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C5a controls TLR-induced IL-10 and IL-12 production independent of phosphoinositide 3-kinase

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The complement system is a classic central player in innate immunity. Most pathogens activate both complement and the toll-like receptor (TLR) pathway. Therefore, to provide a more comprehensive understanding of innate immunity, it is important to understand the crosstalk between these two systems. Mouse macrophages produce IL-12 and IL-10 in response to TLR ligands such as LPS, CpG, Poly I:C and Malp2. The TLR-induced IL-12 production was decreased, while that of IL-10 was increased by concurrent stimulation with a complement fragment C5a. Pharmacological studies have suggested that C5a regulates TLR4-induced IL-12 production in a phosphoinositide 3-kinase (PI3K)-dependent mechanism. In the present study, however, we found that the C5a-mediated changes can be observed in macrophages from mice lacking PI3K p85α or PI3K p110γ. The result indicates that the C5a action is PI3K-independent; neither class IA nor class IB PI3K subtype is involved in this regulation. The actions of C5a were sensitive to pertussis toxin and PD98059, suggesting a role of G protein-mediated activation of the Erk1/2 pathway.

Keywords: C5a/ERK/IL-12/IL-10/phosphoinositide 3-kinase.

Abbreviations: APC, antigen-presenting cells; C5a, complement fragment 5a; IFN, interferon; IL, interleukin; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; Mϕ, macrophage; PTX, pertussis toxin; Poly I:C, polyinosinic-polycytidyluc acid; TLR, Toll-like receptor; TNF, tumor necrosis factor.

Toll-like receptors (TLRs) play a critical role in host defense as well as in the development of adaptive immunity (*1*). The mammalian family of TLRs includes 11 members that recognize a variety of microbial products, such as lipopolysaccharide (LPS), peptidoglycans, flagellin and unmethylated CpG motifs in bacterial DNA (*1*). In antigen-presenting cells (APC), interaction of TLRs with their ligand(s) can lead to the induction of IL-12 transcription. This proinflammatory cytokine plays a significant role in the protection against infections and in anti-tumour immune responses through its effect on the differentiation of Th1 lymphocytes and the activation of NK cells (*2*). Excessive IL-12 production results in tissue damage and toxicity, such as what is seen in Th1-mediated autoimmune diseases (*3*). Thus, homeostatic mechanisms that limit IL-12 production are essential for preventing immune-mediated damage.

APC-derived IL-12 is downregulated by multiple molecules that bind to cell surface receptors, including cytokines such as IL-10, IL-4, IL-13 and TNF-α (*4*). Mice deficient in IL-10, a major anti-inflammatory cytokine, develop inflammatory diseases that are associated with increased IL-12 production (*5*). Also, agonists, such as the complement-derived anaphylatoxin C5a, are known suppressors of TLR-induced IL-12 production via GTP-binding protein-coupled receptor (GPCR) signalling (*6*, *7*). Most pathogens activate both complement and the TLR pathway, suggesting that there is crosstalk between two systems. The physiological significance of such an interaction has been indicated by the observed increase in Th1 responses in C5a receptor-deficient mice (*8*).

IL-12 is a heterodimeric cytokine consisting of two covalently bound subunits, p35 and p40, that are encoded by distinct genes and regulated largely at the transcription level (*3*). In both human peripheral blood monocytes (PBMC) and mouse-derived macrophages (MΦ), C5a suppresses the LPS-induced expression of p40 mRNA (*8*, *9*). As the phosphoinositide 3-kinase (PI3K)-Akt pathway can inhibit IL-12 production (*10*), it is interesting to speculate that C5a suppresses the expression of p40 mRNA by activating PI3K. In agreement with this hypothesis, treatment of cells with a PI3K inhibitor, wortmannin, markedly attenuates the C5a-mediated inhibition of p40 mRNA expression (*8*, *9*).

In the present study, using PI3K knock out mouse-derived MΦ, we demonstrate that the C5a action on IL-12 is not mediated by PI3K. Additionally, C5a also augments TLR-induced expression of IL-10, which is, again, independent of PI3K activity. Collectively, these findings demonstrate that C5a controls the Th1 response by modifying the production of multiple cytokines via a PI3K-independent mechanism.

Experimental Procedures

Materials

Materials were obtained from the following sources: LPS (*Escherichia coli* serotype 0111: B4), pertussis toxin (PTX), poly I:C, PD98059 and C5a (Sigma); wortmannin (Kyowa Medex, Tokyo, Japan); CpG

DNA (HPLC-purified phosphorothioate with the sequence of TCC ATG ACG TTC CTG ATG CT; synthesized by Hokkaido System Science, Sapporo, Japan); RPMI 1640 medium (Invitrogen); Protein assay kit (Bio-Rad); antibodies against pAkt (Ser473) and pErk1/2 (Thr202/Tyr204) (Cell Signalling); anti-Akt1/2, anti-p110γ and anti-p110β (Santa Cruz); anti-p110α and anti-p110δ (BD Transduction Laboratories); anti-IL-10 for neutralization (Peprotech); IL-12/IL23p40 and IL-10 ELISA assay kits (Biolegend); Syber Green Master Mix (Roche) and a synthetic lipopeptide based upon the full-length macrophage activating lipopeptide-2 kDa (Malp-2) was prepared with dipalmitoyl-S-glyceryl cysteine, as described (*11*).

Animals and cell isolation

Female C57BL/6 mice, 8–12-weeks old, were purchased from Japan SLC, Inc. PI3Kγ$^{-/-}$ mice on the C57BL/6 background (*12*) were bred at Akita University (Akita, Japan); PI3Kp85α$^{+/-}$ and PI3Kp85α$^{-/-}$ mice on the BALB/c background (*13*, *14*) were kindly provided by Dr S. Koyasu (Keio University, Tokyo, Japan) via RIKEN BioResorce Center. Mice on a C57BL/6 background were used in all experiments without otherwise specified. Thioglycollate-elicited macrophages were harvested from these mice. Briefly, mice were injected intraperitoneally with 2 ml of 3% thioglycollate broth. After 3 days, the peritoneal exudate cells were collected by washing the peritoneal cavity with ice-cold phosphate-buffered saline (PBS). The cells were seeded at $\sim 5–10 \times 10^5$ cells/well in 24-well plates and incubated in humidified 5% CO_2 at 37°C for 1–2 h in RPMI 1640 medium supplemented with 10% FCS to allow the cells to adhere to the wells. Non-adherent cells were removed by washing with PBS and attached cells were collected.

