

# C5a Negatively Regulates Toll-like Receptor 4-Induced Immune Responses

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## Summary

The complement system and the Toll-like receptors (TLRs) are two central arms of innate immunity that are critical to host defense as well as the development of adaptive immunity. Most pathogens activate both complement and TLRs, suggesting the potential for crosstalk between the two systems. We show here that the complement-derived C5a anaphylatoxin negatively regulates TLR4- and CD40-induced synthesis of IL-12 family cytokines (IL-12, IL-23, and IL-27) from inflammatory macrophages ( $M\phi$ s) by extracellular signal-regulated kinase- and phosphoinositide 3 kinase-dependent pathways. This decreased cytokine response translates into a decreased T helper type 1 (Th1) response in vitro and in vivo. Accordingly, we found enhanced Th1 immunity in C5a receptor-deficient mice, something that conferred protection from *Leishmania major* infection. Our findings identify the negative impact of C5a on IL-12 family cytokines as an important mechanism for regulating Th1 polarization in response to innate and adaptive immune network activation.

## Introduction

The innate immune system recognizes microbes by their expression of unique molecular structures that do not occur in the host. These invariant structures referred to as pathogen-associated molecular patterns (PAMPs) are recognized by pattern recognition receptors (PRRs) of the innate immune system. The complement system and the TLRs are two central arms of innate immunity that have the ability to recognize PAMPs and to destroy microbial invaders.

Lipopolysaccharide (LPS) from gram-negative bacteria is a prototype PAMP that interacts with a cellular LPS-recognition complex. TLR4 plays a dominant signaling role in this complex, as it is able to bind distinct adaptor molecules that drive the cellular response (Akira and Takeda, 2004). In antigen-presenting cells (APCs), ligation of TLR4 induces the production of IL-

12, a heterodimeric proinflammatory cytokine that provides a critical link between innate resistance and adaptive immunity (Trinchieri, 2003). Of the multiple immunomodulatory functions of IL-12, two are of particular importance for the early phase of innate immunity and the later adaptive immune response to pathogens: the induction of interferon- $\gamma$  (IFN- $\gamma$ ) from resting CD8<sup>+</sup> T cells and NK cells and the polarization of naive Th cells toward Th1 effector cells. IFN- $\gamma$  plays a key role in  $M\phi$  activation; which is critical for control of intracellular pathogens (Gordon, 2003).

In addition to IL-12, two other heterodimeric cytokines, IL-23 and IL-27, have recently been described that establish the IL-12 family of cytokines (Trinchieri, 2003). Data accumulated from recent publications suggest that some of the multiple immunoregulatory activities previously accredited to IL-12 are provided by IL-27 and/or IL-23. As such, IL-27 exerts pro- as well as anti-inflammatory properties (Villarino et al., 2004). IL-23 has a major role in mediating and maintaining chronic inflammation (Cua et al., 2003) by activation of a specific IL-23 receptor on inflammatory  $M\phi$ . In contrast to IL-12, IL-23 promotes proliferation of murine memory T cells, suggesting that IL-23 contributes to the maintenance of Th1-committed memory T cells (Oppmann et al., 2000). In addition to TLR4 signaling, CD40 signaling is of critical importance for the induction of IL-12 family cytokines and the development of Th1 adaptive immune responses. CD40 is expressed by APCs and becomes activated by crosslinking with CD40 ligand on T cells as an important feedback mechanism between activated T cells and APCs. Importantly, microbial priming of APCs is required to induce CD40 expression as a prerequisite for CD40 ligand-induced production of IL-12 (Schulz et al., 2000).

Obviously, excessive or inappropriate production of IL-12 family cytokines in response to PRR or CD40 ligand activation can become harmful to the host, resulting in endotoxic shock (Trinchieri, 2003). Further, it can lead to imbalanced Th1-Th2 immune responses affecting antiparasitic immunity (Sacks and Sher, 2002) and the development of autoimmune diseases (Yadav and Sarvetnick, 2003). To prevent such undesirable outcomes, gate-keeping systems have evolved that function as endogenous regulators of TLR signaling pathways, which control the magnitude of IL-12 cytokine family production (Akira and Takeda, 2004). Importantly, this kind of regulation appears to be critical during the second or continuous exposure to microorganisms. In contrast, less is known about pathways that directly impact TLR signaling during first encounters with pathogens.

Complement activation is one of the earliest innate immune responses induced by pathogens. The first evidence of a regulatory link between the complement system and TLR-mediated immune responses was provided by reports demonstrating that activation of complement receptor 3 (CR3) (Marth and Kelsall, 1997) and the complement regulator molecule CD46 (Karp et al., 1996) by C3 cleavage products promotes efficient con-

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trol of PRR-induced synthesis of IL-12 from human monocytes. Further, the C5 cleavage fragment C5a was found to downregulate LPS- and *Staphylococcus aureus* Cowan strain I (SAC)-induced synthesis of IL-12 in human monocytes, but not in monocyte-derived dendritic cells (DCs) (Braun et al., 2000; Wittmann et al., 1999). In contrast, ablation of C5 in murine *Mφs* leads to reduced IL-12 production in response to IFN- $\gamma$  + SAC stimulation, suggesting that C5a can enhance IL-12 production (Karp et al., 2000). These conflicting data suggest a complex, cell-dependent role of C5a in IL-12 regulation. The underlying molecular mechanisms and the consequences of such regulation on adaptive immunity and infection remain elusive.

We show here that C5a has a negative impact on TLR4-induced synthesis of IL-12, IL-23, and IL-27 and provide evidence that C5aR signaling pathways comprising extracellular signal-regulated kinase (ERK) and PI3K are of critical importance for this regulation. In addition to its role in dampening TLR4-driven synthesis, C5a also regulates CD40-induced IL-12 family cytokine production through the signaling intermediate ERK1/2. Our data further identify IFN regulatory factor 1 (IRF-1) and IFN consensus sequence binding protein (ICSBP; IRF-8) as crucial transcription factors downstream of ERK and PI3K pathways, respectively. These data indicate that C5a modulates both innate (TLR4) and adaptive (CD40) immune responses that drive the production of IL-12 family cytokines. These findings translated into a suppressive effect of C5a on Th1 polarization, the in vivo relevance of which was documented by the acquisition of resistance to *Leishmania major* infection by the genetic deficiency of the C5a receptor in normally susceptible BALB/c mice.

## Results

### C5a Downregulates TLR4-Induced Expression of IL-12 Family Cytokines

We investigated the effect of C5a receptor (C5aR) signaling on TLR4-induced IL-12 family cytokine production from thioglycollate-elicited and IFN $\gamma$ -primed *Mφs*. Cells were stimulated with LPS from 1.5 to 24 hr in the presence or absence of C5a (Figure 1A). Consistent with previous data (Trinchieri, 2003), LPS induced the expression of high levels of IL-12p70, with a maximum 16 hr after stimulation. No IL-12p70 secretion was detected in unstimulated *Mφs* or *Mφs* that were treated with C5a alone (data not shown). In accordance with data obtained with human monocytes (Braun et al., 2000; Wittmann et al., 1999), IL-12p70 production in murine *Mφs* was significantly inhibited by preincubation with C5a in a time- (Figure 1A) and dose-dependent (Figure 1B) manner. In addition to C5a, the C3a anaphylatoxin negatively regulated IL-12p70 production (Figure 1C), albeit to a lesser extent than C5a (31% versus 84% at 200 nM). In contrast, the chemokines CCL2 and CXCL2 had no effect, whereas CCL3 significantly increased IL-12p70 production (Figure 1D). C5a had no impact on TLR4-induced release of IL-12p70 in C5aR<sup>-/-</sup> mice (Figure 1C), strongly suggesting that the regulatory effect relates specifically to C5aR signaling and not to the recently described second receptor for C5a C5L2 (Cain and Monk, 2002).

Next, we assessed whether IFN- $\gamma$  priming is a prerequisite for the inhibitory effect of C5a on IL-12p70. We found substantial production of IL-12p70 in *Mφs* in response to LPS without prior IFN- $\gamma$  priming (Figure 1C). Importantly, preincubation with C5a inhibited the synthesis of IL-12p70, suggesting that exogenous IFN- $\gamma$  priming is not a prerequisite for the inhibitory effect of C5a on IL-12p70 production from elicited *Mφs*. Further, TLR4 activation in unprimed *Mφs* induced the release of significant amounts of IFN- $\gamma$ , which were substantially reduced (66%) in the presence of C5a in C5aR-sufficient mice (Figure 1F), but not in C5aR-deficient mice (data not shown).

