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G_i-Protein-Dependent Inhibition of IL-12 Production Is Mediated by Activation of the Phosphatidylinositol 3-Kinase-Protein 3 Kinase B/Akt Pathway and JNK

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Ligands for certain G_i-protein-coupled receptors (GiPCRs) potently inhibit the production of IL-12 by human monocytes. We addressed the intracellular signaling mechanisms by which this occurs using primary human cells. Stimulation with the GiPCR ligands C5a and 1-deoxy-1-[6-[(3-iodophenyl)methyl]amino]-9*H*-purine-9-y1]-*N*-methyl-β-D-ribofuranuronamide (IB-MECA) blocked the production of IL-12 p70 by human monocytes stimulated with LPS and IFN-γ. In addition, C5a reduced the expression of mRNA for IL-12 p35, p40, IL-23 p19, and IL-27 p28. This effect was due neither to a down-regulation of TLR4 or IFN-γ receptor on the cell surface nor to interference with IFN-γ signaling, because IFN-γ-induced up-regulation of HLA-DR and CD40 were unaffected. C5a or IB-MECA activated the PI3K/Akt signaling pathway and induced the phosphorylation of the MAPK p38, ERK, and JNK. Inhibition of the PI3K/Akt signaling pathway with wortmannin or an inhibitor of Akt activity, and inhibition of JNK but not ERK prevented IL-12 and IL-23 suppression by C5a. These data extend observations on IL-12 suppression by C5a to IL-23 and IL-27, and are the first to demonstrate the intracellular signaling events leading to IL-12 and IL-23 inhibition after GiPCR activation. ***The Journal of Immunology,*** **2005, 175: 2994–2999.**

Interleukin-12p70 is a heterodimeric cytokine, consisting of covalently bound p40 and p35 chains, that induces the differentiation of Th-1 lymphocytes and CTLs and activates NK cells. Because of its ability to activate innate immunity and shape adaptive immune responses, ultimately resulting in enhanced production of IFN-γ, IL-12 plays a key role in protection against intracellular bacterial, fungal, protozoan, and certain viral infections as well as in antitumor immune responses (1). Monocytes, macrophages, and dendritic cells produce IL-12 after engagement of TLR by pathogen-associated conserved molecules. In addition, during APC-T lymphocyte interaction, the engagement of CD40 by CD40L expressed by activated T cells reinforces IL-12 production. IFN-γ produced by NK cells and Th1 lymphocytes, not only mediates the inflammatory effects of IL-12, but also acts as a positive feedback signal, strongly enhancing p35 and p40 gene transcription (1). The p40 chain also constitutes, along with the protein p19, the heterodimeric cytokine IL-23 that is an important factor for the amplification of Th1-cell effector functions.

Along with promoting resistance to pathogens, IL-12-dependent IFN-γ production contributes to tissue damage in Th1-mediated autoimmune diseases. Therefore, during protective immune responses, tight regulation of IL-12 production is essential for preventing self-damaging reactions. A number of factors have been shown to down-regulate IL-12 production upon binding to surface receptors, including cytokines such as IL-10 (2), immune complexes acting via FcR (3), thrombospondin acting via CD47 (4), and complement components C3b and iC3b acting via CD46 and CR3 (5–7). In addition, IL-12 inhibition can be achieved by the engagement of G-protein-coupled receptors (8).

We and others previously reported that agonists for certain G_i-protein-coupled receptors (GiPCRs)[3] such as CCR2, C5a receptor (C5aR; CD88), and adenosine A_3 receptor (A_3R) can suppress IL-12 production in an IL-10-independent manner (4, 9–11). Consistent with an inhibitory role for GiPCRs, mice with a deficiency of Gi2α, display increased IL-12 production in response to IL-12 inducers and develop Th1-mediated inflammatory colitis (12). The intracellular events linking the activation of G_i proteins to the failure of APCs to produce IL-12 is currently unknown. In this study we report that the activation of the PI3K/Akt and JNK signaling pathways are necessary for the GiPCR-mediated inhibition of IL-12 production by primary human monocytes.