Mouse bone marrow cells were collected and treated with hypotonic PBS (five-times diluted). The cells were then cultured in RPMI 1640 medium supplemented with 10% FCS and 10 ng/ml of GM-CSF for 7 days. The adherent cells were used to determine cytokine production.

RT–PCR

Total RNA was isolated using RNeasy (Qiagen, Hilden, Germany). Single-strand cDNA was synthesized with M-MLV reverse transcriptase. The cytokine cDNAs were amplified by a PCR method using the specific primers provided in Table I.

Quantitative PCR

The cytokine cDNA prepared as above were amplified with FastStart Universal SYBER Green Master (Rox) and the specific primers provided in Table II by ABI HT-7900. Data analysis was performed by $\Delta\Delta C_T$ methods as described in the Applied Biosystem protocol. A ΔC_T value was determined for each sample using C_T value from input DNA. The C_T value for β-actin was used to normalize loading in the RT–PCRs. A $\Delta\Delta C_T$ values was then obtained by subtracting control ΔC_T values (LPS alone) from the corresponding experimental ΔC_T. The $\Delta\Delta C_T$ values were converted to fold difference compared with the control by raising 2 to the $\Delta\Delta C_T$ power.

In vitro *stimulation*

Macrophages were treated with or without 50 ng/ml of PTX for 4 h prior to stimulation with TLR ligands. Wortmannin and PD98059 were added 15 min before and C5a was added immediately before the TLR ligands were added to the culture. For short-term incubations (<60 min), media (RPMI 1640) from the mouse peritoneal macrophage cultures was aspirated and replenished with the incubation buffer (RPMI 1640 without $NaHCO_3$, supplemented with 20 mM HEPES).

Western blot

Cells were washed with PBS and lysed in 50 μl of lysis buffer containing 25 mM Tris–HCl (pH 7.4), 0.5% Nonidet P-40, 150 mM NaCl, 1 mM sodium orthovanadate (Na_3VO_4), 1 mM EDTA, 0.1% BSA, 20 mM sodium fluoride, 1 mM phenylmethylsulphonyl fluoride, 2 μM leupeptin, 20 μM p-amidinophenylmethylsulphonyl fluoride and 1 mM dithiothreitol. The cell lysates were centrifuged at 15,000 rpm for 10 min. Supernatants were collected and the protein concentration was determined using a Bio-Rad assay kit. Total cell lysates (100 μg of protein) were mixed with 10 μl 5× sample buffer [62.5 mM Tris, (pH 6.8), 1% SDS, 10% glycerol, 5% 2-mercaptoethanol and 0.02% bromophenol blue] and heated at 100°C for 5 min. The proteins were separated by SDS–PAGE and transferred electrophoretically onto a polyvinylidene difluoride (PVDF) membrane (Millipore). The membrane was blocked with 5% skim milk and incubated with the appropriate antibodies. Antibody binding was detected using a chemiluminescent substrate (Perkin-Elmer).

ELISA

Macrophage culture supernatants were used for the quantification of p40 and IL-10 using a commercially available ELISA kit (Biolegend).

Table II. Quantitative PCR primers.

β-actin F	TTTGCAGCTCCTTCGTTGC
β-actin R	TCGTCATCCATGGCGAACT
IL12-p40 F	AATGTCTGCGTGCAAGCTCA
IL12-p40 R	ATGCCCACTTGCTGCATGA
IL-12p35 F	TGTCTCCCAAGGTCAGCGTTCCA
IL-12p35 R	TGCTGGTTTGGTCCCGTGTGA
IL-10 F	GGCGCTGTCATCGATTTCTC
IL-10 R	TGCTCCACTGCCTTGCTCTTA
IFN-γ F	GATATCTGGAGGAACTGGCAAAAG
IFN-γ R	AGAGATAATCTGGCTCTGCAGGAT
iNOS F	CTGGCTCGCTTTGCCACGGA
iNOS R	GCTGCGACAGCAGGAAGGCA
TNF-α F	CCCTCACACTCAGATCATCTTCT
TNF-α R	GCTACGACGTGGGCTACAG

Table I. PCR primers.

Target gene	Primer	Sequence	Position	Product (bp)	Annealing (°C)
β-actin	mβ-actin-Fwd	atcatgtttgagaccttcaacacc	286–309	273	59
	mβ-actin-Rev	gatgtcacgcacgatttccc	558–539		
IL-12p40	mIL-12p40-Fwd	gagtcataggctctggaaagacc	234–256	398	58
	mIL-12p40-Rev	agttgggcaggtgacatcc	631–613		
IL-10	mIL-10-Fwd	gctcctagagctgcggact	195–223	71	55
	mIL-10-Rev	tgttgtccagctggtccttt	265–246		
IL-12p35	mIL-12p35-Fwd	tgtcttagccagtcccgaaac	223–243	545	59
	mIL-12p35-Rev	gagctcagatagcccatcacc	767–747		
iNOS	m iNOS-Fwd	tgggaatggagactgtcccag	779–799	306	64
	m iNOS-Rev	gggatctgaatgtgatgttg	1084–1064		
TNF-α	m TNF-α-Fwd	cgtcgtagcaaaccaccaag	445–464	445	60
	m TNF-α-Rev	gatgaacacccattcccttcac	889–868		
INF-γ	m IFN-γ-Fwd	agacagaagttctgggcttctc	24–45	417	59
	m IFN-γ-Rev	gggttgttgacctcaaacttgg	440–419		

Results and Discussion

C5a Inhibition of LPS-induced IL-12 production is susceptible to PTX

We first examined the effect of C5a on TLR4-induced IL-12 production from thioglycollate-elicited MΦ, which were stimulated with 10 ng/ml LPS for 18 h in the presence or absence of C5a. C5a inhibited the TLR-induced production of IL-12 (Fig. 1A), consistent with previous studies using human PBMC (*9*) and murine MΦ (*8*). The inhibitory effect of C5a was also observed when cells were stimulated with the other TLR ligands (poly I:C, a TLR3 ligand; CpG, TLR9; Malp2, TLR2/6). However, the degree of inhibition was lower in the CpG- and Malp2-stimulated macrophages (data not shown).

