ERK1/2 activation in rat neutrophils, and this was associated with an increase in IL-6 production (46). This contrasts with our present findings for human macrophages. Complement-mediated ERK1/2 activation also inhibited IL-12 production in human monocytes and mouse macrophages (14, 42), whereas the opposite was observed in mouse dendritic cells (47). Such reported variations in effects of C5a on LPS responses reinforce the view that observations of complement-mediated effects on TLR signaling are likely to not only be cell- and species-specific, but also to vary with the inflammatory readout being monitored.

In summary, in this study we have identified C5aR as a regulatory switch that modulates LPS/TLR4 signaling via the Gaii-c-Raf/MEK/ERK signaling axis in primary human macrophages, but not in monocytes. Extrapolating data from mice to humans, although convenient, is often not appropriate (48). Regulatory mechanisms involved in C5aR/TLR4 crosstalk that were previously reported in mouse studies may not be conserved in humans. Differential effects of C5a on monocytes versus macrophages have been demonstrated in this study, consistent with a need to either amplify monocyte proinflammatory responses during systemic danger recognition or attenuate macrophage proinflammatory cytokine responses (without compromising microbicidal activity) during localized infections. Many therapies, including chemotherapy, radiation, cancer vaccines, and small-molecule drugs, already use immune cell–specific treatment strategies (49), but the rational basis for targeting specific cell types frequently lacks a level of understanding necessary to optimize such treatments. Studies such as this one have the potential to unlock secrets relating to interacting human immunoreceptors and control mechanisms that are not obvious in studies targeting one particular receptor on a specific human immune cell type, let alone on immune cells from other mammalian species.

Acknowledgments
We acknowledge the Australian Red Cross for generously providingbuffy coats for human monocyte isolation.

Disclosures
The authors have no financial conflicts of interest.

References
23. Dinakar, C., A. Malur, B. Raychaudhuri, L. T. Buhrow, A. L. Melton,
22. Ariffin, J. K., and M. J. Sweet. 2013. Differences in the repertoire, regulation and
19. Zaal, A., S. N. Lissenberg-Thunnissen, G. van Schijndel, D. Wouters,
29. Scola, A. M., A. Higginbottom, L. J. Partridge, R. C. Reid, T. Woodruff,
42. la Sala, A., M. Gadina, and B. L. Kelsall. 2005. Gi-protein-dependent inhibition