Materials and Methods

Reagents

C5a, pertussis toxin (PT), PI3K inhibitor wortmannin, the Akt inhibitor 1L-6-hydroxymethyl-*chiro*-inositol2-(*R*)-2-*O*-methyl-3-*O*-octadecylcarbonate (Akt inhibitor), and MAPK inhibitor PD98059 were purchased from Calbiochem. SP600125 was obtained from BIOMOL. LPS from *Escherichia coli* serotype 0127:B8 and adenosine A_3 receptor-specific agonist 1-deoxy-1-[6-[(3-iodophenyl)methyl]amino]-9*H*-purine-9-y1]-*N*-methyl-β-D-ribofuranuronamide (IB-MECA) were obtained from Sigma-Aldrich. Abs used in Western blot experiments were obtained from Cell Signaling Technology. Recombinant human (rh)IFN-γ was purchased from R&D Systems.

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[3] Abbreviations used in this paper: GiPCR, G_i-protein-coupled receptor; PT, pertussis toxin; IB-MECA, 1-deoxy-1-[6-[(3-iodophenyl)methyl]amino]-9*H*-purine-9-y1]-*N*-methyl-β-D-ribofuranuronamide; rh, recombinant human; ATF, activating transcription factor.

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Cell culture conditions

Elutriated human monocytes from healthy donors were obtained and cultured as previously described (9). For measurement of IL-12 p70 production, cells were preincubated with medium alone or C5a or IB-MECA for 1 h at 37°C before stimulation with LPS (1 μg/ml) and IFN-γ (100 ng/ml); supernatants were collected 24 h later.

Western blot and cytokine quantification

For Western blot analysis, 10^7 cells per condition were stimulated for the indicated times, resuspended in 100 μl of radioimmunoprecipitation buffer, and 40 μg of cell lysate was subjected to SDS-PAGE and blotted onto nitrocellulose. Cytokine levels in culture supernatants were measured by ELISA using the appropriate DuoSet kit from R&D Systems; samples were assayed in duplicate.

Quantitative PCR

cDNA was synthesized from RNA extracted from human peripheral monocytes and TaqMan RT-PCR was conducted using specific primers for human GAPDH, p35, p40, p19, and p28 from Applied Biosystems (Assay on Demand) according to the manufacturer's instructions.

Statistics

The unpaired two-tailed Student's *t* test was used throughout the paper.

Results

Activation of GiPCRs inhibits IL-12 production via the PI3K-Akt signaling pathway

We used C5a or IB-MECA to stimulate human peripheral monocytes through the C5aR and the A_3R, respectively. Monocytes were pretreated with C5a or IB-MECA for 1 h and then stimulated with LPS and IFN-γ. Both C5a and IB-MECA inhibited LPS and IFN-γ-induced IL-12 p70 production in a dose-dependent manner, achieving a complete block at 20 nM and 50 μM concentrations, respectively (Fig. 1*A*). The inhibition of IL-12 production was prevented by the addition of PT indicating dependence on G_i protein signaling. Furthermore, IL-12 p40 production induced by LPS alone (5.7 $\pm$ 0.7 ng/ml) was reduced below the detection limit of the assay (625 pg/ml) by C5a treatment. In contrast, the expression of TLR4 and the IFN-γ receptor α-chain was unchanged after C5a treatment (Fig. 1*B*), and IFN-γ-dependent up-regulation of HLA-DR and CD40 was not affected by C5a (Fig. 1*C*), suggesting that GiPCR activation can directly affect TLR, but not IFN-γ receptor signaling pathways.

Recent evidence suggests that activation of the PI3K-Akt signaling pathway can inhibit IL-12 p70 production (13). In addition, PI3K activation can occur after GiPCR triggering (14). Therefore, we tested the hypothesis that the PI3K-Akt pathway mediates GiPCR-dependent inhibition of IL-12 production by monocytes. Stimulation with C5a or IB-MECA-induced phosphorylation of Akt (Fig. 2*A*). Peak phosphorylation occurred 30 min after C5a and 60 min after IB-MECA stimulation. PT treatment abolished Akt phosphorylation indicating that PI3K was activated as a result of GiPCR signaling. Next, monocytes were pretreated with the PI3K inhibitor wortmannin, exposed to C5a or IB-MECA, and stimulated with LPS plus IFN-γ. As shown in Fig. 2*B*, inhibition