IL-12 is a heterodimeric cytokine comprising the p35 and the p40 subunits, both of which are largely regulated at the level of transcription (Liu et al., 2003). In addition to IL-12, two other heterodimeric cytokines, named IL-23 (Oppmann et al., 2000) and IL-27 (Pflanz et al., 2002), have been identified. IL-27 consists of an IL-12p40-related protein, EBV-induced gene 3 (EBI3), and the newly discovered IL-12 p35-related protein p28, whereas IL-23 comprises the p40 subunit associated with an IL12 p35-related protein, p19. To determine a possible impact of C5a on the expression of different IL-12 family cytokine genes, we assessed IL-12/IL-23p40, IL-12p35, IL-27p28, IL-23p19, and IL-27EBI3 mRNA levels by quantitative real-time PCR. All IL-12 cytokine genes were upregulated by TLR4 signaling, although to different extents, except IL-27EBI3 (Figure 1G). The strongest effect was on IL-12p40 (6500- to 17,000-fold) followed by IL-12p35 (400- to 1300-fold), IL-27p28 (460- to 900-fold), and IL-23p19 (150- to 490-fold). C5a markedly inhibited the upregulation of all IL-12 family cytokine genes, indicating that C5a regulates IL-12 family expression at the mRNA level. This is not a general suppressive effect of C5a on *Mφs*, as we found no suppressive effect of C5a on other cytokines induced by TLR4 activation such as TNF- $\alpha$  or IL-10 (data not shown).

### C5a Regulates Expression of IL-12 Family Cytokines through Activation of PI3K and ERK1/2

C5a exerts its effector functions by ligating a specific C5aR (CD88), which belongs to the large superfamily of G protein-coupled receptors (GPCRs). Many of the effector functions of C5a are entirely sensitive to blocking of the G $\alpha_i$  subunit by pertussis toxin (PTX); however, inhibition of IL-12 in human monocytes is only partially blocked by this treatment (Braun et al., 2000). We found a similar effect of PTX treatment by using murine *Mφs*, supporting the view that in addition to G $\alpha_i$ , other G $\alpha$  and the  $\beta/\gamma$  subunits contribute to the inhibitory effect of C5a on IL-12p70 expression (data not shown). In human neutrophils, C5aR can couple to G $\alpha_i$  and G $\alpha_{16}$ , resulting in activation of the Ras-Raf-MEK-ERK pathway (Buhl et al., 1995). Further, C5aR triggering in human neutrophils was suggested to activate class I $\beta$  PI3K (PI3K $\gamma$ ) (Perianayagam et al., 2002). PI3K $\gamma$  belongs to a family of lipid kinases involved in generating distinct phosphoinositides that are important second messengers for intracellular signaling (Koyasu, 2003).

To identify the signaling pathways that mediate the inhibition of the different IL-12 family subunits (Figure

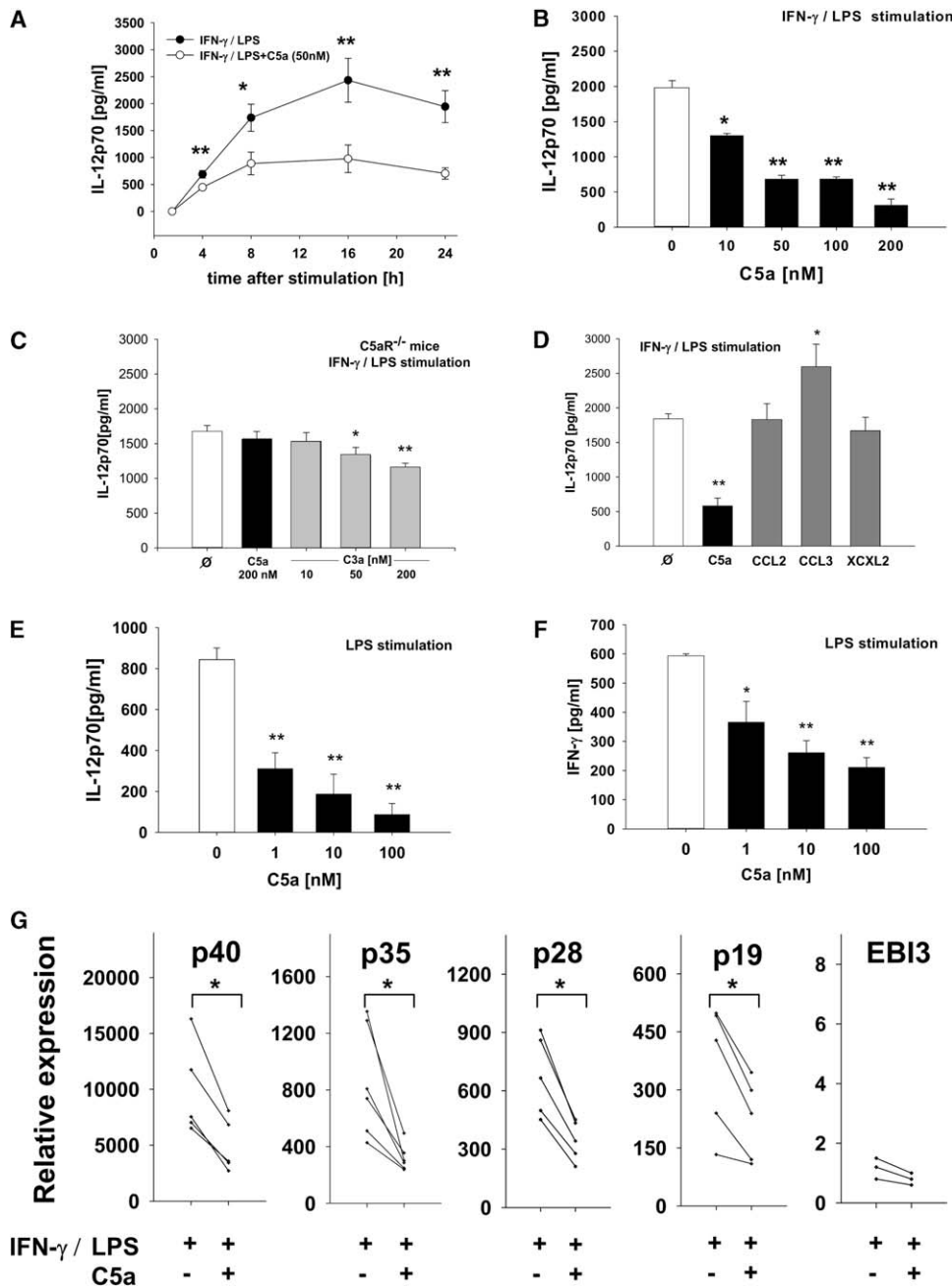


Figure 1. C5a Negatively Regulates TLR4-Induced IL-12 Family Cytokines and IFN- $\gamma$

(A and B) Time- (A) and dose-dependent (B) downregulation of IL-12p70 production from IFN- $\gamma$ -primed M $\phi$ s. Cells were stimulated with LPS (100 ng/ml) in the absence or presence of C5a (50 nM) and incubated for the indicated times.

(C) Impact of C5a and C3a on LPS-induced IL-12p70 production from IFN- $\gamma$ -primed M $\phi$ s of C5aR<sup>-/-</sup> mice.

(D) Impact of C5a, CCL2, CCL3, and CXCL2 on TLR-4-induced IL-12p70 production. All reagents were used at a concentration of 50 nM.

(E and F) Dose-dependent inhibition of IL-12p70 (E) and IFN- $\gamma$  (F) secretion from unprimed M $\phi$ s.

(G) mRNA expression levels of IL-12 family cytokine subunits in IFN- $\gamma$ -primed M $\phi$ s quantified by real-time RT-PCR. Data are depicted as expression levels relative to expression in unstimulated M $\phi$ s.

Values shown are the mean  $\pm$  SEM of at least three experiments. \*p < 0.05; \*\*p < 0.005.