Pertussis toxin (PTX) is known to impair the function of guanine nucleotide-binding proteins (G proteins), such as G_i, by ADP-ribosylating the α-subunit of the heterotrimeric GTP-binding protein. Treatment of MΦ with 50 ng/ml PTX for 4 h increased the LPS-induced production of IL-12 as reported previously (*15*). The inhibitory effect of C5a was completely abolished (Fig. 1B), which was in agreement with a previous study using human monocytes (*9*). However, two other studies using mouse MΦ (*8*) and human monocytes (*7*) demostrated that PTX only partially blocked the inhibitory effect of C5a. The reason for this apparent discrepancy is currently unclear. As shown in Fig. 1C, C5a enhanced the phosphorylation level of Akt, a downstream effector of PI3K. This effect of C5a was additive to the LPS-induced phosphorylation, and was completely abolished by the PTX treatment (Fig. 1C). In contrast, Akt phosphorylation induced by LPS itself was not susceptible to PTX (Fig. 1C). These results indicated that the impact of C5a on IL-12 production and the PI3K-Akt pathway was mediated by G proteins.

PI3K p110γ is not involved in the C5a-mediated inhibition of IL-12 production

Recent evidence, including studies of cells from PI3K-deficient mice, indicates that activation of the PI3K-Akt pathway can negatively regulate IL-12 production (*10*, *16*, *17*). Thus, it was possible that C5a suppressed cytokine production by activating PI3K in a G protein-dependent manner. Of the three major classes of PI3K (I, II, III), class-I kinases transmit signals from cell surface receptors. Class-I PI3K includes four catalytic subunits p110α, p110β, p110δ and p110γ. The former three can transmit signals from receptors functionally coupled to tyrosine kinases or possessing tyrosine kinase activities; the last one is thought to play a major role in signal transduction from G protein-coupled receptors (GPCR). Therefore, we next examined the impact of C5a on MΦ from $p110\gamma^{-/-}$ mice (Fig. 2). The C5a-induced phosphorylation of Akt was completely lost in $p110\gamma^{-/-}$ MΦ (Fig. 2C), consistent with previous reports (*12*). However, C5a was still able to suppress LPS-induced IL-12 production from $p110\gamma^{-/-}$ MΦ

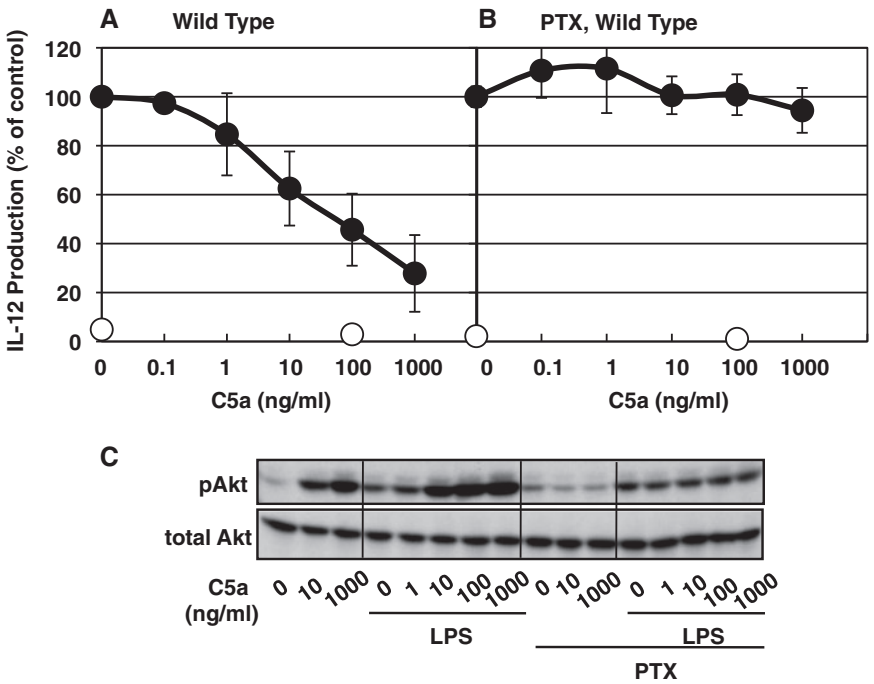

Fig. 1 C5a attenuates LPS-induced IL-12 production and induces Akt phosphorylation in a PTX-sensitive manner. (A and B) Mouse macrophages were incubated in 24-well plates with (closed circle) or without (open circle) the addition of 10 ng/ml LPS. The indicated concentrations of C5a were added to the culture medium immediately before the addition of LPS. After incubation for 18 h, the amount of IL-12 in the medium was determined by ELISA, with samples plated in duplicate. In (B), 50 ng/ml PTX was added 4 h before LPS stimulation. IL-12 in the supernatant of LPS-stimulated cultures (8.66 ± 2.07 and 16.0 ± 5.2 ng/ml in control and PTX-treated cells, respectively) is expressed as 100%. Each point represents the mean ± SD of 4–6 independent experiments. (C) Macrophages were treated or untreated with 50 ng/ml pertussis toxin for 4 h before being stimulated with the indicated concentrations of C5a and LPS for 10 min. Whole cell lysates were analysed by western blot using an antibody against phosphorylated Akt (pAkt, Ser 473) or Akt (total Akt). Representative data are shown from at least three separate experiments.

(Fig. 2A). The inhibitory function of C5a was comparable to the effect of C5a on normal, wild-type cells, and was susceptible to the PTX-treatment (Fig. 2B). These results demonstrated that C5a inhibited IL-12 production through a Gi-mediated, but p110γ-independent, signalling pathway.

PI3K is not involved in the C5a suppression of IL-12 mRNA expression

IL-12 is a heterodimer that consists of the p35 and the p40 subunits, both of which can be regulated at the transcription level (*3*). The inhibitory effect of C5a on IL-12 production has been reported to accompany the suppression of the LPS-induced upregulation of p40 mRNA expression in both human PBMC and elicited MΦ from mice. In the PBMC, a PI3K inhibitor wortmannin abrogated the C5a-mediated effects on both the cytokine production and the p40 mRNA expression (*9*). In contrast, in the MΦ, the inhibitor suppressed C5a-induced changes in mRNA expression without affecting the cytokine production (*8*). According to these findings, it has been argued that the C5a effect on mRNA, but not cytokine production, is dependent on PI3K. Therefore, we then examined the impact of p110γ-deficiency on mRNA expression (Fig. 3). In wild-type MΦ, LPS treatment increased both the p35 and p40 mRNA expression, which was attentuated by C5a. This C5a-mediated inhibition was also observed in p110$\gamma^{-/-}$ cells. The result clearly indicated that the activation of p110γ is not required for the C5a-induced inhibition of mRNA expression.