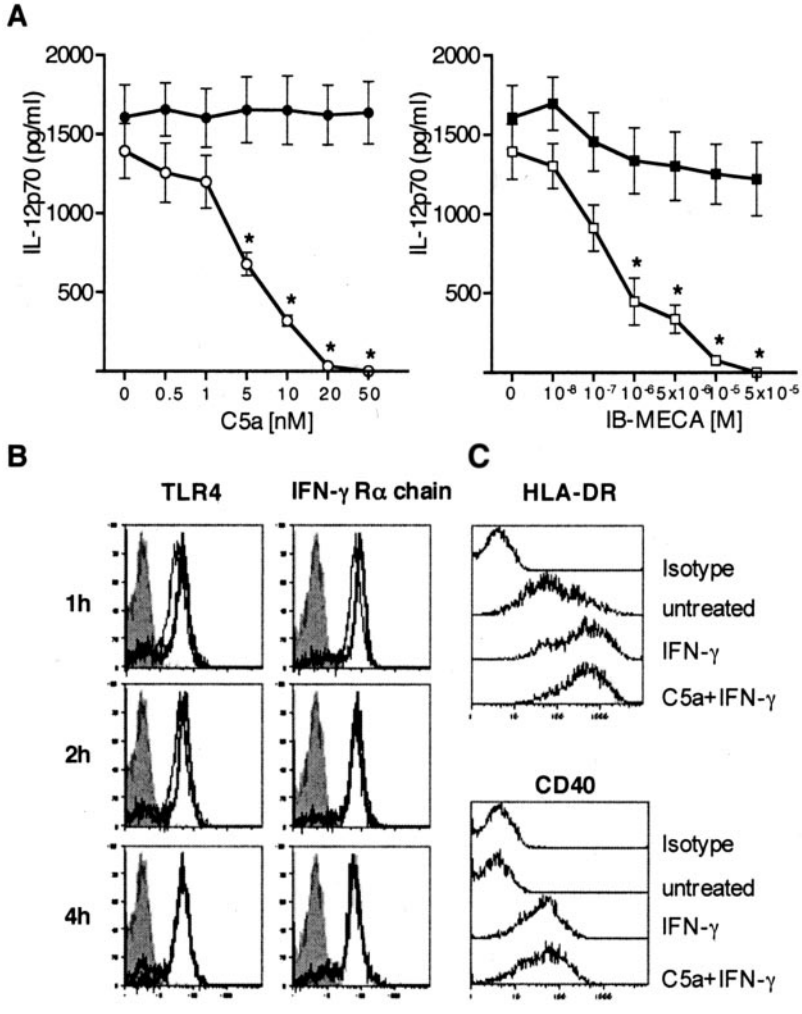

FIGURE 1. Ligand activation of C5aR and A_3R inhibits IL-12 production in a PT-sensitive manner and does not alter TLR4 or IFN-γ receptor expression or IFN-γ stimulatory effects on monocytes. A total of 2×10^6/ml human peripheral monocytes preincubated for 2 h with (●, ■) or without (○, □) 200 ng/ml PT were treated with increasing doses of C5a (*A*, *left*) or IB-MECA (*right*) for one additional hour and subsequently stimulated with LPS (1 μg/ml) plus rhIFN-γ (100 ng/ml) for 24 h. *B*, Flow cytometry of surface expression of TLR4 and IFN-γ receptor α-chain studied at the indicated time points in cells untreated (thin line) or stimulated with C5a (bold line). Shaded histograms represent fluorescence obtained with isotype control Abs. *C*, Lack of effect of C5a on IFN-γ-induced up-regulation of CD40 and HLA-DR in cells stimulated for 16 h with IFN-γ in the absence or presence of C5a. All panels show data calculated from or representative of at least three experiments. Data are expressed as mean $\pm$ SD. *, $p < 0.05$ vs cells exposed to PT.