1G), we pharmacologically targeted PI3K and ERK1/2, as activation of either molecule has been associated with inhibition of TLR-induced IL-12 production. Class I<sub>A</sub> PI3K can act as an endogenous negative regulator of TLR4 signaling in DCs (Fukao et al., 2002), probably by

blocking p38MAPK activation. LPS-activated ERK1/2 can downregulate the expression of the transcription factor IRF-1 (Goodridge et al., 2003), an important inducer of IL-12p35 transcription (Liu et al., 2003). We found that C5a time-dependently activated PI3K (Fig-

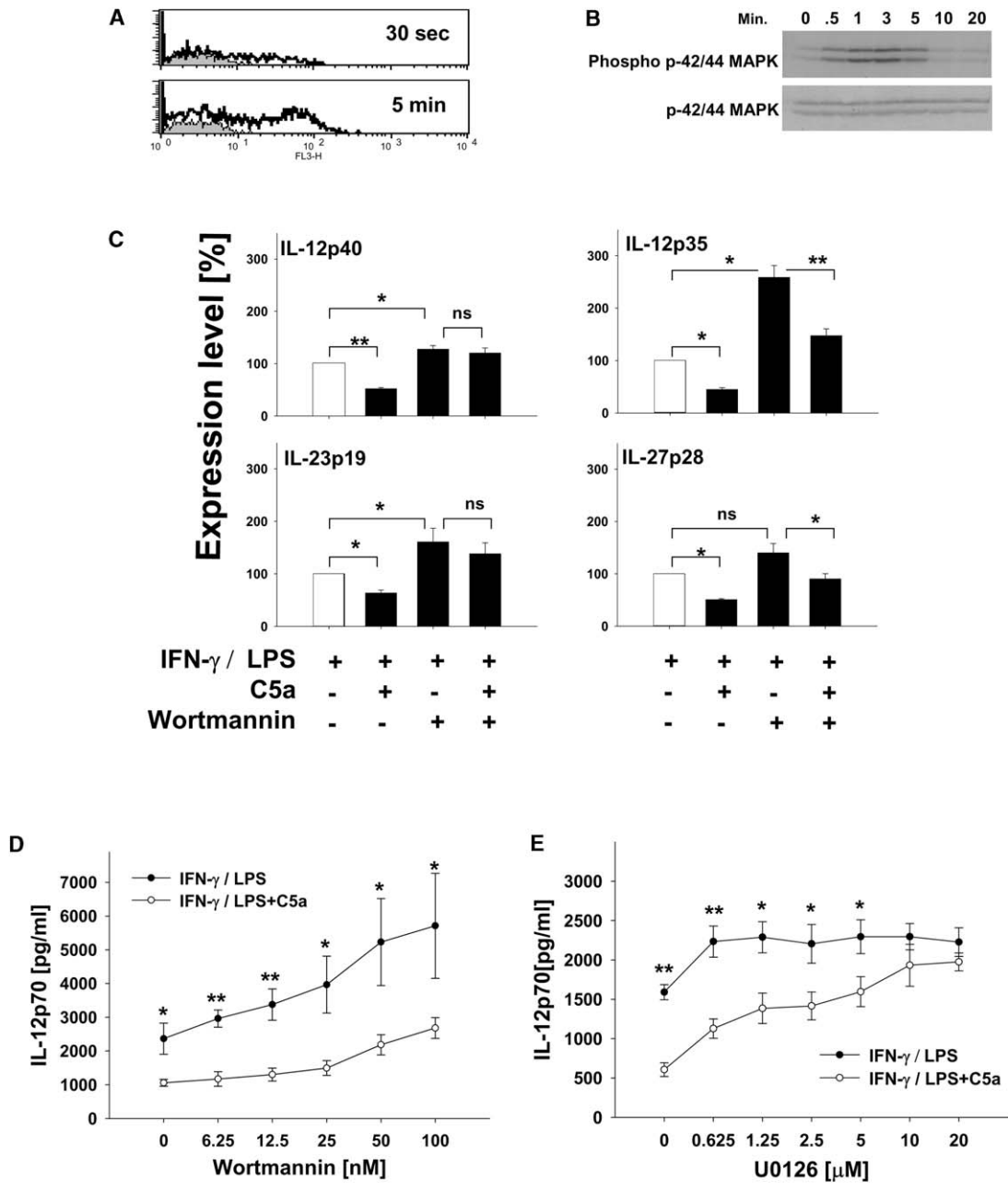


Figure 2. C5a Regulates IL-12 Family Cytokine Expression through Activation of ERK1/2 and PI3K

(A) C5a-induced PIP3 formation. IFN- $\gamma$ -primed M $\phi$ s were stimulated with C5a (50 nM) for 30 s and 5 min, and PIP3 formation was determined by an anti-PIP3 specific mAb (white histogram) in relation to PIP3 formation in unstimulated cells (gray histogram) by flow cytometry. (B) C5a-induced activation of ERK1/2. IFN- $\gamma$ -primed M $\phi$ s were stimulated with C5a (50 nM) for the indicated times, and cell lysates were analyzed by Western blotting using anti-phospho ERK1/2-specific Ab. Equal loading of the gel was confirmed by reprobing the stripped membrane with ERK1/2-specific Ab. (C) Contribution of PI3K to the inhibitory effect of C5a on IL-12 family cytokine subunit mRNA expression. mRNA expression was quantified by real-time RT-PCR. Expression levels in cells stimulated with LPS are set 100%. ns, not significant. (D and E) Contribution of PI3K (D) and contribution of ERK1/2 (E) to the inhibitory effect of C5a on IL-12p70 production. IFN- $\gamma$ -primed M $\phi$ s were treated with the indicated concentrations of the PI3K inhibitor wortmannin or the MEK1/2 inhibitor U0126 1 hr prior to stimulation with LPS (100 ng/ml) or LPS/C5a (200 nM). IL-12p70 levels in supernatants were examined 16 hr after cell stimulation by ELISA. Values shown are the mean  $\pm$ SEM of at least three experiments. \*p < 0.05; \*\*p < 0.005.

ure 2A) and ERK1/2 (Figure 2B), indicating that CD88 signaling may amplify the effect of these endogenous inhibitory pathways. First, to assess the contribution of

PI3K on LPS-induced production of IL-12 family cytokines, we blocked the function of this molecule by using wortmannin. This treatment increased mRNA

synthesis of IL-12/IL-23p40, IL-12p35, and IL-23p19, but not that of IL-27p28, suggesting that PI3K regulates TLR4-induced IL-12 and IL-23 production, but not that of IL-27. Further, PI3K blockade had a strong impact on the inhibitory effect of C5a on IL-12/IL-23p40 and IL-23p19, whereas the inhibitory effect on IL-12p35 and IL-27p28 was not significantly affected (Figure 2C). These data suggest that C5aR signaling has a negative impact on TLR4-induced IL-23 production by a PI3K-dependent pathway. Further, our findings indicate a role for PI3K in C5a-mediated inhibition of IL-12, whereas the regulation of IL-27 is PI3K independent. As only the p40 subunit of biologically active IL-12 was suppressed by C5a-induced PI3K, it remained unclear whether this effect would impact the formation of the IL-12p70 heterodimer. Thus, we determined the expression of IL-12p70 in response to TLR4 activation in the presence of C5a and wortmannin. Similar to what has been found with murine DCs (Fukao et al., 2002), LPS-induced IL-12p70 secretion from M $\phi$ s was dose-dependently enhanced. However, wortmannin treatment had no impact on the inhibitory effect of C5a on TLR4-induced IL-12p70 (Figure 2D). These data suggest that the enhanced production of IL-12p70 in response to PI3K inhibition depends on increased IL-12p35 expression, which is consistent with a previous report that IL-12p70 formation is limited by IL-12p35 (Snijders et al., 1996). Accordingly, the C5a-induced inhibition of IL-12p70 is independent of its PI3K-mediated negative regulation of IL-12/IL-23p40.

In a search for signaling pathways that may account for the effect of C5a on IL-12p70, we focused on ERK1/2. As expected (Hacker et al., 1999), blockade of MEK1/2 dose-dependently increased the production of IL-12p70. Importantly, pharmacological targeting of the MEK-ERK pathway, by using the MEK1/2-specific inhibitor U0126, abrogated the inhibitory effect of C5a (Figure 2E), suggesting that activation of ERK1/2 negatively regulates IL-12p35 expression as the critical mechanism by which C5a impacts IL-12p70. Importantly, the control molecule U0124 had no impact on the negative regulatory effect of C5a, suggesting that the effect was MEK-ERK specific (data not shown). The inhibitory effect of C5a is independent of IL-10, as C5a blocked IL-12p70 production from M $\phi$ s of IL-10-deficient mice and from mice that had been treated with an anti-IL-10R antibody (data not shown).