Since little is known about the effect of C5a on cytokines other than IL-12, the mRNA level of other cytokines was also evaluated (Fig. 3). The LPS-induced upregulation of TNF-α, IFN-γ and iNOS mRNA expression was suppressed by C5a. In contrast, the expression of the anti-inflammatory cytokine IL-10 was increased by C5a. The C5a-enhancement of IL-10 mRNA expression was observed in cells from p110$\gamma^{-/-}$ mice (Fig. 3).

The activity of p110γ is increased by the βγ-subunits of heterotrimeric GTP-binding proteins, thereby enabling the molecular mechanisms to promote p110γ transmission of GPCR signals (*18*). This was originally thought to be specific to the function of p110γ (*18*). However, it has been reported that the βγ-subunits can also activate p110γ in both cell-free and intact-cell systems (*19–21*). Recently, the role of p110β in GPCR signalling system was confirmed by both genetic and pharmacological approaches (*22*, *23*). Thus, the possibility exists that C5a-induced inhibition of mRNA expression was mediated through this PI3K subtype. It is also possible to imagine that C5a activates a tyrosine kinase through a trans-activation mechanism, which in turn, activates class-IA PI3K catalytic subunits (p110α, p110β and p110δ) through the common regulatory subunit p85. To address this idea, we next examined if C5a activity is impaired in cells from p85$^{-/-}$ mice. The levels of all three catalytic subunits decreased severely in these cells (Fig. 4A) as reported previously (*10*). The IL-12 production of p85$^{-/-}$ MΦ (2.33 ± 1.26 ng/ml, $n = 3$) was slightly

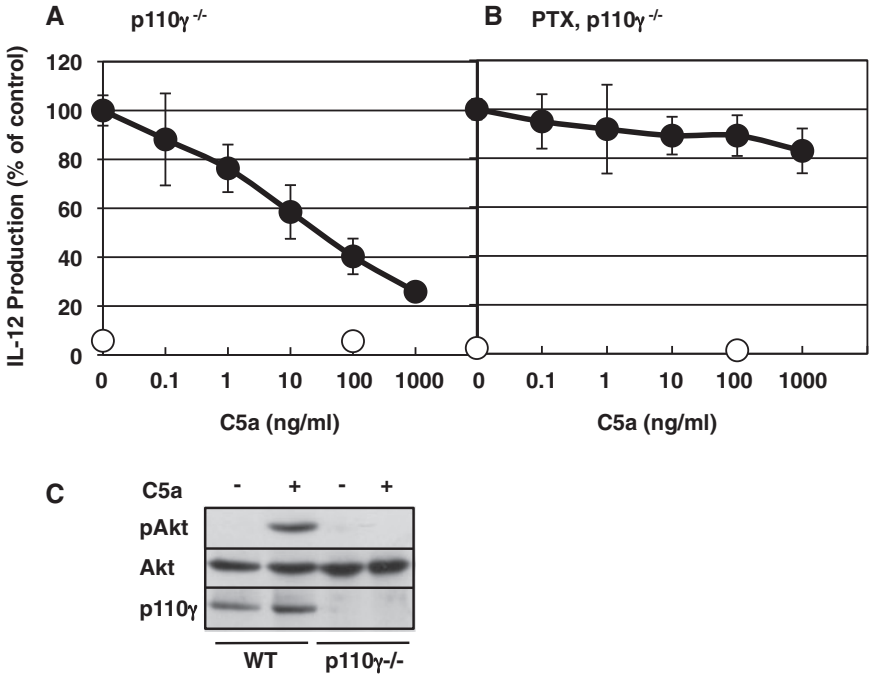

Fig. 2 C5a inhibition of LPS-induced IL-12 production can be observed in p110$\gamma^{-/-}$ cells. (A and B) Macrophages from p110$\gamma^{-/-}$ mice were incubated in 24-well plates with (closed circle) or without (open circle) the addition of 10 ng/ml LPS. The indicated concentrations of C5a were added to the incubation medium immediately before the addition of LPS. After 18 h, the amount of IL-12 in the medium was determined by ELISA, with samples plated in duplicate. In (B), 50 ng/ml PTX was added 4 h before LPS stimulation. IL-12 in the supernatant of LPS-stimulated cultures (7.46 ± 4.42 and 16.0 ± 6.1 ng/ml in control and PTX-treated cells, respectively) is expressed as 100%. Each point represents the mean ± SD of 4–6 independent experiments. (C) Macrophages from wild-type (WT) or p110$\gamma^{-/-}$ mice were incubated with (+) or without (−) 50 ng/mL of C5a for 5 min. Whole cell lysates were analysed by western blot with an antibody against phosphorylated Akt (pAkt, Ser 473), Akt, or p110γ. Representative data are shown from at least three separate experiments.