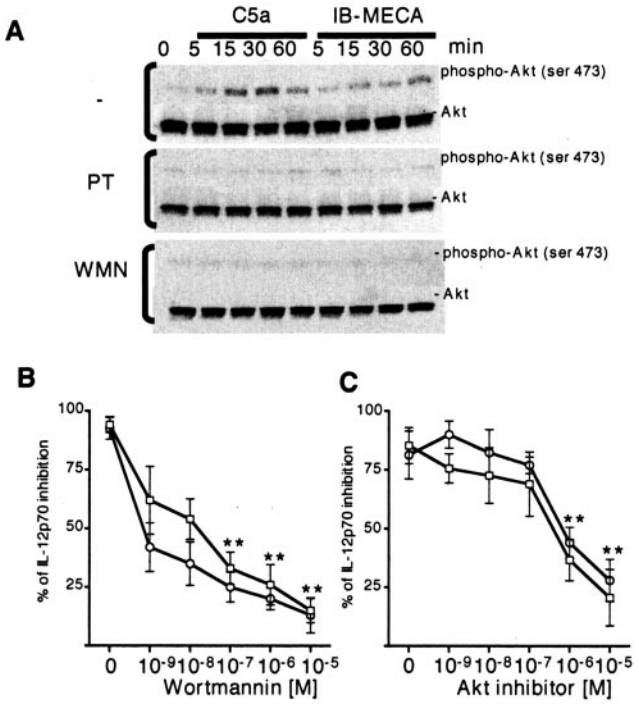

FIGURE 2. C5a and IB-MECA suppression of IL-12 is PI3K- and Akt-mediated. *A*, Lysates from cells preincubated for 2 h with or without 200 ng/ml PT and subsequently stimulated for the indicated time points with 50 nM C5a or 5×10^{-5} M IB-MECA, were subjected to SDS-PAGE followed by immunoblotting using anti-phospho-Akt, or anti-Akt-specific Ab as input control. *B* and *C*, Effect of PI3K/Akt signaling pathway blockage on the ability of C5a or IB-MECA to inhibit IL-12 p70 production. A total of 2×10^6/ml monocytes preincubated for 1 h with indicated doses of wortmannin (*B*) or Akt-inhibitor (*C*) was stimulated with 20 nM C5a (○) or 10^{-5} M IB-MECA (□) for one additional hour and subsequently stimulated with LPS (1 μg/ml) plus rhIFN-γ (100 ng/ml) for 24 h. All panels show data calculated from or representative of at least three experiments. Data are expressed as mean ± SD. *, $p < 0.05$ vs cells not preincubated with the inhibitor.

of IL-12 production by C5a and IB-MECA was reversed by wortmannin in a dose-dependent manner. Between 10^{-8} and 10^{-7} M concentration, 60–70% of IL-12 p70 production was restored. Within this concentration range, the inhibitory effect of wortmannin is highly specific for PI3K. Similar experiments using an Akt-specific inhibitor (Akt inhibitor; $IC_{50} = 5$ μM) demonstrated that inhibition of Akt activity strongly reduces the ability of C5a and IB-MECA to suppress LPS-induced IL-12 in a dose-dependent manner and within a dose range with high Akt-specificity (15, 16) (Fig. 2*C*). In addition, blocking these pathways did not affect the ability of monocytes to release IL-10 (data not shown), consistent with prior studies demonstrating that C5a-mediated IL-12 negative regulation is IL-10 independent (2).

Complement factor 5a suppresses IL-12 production by inhibiting the expression of mRNA for both the p35 and the p40 chains (9). We now extend this analysis to other components of the IL-12 family. First, as shown in Fig. 3, consistent with prior studies (13), blocking PI3K activity by wortmannin in the absence of C5a exposure led to a significant increase in mRNA for p35 (10-fold) and p40 (2- to 3-fold). In addition, increased expression of p19 (2- to 3-fold), but not p28, was found. Pretreatment with C5a inhibited mRNA levels of p35, p40, p19, and p28 induced by LPS and IFN-γ, but when cells were pretreated with wortmannin, the ability of C5a to inhibit p35, p40, and p19, but not p28, expression was abrogated.

Role of mitogen-activated kinases

Because GiPCR can activate MAPK-signaling pathways, we determined whether the activation of the inhibitory MAPKs, ERK and JNK, play a role in IL-12 regulation by C5a. First, C5a induced the phosphorylation of both ERK and JNK in human monocytes in a PT-sensitive manner (Fig. 4*A*). This activation was independent of PI3K or Akt activity, because there was no effect of pretreatment with wortmannin or Akt inhibitor (data not shown). Next, the pretreatment of monocytes with SP600125, a direct inhibitor of JNK activity, decreased phosphorylation of the JNK substrate activating transcription