#### C5a Inhibits TLR4-Induced Upregulation of IRF-1 and ICSBP

In murine M $\phi$ s, activation of TLR4 and TLR9 induces ERK activity through a MEK1/2-dependent pathway (Hacker et al., 1999). Recent data suggest a link between ERK activity and the regulation of IRFs, which have been implicated to be important transcription factors for IFN- $\gamma$ -primed and TLR4-induced IL-12p35 and IL-12/IL-23p40 transcription, respectively (Liu et al., 2003; Wang et al., 2000). Available data from the literature suggest a model in which p35 transcription is mainly regulated by IRF-1 (Liu et al., 2003), whereas the transcription of the IL-12/IL-23p40 subunit depends mainly on ICSBP (Wang et al., 2000) and to some extent to IRF-1 (Maruyama et al., 2003; Wang et al., 2000). Im-

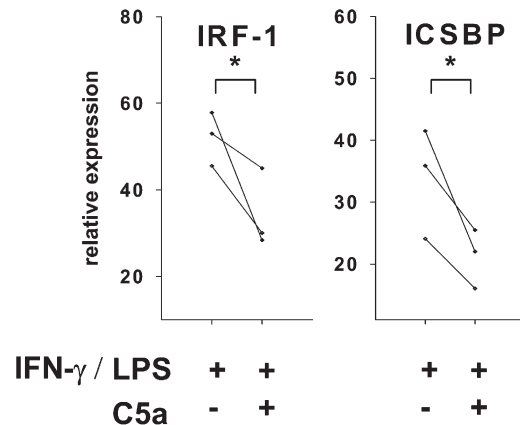


Figure 3. C5a Downregulates the TLR4-Induced Transcription Factors IRF-1 and ICSBP

IFN- $\gamma$ -primed M $\phi$ s were stimulated with LPS (100 ng/ml) or LPS/C5a (50 nM) for 3 hr. mRNA expression was quantified by real-time RT-PCR and is shown as the expression level relative to the expression in unstimulated cells. Values shown are the mean  $\pm$ SEM of at least three experiments. \* $p < 0.05$ .

portantly, ERK phosphorylation has been found to suppress the synthesis of IRF-1 (Goodridge et al., 2003). Based on these data, we hypothesized that C5a suppresses IRF-1 by an ERK1/2-dependent mechanism. As depicted in Figure 3, mRNA expression of IRF-1 was markedly inhibited when IFN- $\gamma$ -primed M $\phi$ s had been stimulated with LPS in the presence of C5a. Further, C5a treatment suppressed the induction of ICSBP. These findings indicate that a C5aR signaling pathway comprising MEK1/2-ERK1/2-IRF-1 promotes inhibition of IL-12p35 transcription, the net effect of which is a reduced production of IL-12p70. Based on our findings that C5a inhibits production of IL-12/IL-23p40 through activation of PI3K (Figure 2C) and the fact that ICSBP regulates transcription of IL-12/IL-23p40 (Wang et al., 2000), we further suggest a C5aR signaling pathway in which PI3K blocks the production of IL-12/IL-23p40 through inhibition of ICSBP.

#### C5a Blocks CD40-Enhanced IL-12p70 Production through an ERK1/2-Dependent Pathway

In addition to TLR4 ligation, CD40 crosslinking on M $\phi$ s and DCs by CD40 ligand expressed by T cells can induce IL-12 production. This pathway critically depends on prior priming through microbial stimuli (Schulz et al., 2000) ensuring that APCs produce high levels of IL-12p70 only during APC-T cell interactions after the appropriate type of infection. We found a tremendous increase of IL-12p70 production in IFN- $\gamma$ -primed M $\phi$ s that had been treated with a combination of LPS and an agonistic anti-CD40 antibody over that observed in M $\phi$ s treated with LPS alone (Figure 4A). Importantly, IL-12p70 production was almost abrogated when C5a was present during TLR4/CD40 stimulation.

CD40 signals through several pathways, including ERK1/2 and PI3K, both of which are relevant to the inhibitory effect of C5a on TLR4-induced production of

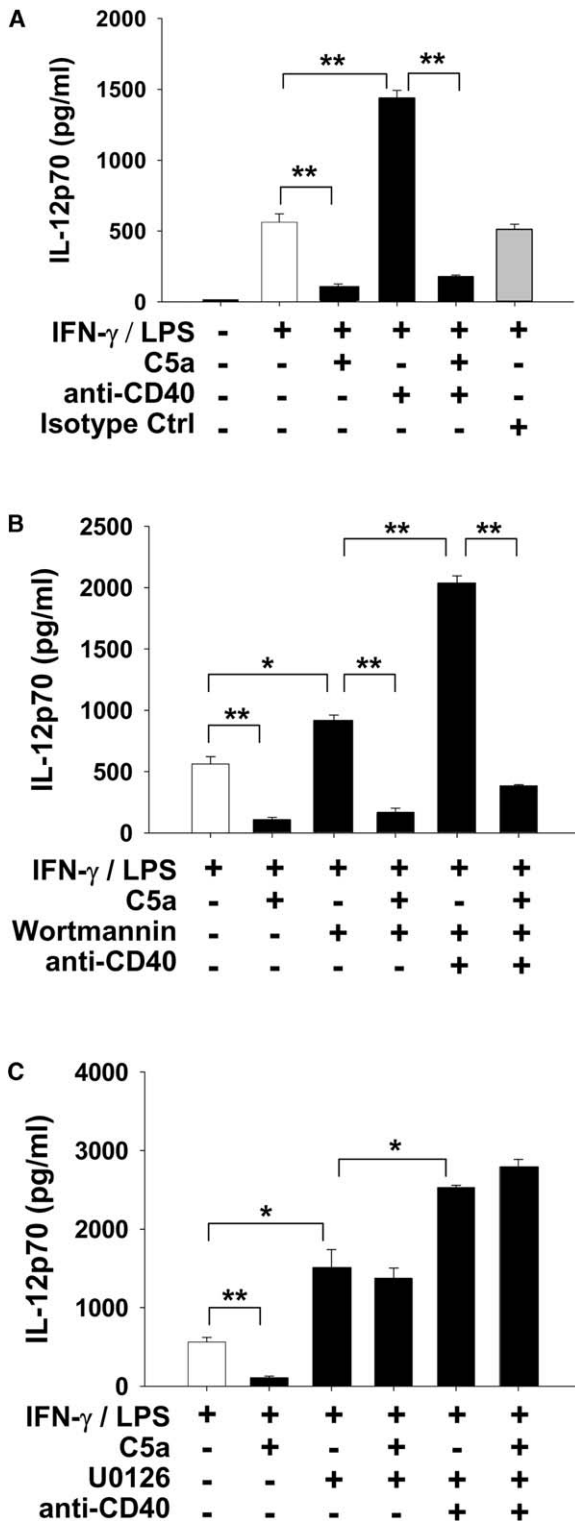


Figure 4. C5a Regulates CD40-Induced IL-12p70 Expression through Activation of ERK1/2

(A) CD40-induced IL-12p70 expression from IFN- $\gamma$ -primed M $\phi$ s stimulated with LPS (100 ng/ml), C5a (200 nM), anti-CD40 antibody (10  $\mu$ g/ml), or an isotype-matched control antibody (Isotype Ctrl). IL-12p70 concentrations were determined from supernatants harvested 16 hr after cell stimulation.

(B and C) Impact of PI3K inhibition (B) and MEK1/2 inhibition (C)

IL-12 family cytokines. Blockade of PI3K resulted in a marked increase of IL-12p70 in IFN- $\gamma$ -primed M $\phi$ s that had been treated with LPS and anti-CD40. Despite this substantial increase in IL-12p70 production, C5a almost completely blocked the production of IL-12p70 in the presence of wortmannin (Figure 4B). Similar to PI3K inhibition, IL-12p70 production substantially increased in response to MEK1/2 blockade with U0126 (Figure 4C). Importantly, MEK1/2 blockade by U0126 abolished the inhibitory effect of C5a (Figure 4C), whereas the administration of the control molecule U0124 had no effect (data not shown). Together, these data suggest that C5aR signaling through MEK1/2-ERK, but not through PI3K, is of critical importance for C5a-mediated inhibition of CD40-induced IL-12p70 production.