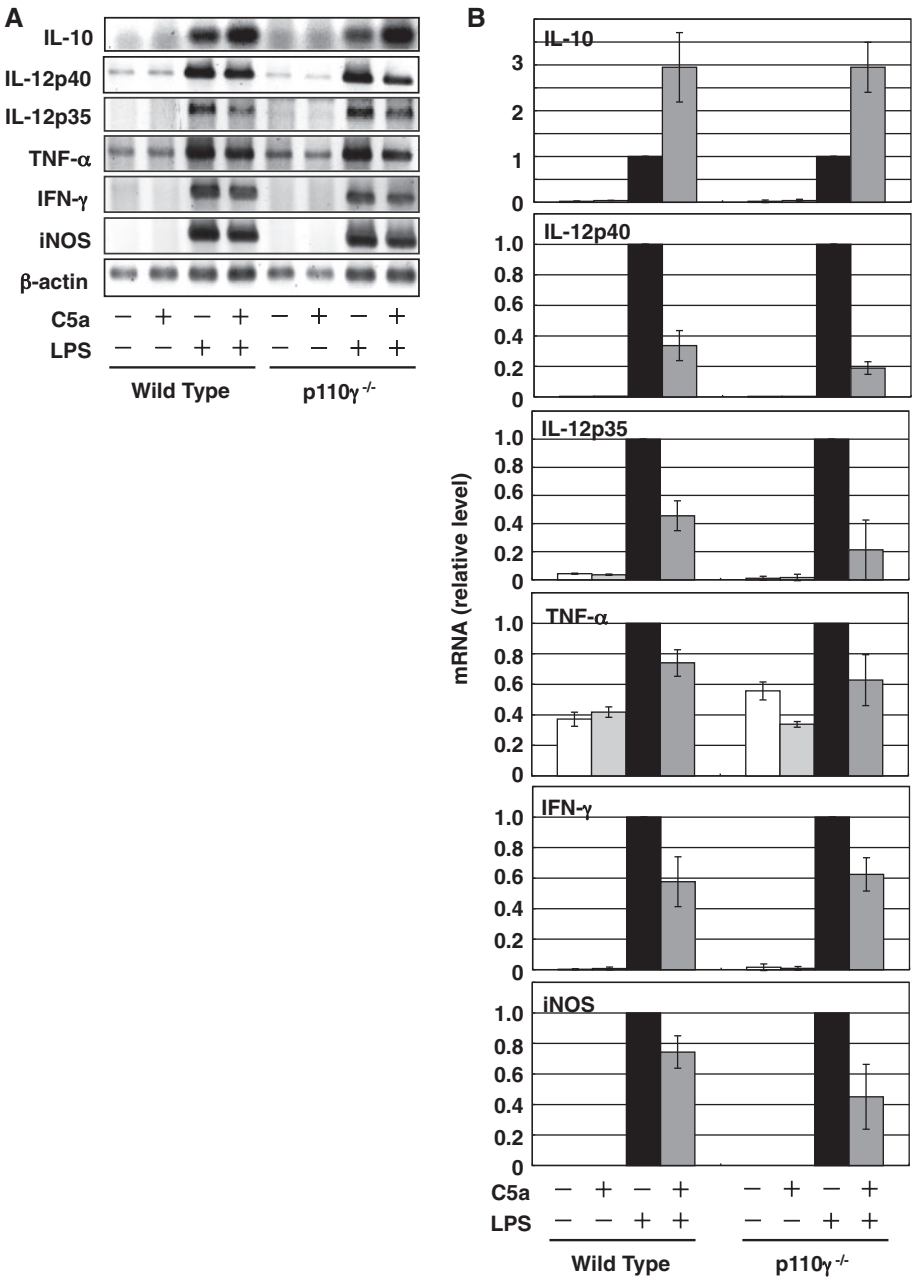

Fig. 3 C5a affects the mRNA expression of multiple cytokines in LPS-stimulated p110$\gamma^{-/-}$ cells. (A) Macrophages from wild-type or p110$\gamma^{-/-}$ mice were incubated in 6-well plates with (+) or without (−) 10 ng/ml LPS for 4 h. Where indicated, 50 ng/ml C5a was also included in the culture. Total RNA was isolated and cDNA was prepared by reverse transcription. The cDNAs of cytokines were amplified by PCR. Similar results were obtained in a repeated experiment. (B) The cDNA prepared as above was analyzed by quantitative PCR as described in the 'Materials and Methods' section. Data were analysed by $\Delta\Delta C_T$ methods where endogenous loading control was β-actin and calibrater was 'LPS alone'. The analyses were performed three times with different preparation of cDNA. Results were expressed as means ± SD.

higher than that of p85$^{+/-}$ cells (1.67 ± 0.57 ng/ml, $n = 3$). Although statistically not significant ($P > 0.05$), this difference may in part reflect the negative regulatory action of class-IA PI3K that has been reported in dendritic cells from these mice (*10*). In p85$^{-/-}$ MΦ, C5a was able to attenuate the LPS-induced IL-12 production (Fig. 4B, upper panel), indicating that this attenuation is not through the activation of class-IA PI3K. The inhibitory effect of C5a on mRNA production of both p35 and p40 was again not impaired in the p85$^{-/-}$ cells (Fig. 4C and D). The C5a-enhancement of IL-10 production was observed in cells from p85α$^{-/-}$ mice as in p110$\gamma^{-/-}$ cells (Fig. 4B, lower panel). This effect was accompanied by increase of IL-10 mRNA expression (Fig. 4C and D).

A PI3K inhibitor wortmannin has been reported to attenuate the C5a inhibition of LPS-induced IL-12 production (*9*). Thus, we next examined the impact of this inhibitor on mouse MΦ. Wortmannin completely inhibited the C5a-induced Akt phosphorylation (Fig. 5A), and augmented the LPS-induced IL-12 production in a dose-dependent manner (Fig. 5B). This effect is at least partly due to the inhibition of