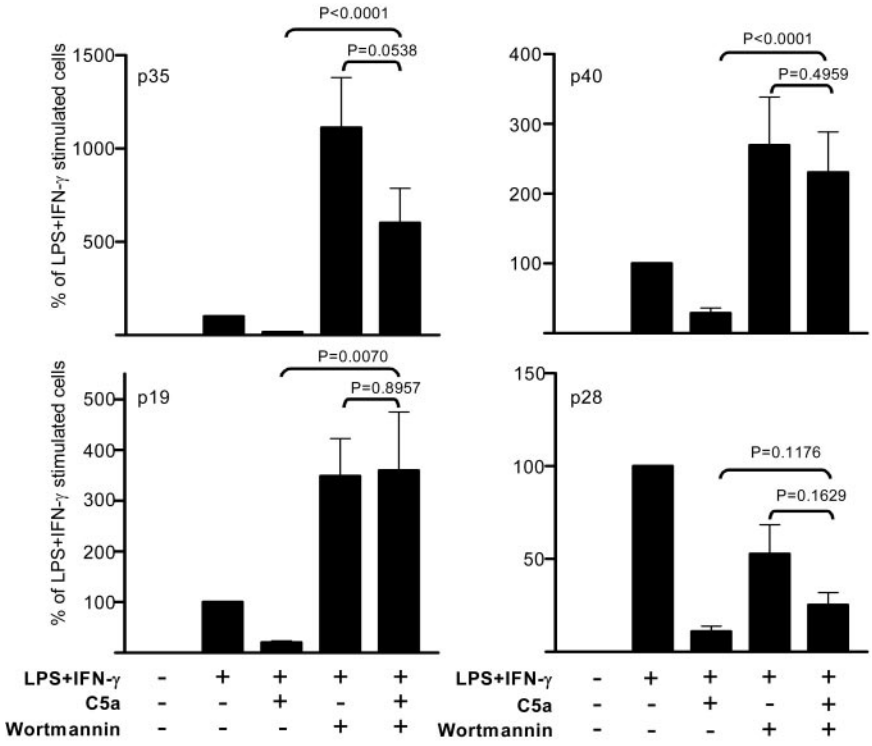

FIGURE 3. C5a inhibits expression of mRNA for IL-12 p35, IL-12 p40, and IL-23 p19 in a PI3K-dependent manner. Monocytes preincubated for 1 h with 2×10^{-7} M wortmannin were stimulated with 20 nM C5a for one additional hour and subsequently stimulated with LPS plus rhIFN-γ. After 6 h, total RNA was subjected to real-time PCR as described in *Materials and Methods*. Results are expressed as the percentage of the value obtained in the sample stimulated with LPS and IFN-γ. Data are expressed as mean ± SD from at least three experiments.