### C5a Negatively Impacts Th1 Polarization In Vitro and In Vivo

The potent inhibition of IL-12 family cytokine production by C5a in response to both TLR4 and CD40 activation prompted us to investigate whether C5a impacts polarization of naive T cells toward a Th1 phenotype. We stimulated naive OVA<sub>323-339</sub>-specific TCR transgenic CD4<sup>+</sup> T cells with IFN- $\gamma$ -primed M $\phi$ s that had been pulsed with different concentrations of OVA<sub>323-339</sub> peptide and LPS in the presence or absence of C5a. We found huge concentrations of IFN- $\gamma$  but no IL-4 in the supernatants of M $\phi$  and CD4<sup>+</sup> T cell cocultures, the magnitude of which was dependent on the amount of OVA<sub>323-339</sub> peptide (Figure 5A). In the presence of C5a, IFN- $\gamma$  secretion was substantially reduced. No IL-4 production was measurable. Culturing M $\phi$ s or CD4<sup>+</sup> T cells alone did not result in any measurable IFN- $\gamma$  release. Similarly, intracellular production of IFN- $\gamma$  in OVA-specific CD4<sup>+</sup> T cells was reduced from 22.9% to 14.8%. Very few cells produced IL-4 (0.9%), the percentage of which remained unchanged (0.7%) after C5a treatment (Figure 5B). Thus, C5a attenuates M $\phi$ -induced Th1 skewing in vitro.

In vivo, IL-12-dependent Th1 immune responses are critical for protective immunity against intracellular pathogens such as *Leishmania major*. Importantly, M $\phi$ s infected with *Leishmania* have lost their ability to produce IL-12 in response to TLR4 and CD40 activation (Sacks and Sher, 2002). Based on our in vitro data, we hypothesized that C5aR signaling contributes to the impaired IL-12 production and the consecutive failure of mounting protective Th1 immunity in response to *Leishmania* infection. We tested this hypothesis in the well-characterized murine model of dermal *L. major* infection (Belkaid et al., 2002a) by using C5aR-deficient mice (C5aR<sup>-/-</sup>) backcrossed to the highly susceptible BALB/c background. First, we determined the impact of C5aR signaling on the cutaneous inflammatory response. C5aR<sup>-/-</sup> mice were more resistant to *L. major* infection as compared with wild-type (wt) BALB/c mice as demonstrated by the absence of necrotizing dermal

on CD40-induced IL-12p70 production. Cells were incubated with the PI3K inhibitor wortmannin (100 nM) or MEK1/2 inhibitor U0126 (10  $\mu$ M) 1 hr prior to stimulation.

Values shown are the mean  $\pm$ SEM of at least three experiments.

\*p < 0.05; \*\*p < 0.005.

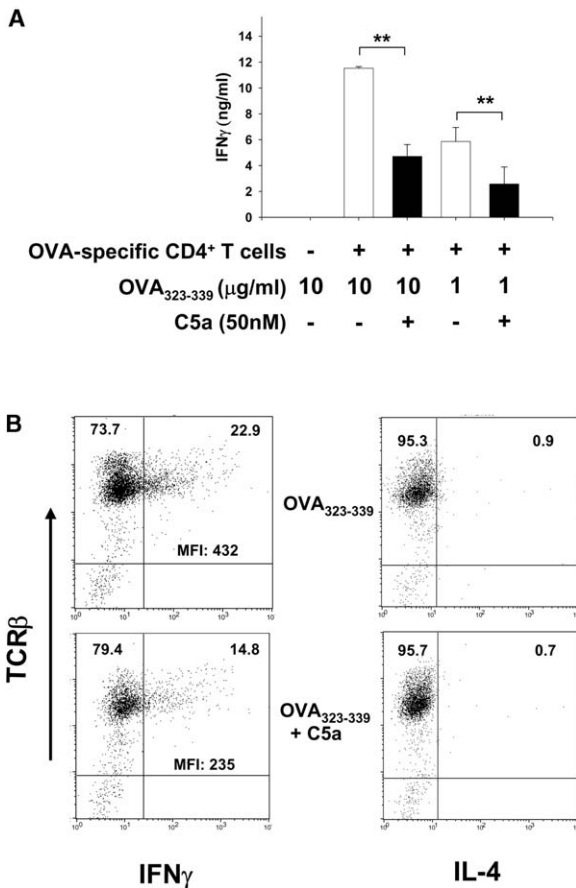


Figure 5. C5a Inhibits Th1 Polarization In Vitro

(A) Impact of C5a on IFN- $\gamma$  production from OVA-specific CD4<sup>+</sup> T cells isolated from TCR-DO11.10/RAG2 [KO] mice. T cells were co-cultured with OVA<sub>322-339</sub> pulsed and LPS (100 ng/ml) stimulated M $\phi$ s for 5 days and restimulated with freshly pulsed M $\phi$ s for another 3 days in the absence or the presence of C5a. C5a and OVA<sub>322-339</sub> were used at the indicated concentrations. Cytokine production was determined by ELISA.

(B) As in (A) except that the cytokine profile was determined by intracellular cytokine staining for IFN- $\gamma$  and IL-4 using flow cytometry.

Values shown are the mean  $\pm$  SEM of at least three experiments. \*\**p* < 0.005.

lesions (Figure 6A). This protective effect was associated with a significant reduction in parasite numbers in the ear (10-fold) and in the draining lymph nodes (3-fold; Figure 6B). Further, numbers of CD4<sup>+</sup> and CD8<sup>+</sup> T cells were significantly increased in draining lymph nodes of C5aR<sup>-/-</sup> mice as compared with BALB/c controls (Figure 6C). Recall of lymph node cells with *L. major* Ag induced considerable production of the Th1 cytokine IFN- $\gamma$  in C5aR<sup>-/-</sup> mice, but not in BALB/c controls. In contrast, the production of the Th2 cytokine IL-4 from lymph node cells of C5aR<sup>-/-</sup> was reduced as compared with the production of cells from BALB/c controls (Figure 6D). Although our data provide no direct evidence that Th1 skewing is caused by the lack of C5aR-signaling specifically in M $\phi$ s, they strongly support the view that C5a plays a critical role in nega-

tive regulation of IL-12 family cytokines in vivo, which ultimately controls the development of type 1 immunity in *L. major* infection.

## Discussion

The production of IL-12 in response to pathogen-induced activation of TLR4 provides a critical link between innate and adaptive immune responses. Clearly, the pleiotropic activities of IL-12 on NK cell, T cell, and B cell function warrant tight control to assure appropriate innate and adaptive immune responses. In addition to the multiple endogenous inhibitory pathways downstream of TLR4 signaling (reviewed in Akira and Takeda [2004]), activation of complement receptors that bind C3 cleavage products have been described to control TLR4-mediated IL-12 release (Karp et al., 1996; Marth and Kelsall, 1997). More recently, C5aR signaling has also been implicated in negative regulation of TLR-induced IL-12 synthesis (Wittmann et al., 1999), although opposite effects have been reported as well (Karp et al., 2000). Our data support the view that C5a is a negative regulator of IL-12 production. In contrast to previous data obtained with human monocytes, we found that the negative regulatory effect of C5a in murine M $\phi$ s is independent of IFN- $\gamma$  priming, suggesting distinct species and/or cell-specific mechanisms (signaling pathways). Our data are in apparent contrast to findings that suggest a positive impact of C5aR signaling on IL-12 production by human monocytes and murine M $\phi$ s in response to SAC stimulation (Karp et al., 2000). SAC is a crude mixture of molecules that is likely to activate TLR2 and multiple other, poorly defined signaling pathways. C5a may synergize with TLR-independent pathways that are important for SAC-induced production of IL-12. Notably, our data demonstrate that C3a also negatively regulates IL-12 production (although at a lower potency), suggesting that C3aR shares to some extent a G $\alpha_i$  signaling pathway with the C5aR in murine M $\phi$ s. CCL2, CCL3, and CXCL2, all of which activate GPCR (CCR2, CCR5, and CXCR2) that can couple to G $\alpha_i$ , had either no impact or did increase (CCL3) IL-12p70 production in murine M $\phi$ s. Our findings are in contrast to data showing that CCL2 negatively regulates bacterial-induced IL-12 production in human monocytes (Braun et al., 2000) but are in agreement with the finding that parasite-induced activation of CCR5 promotes IL-12 production in murine DCs (Aliberti et al., 2003). Apparently, a tremendous plasticity of cell and/or species-specific coupling of distinct G protein has evolved, determining activation of distinct signaling cascades that eventually lead to positive or negative regulation of microbial-driven IL-12 production.