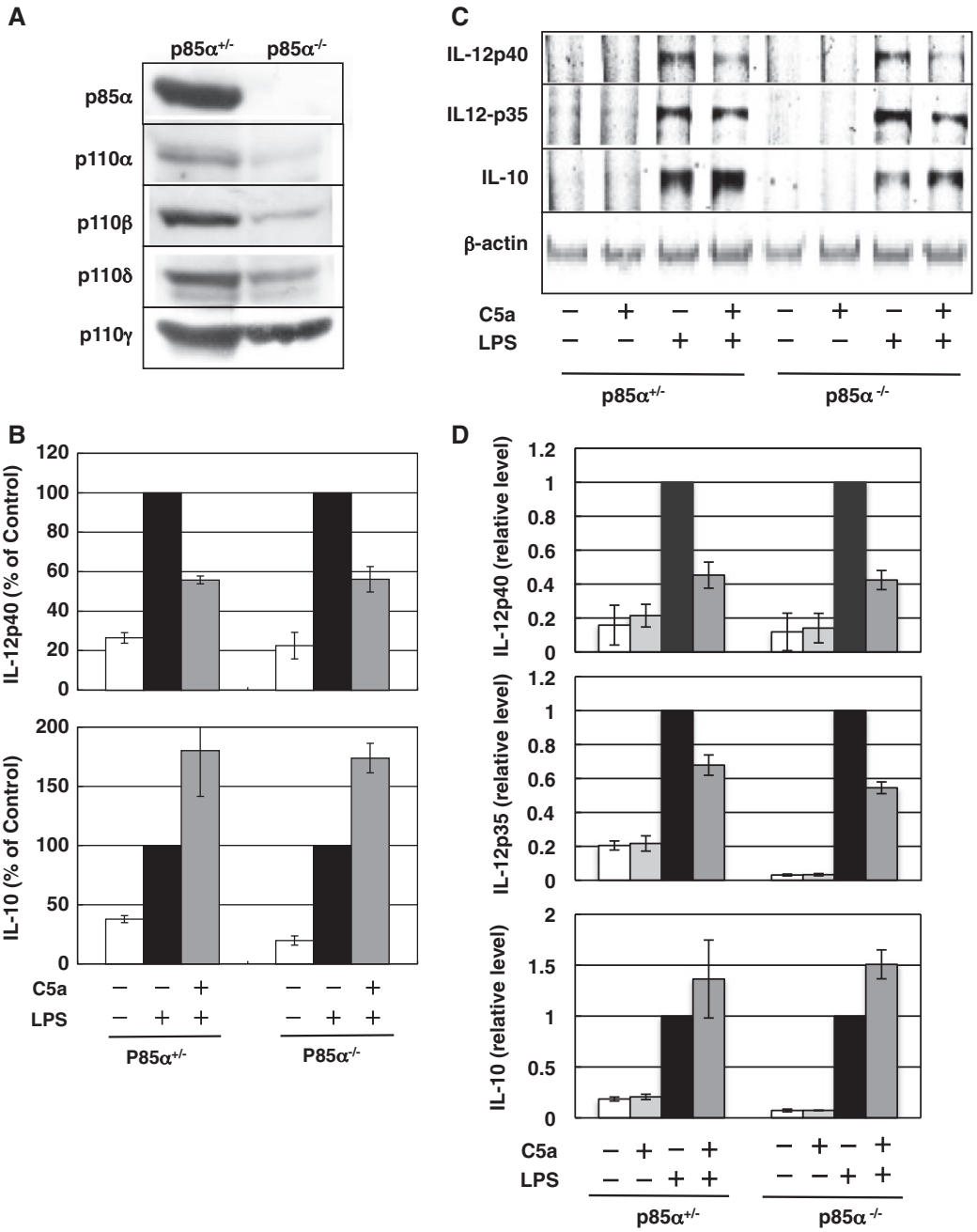

Fig. 4 C5a-mediated regulation of LPS-induced production of multiple cytokines is maintained in p85α$^{-/-}$ cells (Balb/c background). (A) Whole cell lysates were analyzed by western blot using antibodies against p85α and class I PI3Ks. (B) Macrophages were incubated in 24-well plates with (+) or without (−) the addition of 10 ng/ml LPS. C5a (200 ng/ml) were added to the culture medium immediately before the addition of LPS. After incubation for 18 h, the amount of IL-12 and IL-10 in the medium was determined by ELISA. Cytokines in the supernatant of LPS-stimulated cultures is expressed as 100%. Each bar represents the mean ± SD of triplicate determination. Similar results were obtained in a repeated experiment. (C) Cells were incubated in 6-well plates with (+) or without (−) 10 ng/ml LPS for 4 h. Where indicated, 50 ng/ml C5a was also included in the culture. Total RNA was isolated and cDNA was prepared by reverse transcription. The cDNAs of cytokines were amplified by PCR. Similar results were obtained in a repeated experiment. (D) The cDNA prepared as above was analysed by quantitative PCR as described in the 'Materials and Methods' section. Data were analyzed by $\Delta\Delta C_T$ methods where endogenous loading control was β-actin and calibrater was 'LPS alone'. Results were expressed as means ± SD of triplicate samples.

class-IA PI3K subtypes as has been demonstrated in MΦ from p85α$^{-/-}$ mice (*10*). However, wortmannin treatment had no impact on the inhibitory effect of C5a on LPS-induced IL-12 secretion (Fig. 5B).

C5a actions are dependent on MEK-Erk pathway

The above results indicated that PI3K is not required for the modulatory effect of C5a on both p35 and p40 mRNA or on IL-12 productions in mouse MΦ. Erk1/2 is another signalling pathway that has been suggested to mediate effects downstream of the C5a receptor (*8*). In fact, C5a increased Erk1/2 activity in a PTX-sensitive and wortmannin-insensitive manner in the thioglycollate-elicited MΦ (data not shown). As shown in Fig. 5A, a MEK inhibitor, PD98059 completely inhibited C5a-induced phosphorylation of Erk1/2. Similar to the