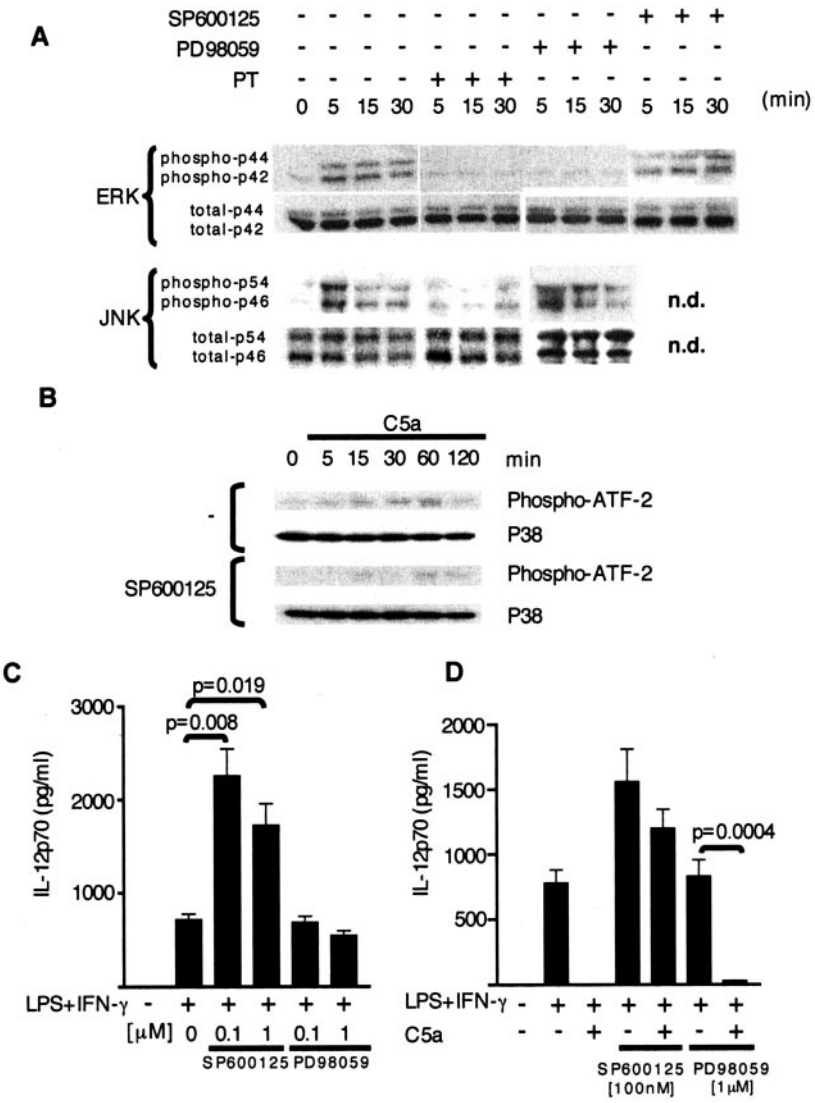

FIGURE 4. JNK activity is required for C5a-dependent IL-12 inhibition. *A*, Cell lysates from cultures preincubated for 2 h with PT (200 ng/ml) or PD98059 (10^{-5} M) and subsequently stimulated with 20 nM C5a were used for Western blot analysis using specific Abs against phosphorylated form of ERK or JNK MAPK. Membranes were stripped and reblotted using Ab against total ERK or JNK as input control. *B*, Cells preincubated for 1 h with or without SP600125 were stimulated with C5a for the indicated times, and phosphorylation of ATF-2 was analyzed by Western blot. Membranes were stripped and reblotted using Ab against total ATF-2 as input control. *C* and *D*, Monocytes were incubated for 1 h with PD98059 or SP600125 and subsequently stimulated with LPS plus rhIFN-γ. Where indicated, cells were also incubated with 20 nM C5, 1 h before the addition of LPS and rhIFN-γ. IL-12 p70 was measured in cell culture supernatants by ELISA after 24 h. All panels show data calculated from or representative of at least three experiments. ELISA results are expressed as mean ± SD.

factor-2 (ATF-2) in response to C5a, as expected (Fig. 4*B*), and significantly increased IL-12 p70 production induced by LPS and IFN-γ (Fig. 4*C*). Inhibition of JNK with SP600125 also significantly reversed the C5a-induced suppression of IL-12 (Fig. 4*D*). Conversely, pretreatment with PD98059, which prevents the phosphorylation of ERK by inhibiting the upstream MEK2, completely abolished C5a-induced ERK phosphorylation (Fig. 4*A*), yet did not affect the induction of IL-12 by LPS and IFN-γ (Fig. 4*C*) or the ability of C5a to suppress IL-12 (Fig. 4*D*).

Discussion

IL-12 production by human monocytes is inhibited by the triggering of certain GiPCR, particularly CD88 (C5aR), the high-affinity formyl-peptide receptor, and the adenosine A3 receptor (8–10). In this study, we extend these findings by demonstrating a suppressive effect of C5a on the production of two other members of the IL-12 family, p19 and p28, and provide the first experimental evidence identifying the intracellular signaling events responsible for such inhibition in primary human peripheral blood monocytes.

In prior studies, we demonstrated that GiPCR-mediated inhibition of IL-12 is not dependent on autocrine secretion of known negative regulators of IL-12 production, such as IL-10, TGF-β, or PGE_2 (9). We now provide evidence that TLR4 and IFN-γ receptor expression was unaffected by GiPCR triggering, and that interference with IFN-γ signaling by GiPCR is unlikely, because IFN-γ-induced up-regulation of HLA-DR and CD40 membrane expression was not affected by C5a. Together these data suggest that GiPCR activation can directly affect TLR, but not IFN-γ receptor signaling pathways.

We further demonstrated a critical role for PI3K in the GiPCR-induced inhibition of IL-12 production by human monocytes. The activation of the PI3K-Akt pathway can occur as a result of G-protein-coupled receptor triggering: PI3Ks are stimulated through direct interaction with the active G protein βγ subunit or indirectly, by α subunit-induced tyrosine kinase activity (14). PI3K catalyzes the phosphorylation of membrane phosphatidylinositols generating phosphatidylinositol mono-, bis-, and tris-phosphate (17). These products recruit the protein kinase Akt by interacting with its PH domain and facilitate its phosphorylation on threonine 308 and serine 473 by phosphoinositide-dependent kinase 1 and 2 (18). We demonstrated that the GiPCR agonists C5a and IB-MECA activate the PI3K pathway, and the activity of both PI3K and Akt is required for IL-12 down-regulation. Furthermore, exposure to C5a inhibited mRNA expression not only of IL-12 p35 and p40 but also of other IL-12 family members, IL-23 p19 and IL-27 p28. The fact that the suppression of all IL-12 family members except for p28 were reversed by PI3K inhibition suggests that

PI3K plays a role in the C5a-mediated suppression of IL-12 and IL-23, but not IL-27.