In light of the recent discovery of IL-23 and IL-27, it is now clear that IL-12 is only one member of a growing family of heterodimeric cytokines at the interface between innate and adaptive immunity. Importantly, IL-23 and IL-27 are not mere copycat cytokines that share many functions with IL-12 but have unique functions that are not redundant with IL-12. Data obtained with mice lacking the IL-23-specific subunit p19 (Cua et al., 2003) emphasize a major role for IL-23 in macrophage-

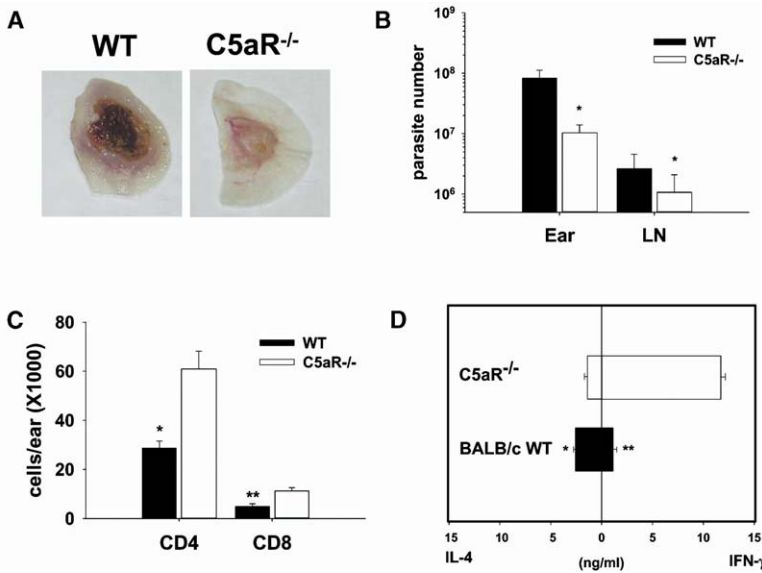


Figure 6. C5aR-Deficient BALB/c Mice Are Almost Resistant and Show a Th1 Response in Response to *L. major* Infection

(A) Appearance of lesions at the inoculation site 7 weeks postinfection.

(B) Number of parasites in *L. major*-infected C5aR<sup>-/-</sup> and wt mice. Parasite numbers in ear tissue and draining lymph nodes from *L. major*-infected mice were determined 4 weeks postinfection.

(C) Numbers of CD4<sup>+</sup> and CD8<sup>+</sup> T cells in ear tissue from *L. major*-infected mice. Cell numbers were calculated from total cell numbers in the ear.

(D) Cytokine secretion from lymph node cells 4 weeks postinfection. Cells from draining lymph nodes were restimulated with soluble *Leishmania* antigen for 72 hr. Cytokine production in the supernatants was examined by specific ELISA.

Values shown are the mean ±SEM of at least three experiments. \*p < 0.05; \*\*p < 0.005.

mediated inflammation, but not in Th1 polarization, providing an explanation for the finding that p40-deficient mice (lacking IL-12 and IL-23) and IL-12Rβ1-deficient mice (lacking the common subunit of IL-12 and IL-23 receptors) are protected from autoimmune disease, whereas p35- and IL-12Rβ2-deficient mice (the latter lacking the IL-12R-specific subunit) are more susceptible than wt mice (Trinchieri, 2003). Our data demonstrate that in addition to its role as a negative regulator of IL-12 production, C5a also suppresses TLR4-induced synthesis of IL-23 and IL-27. Such broad inhibition is likely to affect the host response to infection and to modulate autoimmunity. In support of this view, a central role of C5/C5a was suggested in the pathogenesis of autoimmune arthritis (Ji et al., 2002), systemic lupus (Wang et al., 1996), DTH responses (Tsuji et al., 2000), and allergy (Karp et al., 2000), as well as in resistance to *Listeria* (Gervais et al., 1989) and to blood stage-malaria infection (Sam and Stevenson, 1999). Common to all of these models is their dependency on or exacerbation by IL-12 family cytokines.

The signaling pathways underlying the synthesis of the different subunits of IL-12 family cytokines downstream of TLR4 activation are complex and involve several distinct adaptor molecules, the assembly of which activates the NF-κB family of transcription factors and/or MAPKs (Akira and Takeda, 2004). The contribution of PI3K in this signaling pathway is controversial. Activated class I<sub>A</sub> PI3K has the ability to form a complex with tyrosine-phosphorylated adaptor molecule myeloid differentiation primary-response protein (MyD88) in response to TLR4 triggering (Akira and Takeda, 2004). A study using a dominant negative mutant of the p85 subunit of PI3K suggested that class I<sub>A</sub> PI3K is a positive mediator of TLR4 signaling that activates NF-κB (Li et al., 2003). In contrast, targeting class I<sub>A</sub> PI3K increases the activity of p38 MAPK, which is critical for transcriptional activation of both IL-12p35 and IL-12/IL-23p40 genes (Fukao et al., 2002; Lu et al., 1999). Accordingly, IL-12/IL-23p40 and IL-12p35 production was

shown to be markedly enhanced in DCs of class I<sub>A</sub> PI3K-deficient mice (Fukao et al., 2002), suggesting negative feedback regulation of IL-12 production by class I<sub>A</sub> PI3K. Our data support the latter finding, as pharmacological targeting of PI3K in murine Mφs increased mRNA synthesis of IL-12/IL-23p40, IL-12p35, and IL-23p19. Importantly, we found that C5aR signaling induces class I<sub>B</sub> PI3K activation in inflammatory Mφs, the blockade of which suppressed the inhibitory effect of C5a on IL-12/IL23p40 and IL-23p19. These data suggest that both PI3K isoforms, class I<sub>A</sub> and class I<sub>B</sub>, control the production of IL-12p40. Further, our data identify C5aR signaling as an important upstream mechanism to activate PI3K, which negatively regulates TLR4-induced synthesis of IL-12p40 and IL-23.

In addition to PI3K activation, ERK activation was described to selectively inhibit TLR-induced production of IL-12 in Mφs (Hacker et al., 1999). In agreement with the inhibitory role of ERK, we found increased IL-12p70 production in response to pharmacological targeting of MEK1/2. Importantly, targeting MEK1/2 abrogated the negative regulatory effect of C5a on IL-12p70 production, indicating that ERK1/2 phosphorylation is crucial to this effect. In contrast to data suggesting that the inhibitory effect of ERK on IL-12 production depends on the production of IL-10 (Yi et al., 2002), we found the negative impact of C5a on IL-12 production in Mφs to be independent of IL-10, as suggested previously (Braun et al., 2000; Wittmann et al., 1999).

The strong, negative impact of complement cleavage products on IL-12 production suggests an important role of complement in the control of T cell-mediated adaptive immunity, in particular in Th cell lineage commitment. To date, no data are available that link CR3-, CD46-, or C5a-mediated suppression of IL-12 to impaired Th1 skewing. By using the well-established model of OVA-induced Th1 polarization, we found that C5a suppresses Th1 lineage commitment. In addition to microbial priming via TLR, the concentration and the nature of the antigen, Th cell lineage commitment criti-

cally depends on bidirectional signaling between APCs and T cells. As such, CD40 acts as an important co-stimulatory molecule on APCs to activate Th cells. Vice versa, activated Th cells stimulate APCs through CD40-CD40 ligand interaction as an important positive feedback mechanism amplifying IL-12 production from APCs. Our data provide evidence that this positive feedback mechanism is restrained in the presence of C5a as a second means (in addition to the effect on TLR4) by which C5a suppresses Th1 polarization. Importantly, our data are in contrast to a previous report in which C5a was found to have no impact on CD40 ligand-induced IL-12 production in human monocyte-derived DCs, despite C5aR expression (Braun et al., 2000). Distinct, cell-specific signaling pathways in DCs and M $\phi$ s are likely to account for these differences. The strong impact on Th cell polarization suggests that C5a acts not only at sites of inflammation to suppress IL-12 and IFN- $\gamma$  but also plays an important role in the lymph node to affect T cell priming.

Importantly, we demonstrate that activation of ERK1/2 is critical for the C5a effect on CD40 signaling, a pathway that has been recently implicated in negative regulation of CD40-induced IL-12 production in *Leishmania* infection (Mathur et al., 2004) (see below). Although intriguing, it remains to be determined whether the inhibitory effect of C5a in vivo requires ERK1/2 signaling.