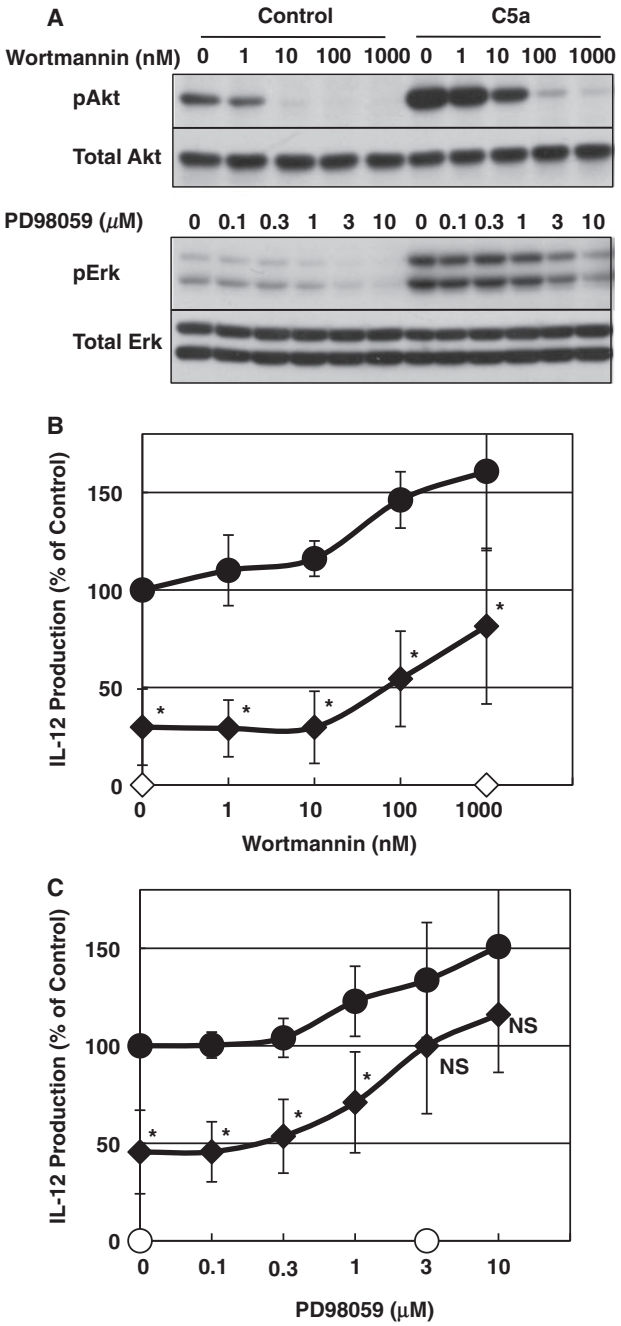

Fig. 5 The C5a-mediated inhibition of IL-12 production is not attenuated by treatment with wortmannin. (A) Macrophages were treated with the increasing concentrations of wortmannin (upper panel) or PD98059 (lower panel) for 15 min followed by the stimulation with 200 ng/ml C5a or vehicle for 3 min. Whole cell lysates were analysed by western blot using an antibody against phosphorylated Akt (pAkt, Ser 473), Akt (total Akt), phosphorylated Erk1/2 (pErk) or Erk1/2 (total Erk). Representative data are shown from at least three separate experiments. (B and C) Mouse macrophages were incubated with (closed symbols) or without (open symbols) 10 ng/ml LPS in 24-well plates and with (diamonds) or without (circles) the addition of 200 ng/ml C5a. The indicated concentration of wortmannin (B) or PD98059 (C) were added to the culture 15 min before LPS stimulation. After incubation for 18 h, the amount of IL-12 in the supernatant was determined by ELISA. Each point represents the mean ± SD of 4–6 independent experiments. $^*P < 0.05$; NS, not significant.

wortmannin treatment, treatment with PD98059 resulted in an increase of LPS-induced IL-12 production. The inhibitory effect of C5a was attenuated at higher concentrations of PD98059 (Fig. 5C), suggesting that G protein-mediated activation of the Erk1/2 pathway is involved in the C5a action.

The C5a-enhancement of IL-10 mRNA expression was observed in cells from both p110$\gamma^{-/-}$ and p85$\alpha^{-/-}$ mice (Figs 3B and 4B, respectively). This effect was accompanied by increased accumulation of IL-10 in the culture medium (Fig. 6A and B). The enhancing effect of C5a on IL-10 was completely abolished by PTX-treatment indicating that the augmentation was mediated through Gi protein (Fig. 6C). It has been reported that LPS-induced IL-10 production is increased by activation of ERK1/2 (*24*, *25*). Consistent with this, PD98059 slightly inhibited the LPS-induced IL-10 production (Fig. 6D). In addition, the enhancing effect of C5a was attenuated by PD98059 (Fig. 6D). Thus the C5a-mediated effects on both IL-10 and IL-12 are considered to depend on the MEK-Erk1/2 pathway.

The C5a effect can be observed in the presence of wortmannin (Fig. 6D), indicating that this C5a action is again independent of the PI3K-Akt pathway. Interestingly, the effect of LPS alone was increased by wortmannin (Fig. 6D). Both positive and negative regulation of IL-10 production by PI3K has been reported. LY294002, an inhibitor of PI3K attenuates LPS-induced IL-10 production in human PBMC (*26*). Conflictingly, wortmannin administration to sepsis model mice increased serum IL-10 level (*27*). One possible reason for this discrepancy is the inhibitor specificity, because the opposite effects of LY294002 and wortmannin on LPS-mediated cellular responses have been reported (*28*). Alternatively, the role of PI3K on IL-10 production is varied depending on cell types.

+IL-10 is known to attenuate the production of IL-12 from APC (*29*, *30*). Therefore, we determined if the increase in IL-10 was the basis of C5a-induced inhibition of IL-12. Anti-IL-10 antibody increased the basal and LPS-induced IL-12 production (Fig. 7). This action of the neutralizing antibody was concentration-dependent and its full effect was obtained at 2 μg/ml (data not shown). The inhibitory effect of C5a was still observed in the presence of 2.5 μg/ml of the antibody (Fig. 7). Therefore, increased IL-10 release cannot fully explain the inhibitory activity of C5a on IL-12 production.

Because MΦ display remarkable plasticity depending on their environment (*31*), we next tested the effect of C5a on LPS-induced cytokine production in GM-CSF-treated bone marrow cells. C5a actions on IL-12 and IL-10 production were observed similarly in these cells (Fig. 8).

Concluding Remarks

Because most pathogens activate both complement and the TLR pathways, we examined the crosstalk between these two innate systems. C5a has been shown to attenuate LPS-induced production of the