These data are consistent with several studies demonstrating a suppressive role for PI3K in the production of IL-12. First, ligation of CD47 inhibited IL-12 production in response to TLR ligands and IFN-γ in a PI3K-dependent manner (4). Interestingly, it was recently recognized that CD47 can signal via a G_i-protein pathway; however, it is not known whether CD47-mediated suppression of IL-12 production is sensitive to PT. Second, it has been shown that the PI3K-Akt pathway is a negative feedback mechanism for the control of IL-12 production induced via TLR engagement in the absence of GiPCR signaling (13, 19). Because C5a and IB-MECA do not drive IL-12 production on their own (with or without IFN-γ), GiPCR-signaling by these ligands appears to mimic the suppressive, but not the activating, signals that normally govern TLR-induced IL-12 production.

We also studied the role of MAPK in mediating GiPCR IL-12 inhibition. Several studies have assessed the role of the ERK, the JNK, and p38 MAPK in IL-12 regulation, primarily by determining their effects on IL-12 p40 gene transcription. In particular, a genetic deficiency of MAP kinase kinase 3, which is responsible for p38 phosphorylation, is associated with defective IL-12 production by macrophages and dendritic cells in vitro and with suppressed IFN-γ production in vivo after immunization with protein Ags (20). In addition, direct inhibition of p38 activity in vitro reduces IL-12 production at the level of mRNA expression in response to LPS in monocytes, macrophages, and monocytic cell lines, suggesting that p38 activity promotes IL-12 production (21, 22). Conversely, in primary monocytes, the activities of ERK and JNK have been shown to down-regulate LPS-induced IL-12 p40 mRNA transcription (21, 23–25), suggesting opposing regulatory effects by p38 and ERK and JNK pathways.

Our data show that JNK activation is necessary for the down-regulation of IL-12 production upon GiPCR triggering. The lack of an inhibitory effect of ERK on IL-12 production may be due to the presence of IFN-γ or to the use of primary human monocytes, because prior studies were performed with cell lines that may involve different, and possibly less relevant, regulatory mechanisms or with cells stimulated with LPS alone. Taken together, these data demonstrate that JNK activity is necessary for the negative regulation of IL-12 production by C5a in primary human monocytes.

The events downstream of PI3K/Akt and JNK activation that are relevant for IL-12 regulation are not yet clear. It is likely that JNK mediates its suppressive effect via the transcription factor AP-1, which is activated by JNK-mediated phosphorylation of c-Jun, and is a transcriptional inhibitor for the IL-12 p40 gene (26, 27). Because C5a induces the phosphorylation of ERK and JNK, which activate c-Fos and c-Jun, respectively, to become components of AP-1, it is not clear why ERK inhibition does not reverse IL-12 inhibition in the presence of IFN-γ. However, this is consistent with studies demonstrating that genetic deficiency of c-Fos renders macrophages more potent producers of IL-12 in response to LPS but not to IFN-γ plus LPS (28). Because c-Fos may be suppressed by the presence of IFN-γ (28), the results presented here suggest that c-Jun can be inhibitory in conditions of limited c-Fos activity. Furthermore, PI3K-Akt does not appear to be directly involved in JNK activation, because the phosphorylation of JNK by C5a is inhibited by PT but not by wortmannin or the Akt-inhibitor (data not shown). Therefore, PI3K-Akt and JNK pathways are both induced by G_i-mediated signaling, and both are required for maximal IL-12 suppression.

Because C5a and the natural A_3R-agonist adenosine are present at high levels at sites of inflammation (29, 30), it is likely that one of their many functions is to limit IL-12 and IL-23 production by APCs in the context of ongoing inflammation, thus preventing sustained self-harmful Th1 responses. The fact that inhibitory regulation of IL-12 and IL-23 regulation by LPS also involves PI3K-signaling pathways suggests that activation of PI3K pathways may represent physiological negative feedback signals to control untoward inflammation.

Disclosures

The authors have no financial conflict of interest.

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