Several intracellular pathogens like *Leishmania*, *Mycobacteria*, *Listeria*, *Histoplasma*, and *HIV* activate the complement cascade, become opsonized by C3b/iC3b, use CR3-mediated phagocytosis to enter M $\phi$ s, and induce the generation of C5a. One of the earliest events in *Leishmania* infection is the binding of promastigotes to natural antibodies in blood and activation of the classical pathway of complement (Dominguez et al., 2002), resulting in C3 opsonization. Opsonized parasites can bind to CR1 on red blood cells (immune adherence) and finally enter their preferred host cell, the M $\phi$ , through CR3-mediated uptake (Sacks and Sher, 2002). This uptake through CR3 is considered an important mechanism that contributes to the selective failure of infected M $\phi$ s to produce IL-12 (Sacks and Sher, 2002). In addition to the activation of complement, recent data suggest that *Leishmania* infection activates TLR4 as an important mechanism to control parasite replication (Kropf et al., 2004). Further, CD40-mediated production of IL-12 and the production of IFN- $\gamma$  from Th1-polarized T cells are considered crucial for parasite killing (Sacks and Sher, 2002). No data exist as to whether *Leishmania* parasites exploit C5aR signaling for immunoregulatory properties. Given the role of C5aR signaling in regulating IL-12 family cytokine and IFN- $\gamma$  production, we hypothesized that *Leishmania* takes advantage of the generation of C5a as an evasion mechanism to avoid killing by the adaptive immune system. Our data support this hypothesis by showing that the local *L. major* infection is substantially reduced in C5aR<sup>-/-</sup> mice as evidenced by reduced dermal lesion and reduced parasite burden. Mechanistically, disease improvement was associated with increased numbers of CD4<sup>+</sup> as well as CD8<sup>+</sup> cells and a Th1-polarized immune response. Importantly, IFN- $\gamma$  release by CD8<sup>+</sup> *L. major*-specific T cells was recently found to promote

development of protective immunity (Belkaid et al., 2002b).

In addition to IL-12, recent reports suggest a role for IL-23 and IL-27 in leishmanial disease pathogenesis. Mice deficient in the IL-27 receptor WSX-1 are highly susceptible to *L. major* infection and suffer from impaired IFN- $\gamma$  production early in the infection (Yoshida et al., 2001). The critical role of IL-23 in macrophage-mediated inflammation (Cua et al., 2003) indicates a possible role of this cytokine in intracellular parasite killing, although the contribution of IL-23 to infection still awaits to be fully addressed. A recent study suggests that IL-23 can enhance resistance to *Toxoplasma* infection in the absence of IL-12 (Lieberman et al., 2004). Thus, it is tempting to speculate that the beneficial effect of C5aR deficiency on *L. major* infection is not only entirely dependent on rebalanced IL-12 but also on rebalanced IL-27 and/or IL-23 production. Whatever the contribution of each of the IL-12 family cytokines is, our data imply that *L. major* takes advantage of the activation of one important defense mechanism of innate immunity (the complement system) to suppress cell-mediated immunity induced by another crucial arm of innate immunity, i.e., the TLR system. This immune evasion strategy may be used by other intracellular pathogens as well, such as *Mycobacteria*, *Listeria*, *Histoplasma*, or even *HIV*. Accordingly, specific blockade of the C5aR may prove useful to prevent pathogen-induced deprivation of IL-12 family cytokines as a novel therapeutic immunointervention strategy.

#### Experimental Procedures

##### Mice

B10.D2/nSnJ and BALB/c mice were purchased from Jackson Laboratories (Bar Harbor), and TCR transgenic DO11.10/RAG2 [KO] mice were purchased from Taconic (Germantown). C5aR-deficient mice (Gerard and Gerard, 1991) were backcrossed to the BALB/c background (n = 7). All animals were held under SPF conditions. Experimental groups were sex matched and 8–12 weeks of age. All animal experiments were approved by the institutional animal care and use committee of Cincinnati Children's Hospital.

##### Generation of Elicited Peritoneal M $\phi$ s

B10.D2/nSnJ mice were injected i.p. with 2 ml thioglycollate (0.3%). Elicited cells were harvested after 72 hr by peritoneal lavage with cold PBS, washed twice with PBS, and resuspended in complete medium (DMEM + FBS [10%] + 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin). In some experiments, M $\phi$ s were purified from lavage cells by flow cytometry. Briefly, cells were incubated with 2  $\mu$ l anti-F4/80 or isotype control (conjugated to PE, Serotec) per 1  $\times$  10<sup>6</sup> cells on ice for 30 min and sorted for FSC/SSC characteristics and F4/80<sup>bright</sup> expression on a FACSVantage cell sorter to a purity of >95%.

##### Flow Cytometric Analysis of Elicited M $\phi$ s

Expression of surface markers on sorted F4/80-positive M $\phi$ s was analyzed by flow cytometry. Fc receptors were blocked with 2.4G2 antibody (5  $\mu$ g per 1  $\times$  10<sup>6</sup> cells in PBS-FBS 0.1%) for 15 min on ice. Cells were stained for 60 min on ice with the following antibodies or the appropriate isotype controls (400 ng antibody per 1  $\times$  10<sup>6</sup> cells in PBS-FBS 0.1%): CD11b (clone M1/70), CD11c (clone HL3), Ly6G (clone RB6-8C5; all antibodies were conjugated to FITC, BD Bioscience), CD88 (conjugated to Alexa-488, a kind gift of Dr. Jörg Zwirner, Göttingen), or biotinylated antimouse I-A<sup>d</sup> (clone AMS-32.1) stained with Streptavidin-CyChrome (1:3000, both from BD Bioscience). Data were collected and analyzed by using

CELLQuest software and a FACSCalibur flow cytometer (Becton Dickinson).

#### Stimulation of M $\phi$ s

Elicited M $\phi$ s ( $5 \times 10^5$  cells) were incubated in 250  $\mu$ l complete medium in 48-well plates for 2 hr at 37°C, 5% CO<sub>2</sub>. Endotoxin levels of all reagents were <0.03 IU as determined by LAL test (Bio Whittacker). Nonadherent cells were removed by washing twice with PBS. Cells were primed with murine IFN- $\gamma$  (40 ng/ml, Sigma-Aldrich) for 2 hr in a total volume of 250  $\mu$ l and stimulated with LPS (100 ng/ml, Sigma, *E.coli* O127:B8). In some experiments, cells were stimulated simultaneously with LPS and an agonistic anti-CD40 antibody (10  $\mu$ g/ml, clone FGK45). Further, repurified TLR4-specific LPS (Hirschfeld et al., 2000) (a kind gift of S.N. Vogel, Baltimore) was used for stimulation of M $\phi$ s in some experiments. Results obtained with repurified LPS were indistinguishable from data obtained with commercially available LPS. Recombinant human C5a (Sigma), human C3a (Advanced Research Technologies), CCL2, CCL3, and CXCL2 (all at 50 nM; all from PreproTech) were added to the culture 10 min prior to LPS challenge. Inhibitors for PI3K (Wortmannin, 6.25–100 nM), MEK1/2 (U0126, 0.625–20  $\mu$ M), and PTX (2  $\mu$ g/ml; all from Calbiochem) were added 1 hr before stimulation. To ascertain that the effect of C5a was M $\phi$  derived, we repeated some experiments obtained with adherent M $\phi$ s with M $\phi$ s gated on F4/80<sup>high</sup> expression and typical FSC/SSC characteristics. We found that the majority of such purified M $\phi$ s was CD11b<sup>+</sup> (92.8%) and CD88<sup>+</sup> (82.7%). Some cells expressed MHC II (22.8%), and a minor portion was CD11c<sup>+</sup> (7.2%). All cells were Ly6G<sup>-</sup>. Results obtained with purified M $\phi$ s were indistinguishable from those obtained with adherent M $\phi$ s, suggesting that the observed effects can be attributed to M $\phi$ s.

#### Determination of Gene Expression Levels in Elicited M $\phi$ s

2–4  $\times 10^6$  IFN $\gamma$ -primed M $\phi$ s were stimulated with LPS and treated with or without C5a (200 nM) or wortmannin (100 nM) for different times as described above. Cells were washed with PBS, and mRNA was prepared with Trizol reagent (Invitrogen). cDNA was prepared from 2  $\mu$ g of DNase-treated RNA by using SuperscriptII Reverse Transcriptase (Invitrogen) per the manufacturer's protocol. Gene expression levels were determined by real-time RT-PCR using iQ-SYBRgreen reaction mix (BioRad) containing 5  $\mu$ l cDNA and 500 nM primer. The following primers were used with an annealing temperature of 58°C: Actin- $\beta$  (183bp) (f) 5'-GAAATCGTGCGTGACATCAAG-3', (r) 5'-CCAAGAAGGAAGGCTGAAAAG-3'; IL-12p40 (103bp) (f) 5'-CAGAAGCTAACCATCTCTCTGG-3', (r) 5'-CAACATCTCCACCTGACC TGA-3'; IL-12p35 (256bp) (f) 5'-GATCATGAAGACATCACACGG-3', (r) 5'-GTTGGTAGTCGTCTAGTAAGA-3'; and IL-23p19 (133bp) (f) 5'-AATAATGTGCCCGTATCCAG-3', (r) 5'-GAAGATGTCAGAGTCAA GCAG-3'. Primers for IL-27p28,EBI-3 (Pflanz et al., 2002), IRF-1, IRF-2, and ICSBP (Barber et al., 1995) were used as described. Samples were analyzed on an iCycler Real-Time PCR System (BioRad). Gene expression levels were normalized to Actin- $\beta$  expression, and induction of gene expression was calculated relative to unstimulated cells.