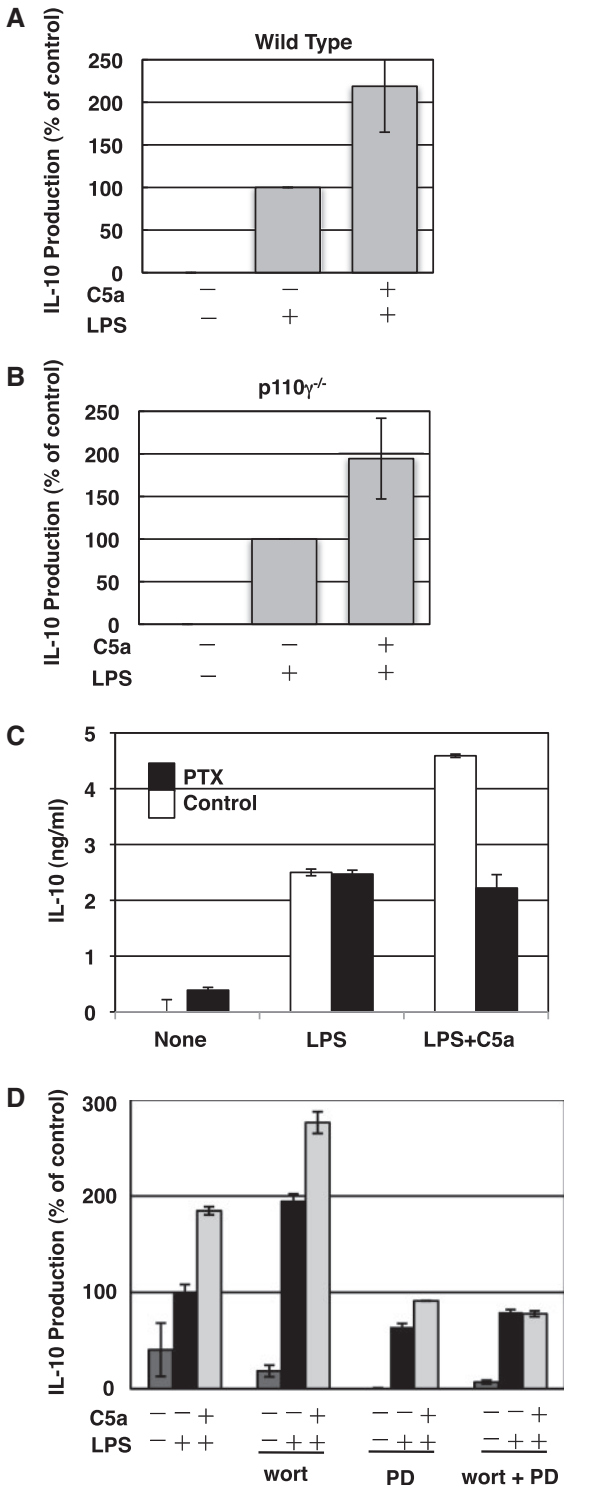

Fig. 6 C5a-mediated changes in LPS-induced IL-10 production is PI3K-independent. (A and B) Macrophages from wild-type (A) or p110$\gamma^{-/-}$ (B) mice were incubated in 24-well plates with (+) or without (−) 10 ng/ml LPS. Where indicated, 200 ng/ml C5a was also added to the culture. After incubation for 18 h, the amount of IL-10 in the supernatant was determined by ELISA. IL-10 in the supernatant of LPS-stimulated cultures (3.54 ± 0.27 and 2.53 ± 0.77 ng/ml in wild-type and p110$\gamma^{-/-}$ cells, respectively; $n = 10$). Each bar indicates the mean ± SD of four independent experiments. (C) About 50 ng/ml PTX was added 4 h before LPS stimulation. Where indicated, 200 ng/ml C5a was also added to the culture immediately before the LPS addition. IL-10 in the supernatant was determined by ELISA. Each point represents the mean ± SD of triplicate observation. A similar result was obtained in a repeated experiment. (D) Macrophages from wild-type mice were incubated with (+) or without (−) the addition of 10 ng/ml LPS and 50 ng/ml C5a. Where indicated, 1 μM wortmannin (wort) and/or 5 μM PD98059 (PD) was added to the culture 15 min before LPS. Representative data are shown from at least three separate experiments.

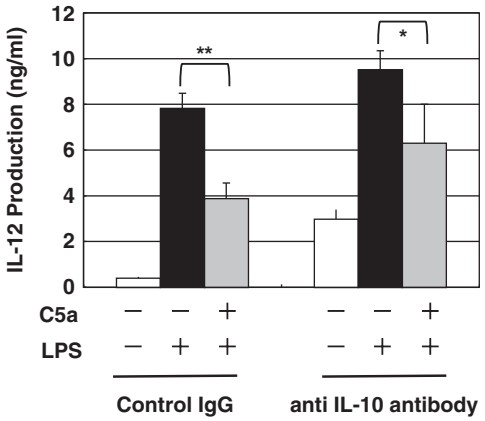

Fig. 7 Neutralizing anti-IL-10 antibody does not attenuate C5a-mediated inhibition of IL-12. Mouse macrophages were plated in 24-well plates and incubated in the presence or absence of an anti-IL-10 antibody (2.5 μg/ml). The cells were stimulated with 10 ng/ml LPS and 200 ng/ml C5a for 18 h. The amount of IL-12 protein in the supernatant was determined by ELISA. Each bar represents the mean ± SD of three independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$.

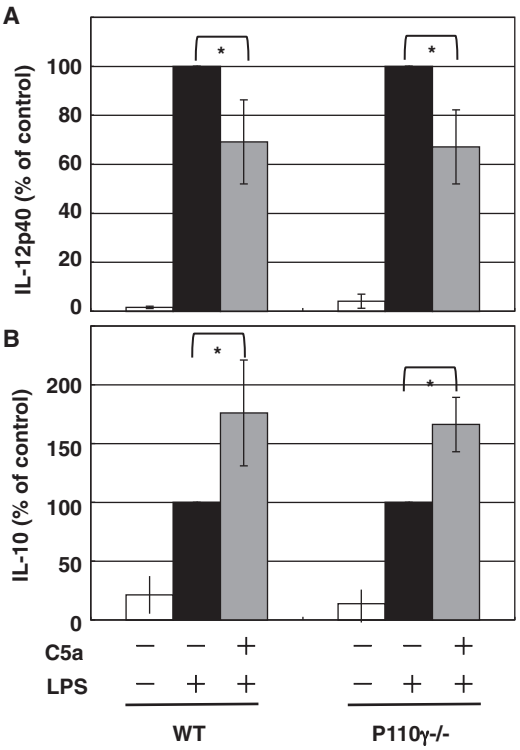

Fig. 8 C5a regulates cytokine production also in GM-CSF-treated bone marrow cells. Mouse bone marrow cells were treated with 10 ng/ml GM-CSF for 7 days. The cells were stimulated with 10 ng/ml LPS and 200 ng/ml C5a for 18 h. The amounts of IL-12 (A) and IL-10 (B) in the supernatant were determined by ELISA. (A) IL-12 in the supernatant of LPS-stimulated cultures (10.3 ± 3.6 and 8.46 ± 2.10 ng/ml in wild-type and p110$\gamma^{-/-}$ cells, respectively; $n = 3$) is expressed as 100%. (B) IL-10 in the LPS-stimulated cultures (2.42 ± 1.55 and 3.46 ± 1.43 ng/ml in wild-type and p110$\gamma^{-/-}$ cells, respectively; $n = 3$) is expressed as 100%. $^{*}P < 0.05$.

IL-12 cytokine family and pharmacological approaches have indicated that PI3K is involved in this regulation (*8*, *9*). In the present study, however, we found that the C5a inhibition of both IL-12 mRNA and protein was maintained in PI3K-deficient cells.

Another novel finding of the present study was the enhanced IL-10 production induced by C5a. We also found that the C5a-mediated changes in the expression of both IL-12 and IL-10 were sensitive to PD98059, and possibly dependent on the Erk1/2 pathway. However, the underlying mechanisms for Erk1/2 involvement in complement-mediated cytokine regulation is still largely unknown. It has been reported that FcγR ligation enhances LPS-induced IL-10 production through chromatin modification, which is susceptible to PD98059 (*25*). Therefore, it is intriguing to consider that a similar mechanism is involved in the PI3K-independent regulation of cytokines by C5a.

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Conflict of interest

None declared.

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