#### Cytokine ELISA

IL-12p70, IFN- $\gamma$ , and IL-4 concentrations in supernatants from stimulated M $\phi$ s or restimulated lymph node cells were determined by using Duo Set ELISA kits (R&D Systems) per the manufacturer's protocol. Detection limits were 23.4 pg/ml (IL-12p70), 31.2 pg/ml (IFN- $\gamma$ ), and 15.6 pg/ml (IL-4).

#### Determination of ERK1/2 phosphorylation

2  $\times 10^6$  IFN- $\gamma$ -primed M $\phi$ s were stimulated with C5a (50 nM) for 0.5–20 min. Cells were washed twice with cold PBS/orthovanadate (2 mM), lysed in 50  $\mu$ l lysis buffer (1% Triton X-100, 5 mM EDTA, 50 mM NaCl, 50 mM NaF, 10 mM Tris/Cl [pH 7.6], 0.1% BSA, 10  $\mu$ g/ml Aprotinin, and 10  $\mu$ g/ml Leupeptin) and incubated 5 min on ice. Lysates were cleared by centrifugation (25 min, 15,000  $\times$  g at 4°C), applied to 12% SDS PAGE, and analyzed by Western blotting using a Phospho-p44/42 MAPKinase antibody (1/2000, clone 9101, Cell Signaling) and anti-rabbit IgG HRP (1/2000, Cell Signaling). Signals were visualized by ECL detection system (Amersham). Equal load-

ing of the gel with protein was confirmed by reprobing the stripped membrane with p44/42 MAPKinase antibody (1/2000, clone 9102, Cell Signaling).

#### Determination of PI3K Activation

1  $\times 10^6$  IFN $\gamma$ -primed M $\phi$ s were stimulated for 0–10 min with C5a (50 nM). Cells were fixed with paraformaldehyde (4%), permeabilized with saponin (0.3%), incubated for 30 min with anti-PIP3 mAb (1/100, clone RC6F8, Molecular Probes), and stained with an anti-mouse IgM-Cy3 conjugate (1/1000, Jackson).

#### Stimulation of OVA-Specific CD4<sup>+</sup> T Cells with M $\phi$ s

IFN- $\gamma$ -primed M $\phi$ s ( $2.5 \times 10^5$ ) were cultured in 250  $\mu$ l complete medium in 48-well plates and stimulated with LPS (100 ng/ml) and 10 or 1  $\mu$ g/ml OVA<sub>323–339</sub> (Jerini Peptide Technology, Berlin) for 48 hr. In some experiments, C5a (50 nM) was added 10 min prior to stimulation. DO11.10/RAG2 [KO] mice (Taconic) transgenic for an OVA<sub>323–339</sub>-specific  $\alpha\beta$ TCR and deficient in the recombinase activating gene 2 (*Rag2*) were used as a source of antigen-specific T cells. CD4<sup>+</sup> T cells were positively selected from spleen cells by using CD4 (L3T4) MicroBeads (Miltenyi) according to the manufacturer's protocol. Staining with anti-CD4-FITC revealed a single population of CD4<sup>+</sup> cells. Stimulated M $\phi$ s were washed twice with complete medium prior to the addition of purified CD4<sup>+</sup> T cells ( $1 \times 10^6$ ). After 5 days of incubation, nonadherent cells were transferred to freshly stimulated M $\phi$ s (treated as above) and incubated for another 3 days. IFN- $\gamma$  concentrations in the supernatants were determined by ELISA. To directly determine cytokine production from CD4<sup>+</sup> T cells, cells were harvested, washed, counted, and restimulated with PMA (50 ng/ml) and ionomycin (500 ng/ml) for 2 hr. Then, brefeldin A (10  $\mu$ g/ml) was added, and cells were incubated for another 2 hr followed by fixation, permeabilization, and analysis with flow cytometry as above.

#### Leishmania infection

C5aR<sup>-/-</sup> or BALB/c mice were infected intradermally with *L. major* promastigotes as described (Mendez et al., 2004). In brief, *L. major* clone V1 (MHOM/IL/80/Friedlin) promastigotes were grown at 26°C in medium 199 supplemented with 20% Hi-FCS (Hyclone), 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, 2 mM L-glutamine, 40 mM Hepes, 0.1 mM adenine (in 50 mM Hepes), 5  $\mu$ g/ml hemin (in 50% triethanolamine), and 1  $\mu$ g/ml 6-biotin (M199/S). Infective-stage promastigotes (metacyclics) of *L. major* were isolated from 4- to 5-day-old stationary cultures by negative selection of infective forms using peanut agglutinin (Vector Laboratories). Mice were infected in the ear dermis with 10<sup>6</sup> *L. major* metacyclic promastigotes using a 27½G needle in a volume of 10  $\mu$ l.

#### Parasite Quantitation

Parasite loads in the ears were determined 4 weeks postinfection as described (Mendez et al., 2004). In brief, the ventral and dorsal sheets of the infected ears were separated and deposited dermal side down in RPMI containing 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, and 50  $\mu$ g/ml of liberase CI enzyme blend (Boehringer). Ears were incubated for 40 min at 37°C. The sheets were dissociated in RPMI with 10% serum and 0.05% DNase I (Sigma), and tissue homogenates were filtered with a 70  $\mu$ m cell strainer (Falcon) and serially diluted in a 96-well flat-bottom microtiter plate containing biphasic medium (50  $\mu$ l NNN medium, 20% of defibrinated rabbit blood overlaid with 100  $\mu$ l M199/S). The number of viable parasites in each ear and from local-draining lymph nodes was determined from the highest dilution at which promastigotes could be grown out after 7 days of incubation at 26°C.

#### Cytokine production after Restimulation of Lymph Node

##### Cells from *L. major*-Infected Mice

For cytokine measurements in culture supernatants, cell suspensions from lymph nodes of individual mice were taken 4 weeks postinfection and resuspended in RPMI containing FBS/pen/strept at 6  $\times 10^6$  cells/ml, and 0.1 ml was plated in 96-well U-bottom plates. Cells were incubated at 37°C in 5% CO<sub>2</sub> and stimulated for 72 hr with 25  $\mu$ g/ml soluble leishmanial antigen. IFN- $\gamma$  and IL-4 levels were analyzed as described above.

### Analysis of Dermal Lymphocytes

Single cell suspensions from the ear dermis were obtained as described above. For the analysis of surface markers, cells were fixed in 4% paraformaldehyde. Before staining, cells were incubated with mAb 2.4G2 to block IgG Fc receptors and 10% normal mouse serum in PBS containing 0.1% BSA, 0.01% NaN<sub>3</sub>. Cells were stained for the surface markers TCR- $\beta$  (H57-597, APC conjugated), CD4 (GK1.5 FITC conjugated), and CD8 (RM4-5 and 53-6.7, cyochrome conjugated). Incubations were performed for 30 min on ice. The isotype controls used were rat IgG<sub>2b</sub> (A95-1) and rat IgG<sub>2a</sub> (R35-95) (all from BD Biosciences). The frequency of CD4<sup>+</sup> and CD8<sup>+</sup> T cells was determined by gating on TCR- $\beta$ <sup>+</sup> cells. For each sample, at least 100,000 cells were analyzed.

### Statistical Analysis

Statistical analysis was performed with the SigmaStat version 2.0 statistical package (Jandel, Germany). With regard to the small sample sizes, normal distribution was assumed. To analyze differences between two normally distributed groups, an unpaired t test was used. Comparisons of the means of more than two normally or nonnormally distributed groups were done by one way analysis of variance (ANOVA) and ANOVA on ranks. When the mean values of the groups showed a significant difference, pairwise comparison was performed by using the Tukey test (ANOVA) or Dunn's method (ANOVA on ranks).  $p < 0.05$  was considered a significant difference,  $p < 0.005$  was considered to be highly significant. If not stated otherwise, data were taken from three to five individual experiments and expressed as mean values  $\pm$  SEM.

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