

# Adenylate cyclase toxin of *Bordetella pertussis* inhibits TLR-induced IRF-1 and IRF-8 activation and IL-12 production and enhances IL-10 through MAPK activation in dendritic cells

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**Abstract:** Adenylate cyclase toxin (CyaA) of *Bordetella pertussis* binds to CD11b/CD18 on macrophages and dendritic cells (DC) and confers virulence to the bacteria by subverting innate immune responses of the host. We have previously demonstrated that CyaA promotes the induction of IL-10-secreting regulatory T cells in vivo by modulating DC activation. Here, we examine the mechanism of immune subversion, specifically, the modulation of TLR signaling pathways in DC. We found that CyaA synergized with LPS to induce IL-10 mRNA and protein expression in DC but significantly inhibited IL-12p70 production. CyaA enhanced LPS-induced phosphorylation of p38 MAPK and ERK in DC, and inhibitors of p38 MAPK, MEK, or NF- $\kappa$ B suppressed IL-10 production in response to LPS and CyaA. However, inhibition of p38 MAPK, MEK, and NF- $\kappa$ B did not reverse the inhibitory effect of CyaA on TLR agonist-induced IL-12 production. Furthermore, CyaA suppression of IL-12 was independent of IL-10. In contrast, CyaA suppressed LPS- and IFN- $\gamma$ -induced IFN-regulatory factor-1 (IRF-1) and IRF-8 expression in DC. The modulatory effects of CyaA were dependent on adenylate cyclase activity and induction of intracellular cAMP, as an enzyme-inactive mutant of CyaA failed to modulate TLR-induced signaling in DC, whereas the effects of the wild-type toxin were mimicked by stimulation of the DC with PGE<sub>2</sub>. Our findings demonstrate that CyaA modulates TLR agonist-induced IL-10 and IL-12p70 production in DC by, respectively, enhancing MAPK phosphorylation and inhibiting IRF-1 and IRF-8 expression and that this is mediated by elevation of intercellular cAMP concentrations. *J. Leukoc. Biol.* 84: 234–243; 2008.

**Key Words:** bacterial immunity · immune suppression · TLR signaling

## INTRODUCTION

Activation of TLR signaling pathways in innate immune cells leads to the production of inflammatory cytokines and other

immune mediators that play a critical role in host protective immunity against infectious agents. However, pathogens have evolved strategies for subverting host immune responses by inhibiting the inflammatory molecules and effector cells and by the induction of reciprocal anti-inflammatory cytokines and regulatory cells [1]. These immunosuppressive effects are often mediated through direct interference with TLR signaling molecules or the interaction of viral or bacterial proteins with specific cell surface receptors on immune cells, especially cells of the innate immune system. One such receptor targeted by a number of bacterial virulence factors is the  $\alpha_M\beta_2$  integrin CD11b/CD18, expressed on macrophages and dendritic cells (DC).

Adenylate cyclase toxin (CyaA) is a major virulence factor of *Bordetella pertussis*, which binds to CD11b/CD18 and subverts host immunity to *B. pertussis* by inhibiting chemotaxis, phagocytosis, and superoxide production and thereby promotes bacterial colonization and persistence [2–4]. More recently, we have demonstrated that CyaA can also subvert innate and adaptive immunity by modulating TLR activation of DC, enhancing IL-10 and inhibiting IL-12 production, and as a consequence, promote the induction of IL-10-secreting regulatory T (Treg) cells [5, 6]. IL-12-induced IFN- $\gamma$  production by NK cells and Th1 cells plays a critical role in protective immunity to *B. pertussis* [7]. However, IL-10-secreting Treg cells are also induced during infection with *B. pertussis* [8]. We found that antigen-specific, IL-10-secreting Treg cell clones generated from the lungs of mice during acute *B. pertussis* infection were capable of suppressing IFN- $\gamma$  production by protective *B. pertussis*-specific Th1 cells [8]. Thus, it appears that induction of anti-inflammatory cytokines and Treg cells is not only an important host protective mechanism to limit immunopathology during infection but also can be exploited by pathogens as an immune subversion strategy to suppress Th1 cells and prolong their survival in the host [1]. The induction of IL-10-secreting Treg cells is driven by semi-mature DC that secrete IL-10 but in which IL-12 production is suppressed, a regulatory pheno-

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type observed following stimulation of DC with CyaA and other immunomodulatory molecules [5, 6, 8, 9].

The induction of IL-10 production by macrophages and DC in response to TLR agonists has been linked with activation of NF- $\kappa$ B as well as ERK and p38 MAPK signaling pathways [10–14]. Activation of NF- $\kappa$ B and p38 MAPK has also been implicated in TLR-induced IL-12 production [15, 16]. However, recent reports have suggested that IFN-regulatory factors (IRF), which mediate antiviral responses through transcription of IFN genes, also play a critical role in signaling for IL-12 production. It has been demonstrated that expression of hepatitis C virus core protein suppresses IRF-1 and IL-12 promoter activity in Huh-7 cells [17]. Furthermore, it has been reported that infection of human DC with a mutant *B. pertussis* lacking CyaA enhanced expression of IRF-1 and IRF-8 over that seen with the wild-type bacteria [18].

CyaA is composed of a repeat in toxin hemolysin moiety, which mediates cell binding, and a catalytic adenylate cyclase domain, which is activated by calmodulin and catalyzes the conversion of ATP to cAMP [19, 20]. The toxic effects of CyaA are mediated by enhancement of intracellular cAMP concentrations. However, the mechanism of immunomodulation, in particular, the possible interference with cell signaling, has received little attention. In this study, we have examined the role of CyaA in modulating TLR-activated DC at the signaling level. Our data demonstrate that CyaA enhances IL-10 and inhibits IL-12 at the protein and mRNA level by independently targeting distinct signaling pathways in DC. Induction of IL-10 was mediated through enhancement of ERK and p38 MAPK phosphorylation, whereas IL-12 was associated with inhibition of TLR-activated IRF-1 and IRF-8. Furthermore, the immunomodulatory effects were dependent on enhancement of intracellular cAMP and were mimicked by PGE2.

## MATERIALS AND METHODS

### Purification of CyaA

CyaA and enzymatically inactive CyaA (iAC-CyaA) proteins were expressed and purified from *Escherichia coli* XL-1 blue cells carrying plasmid pJR2 (expressing His-CyaA and CyaC) or pNM2 (expressing His-iAC-CyaA and CyaC) as described previously [5]. N-terminal His-tagged proteins were purified from inclusion bodies by DEAE-Sepharose and  $\text{Ni}^{2+}$ -agarose chromatography [6]. LPS was removed from CyaA by dialysis, first against Dulbecco's PBS (Biosera, Ringmer, UK) containing 1 mM EDTA and 1 M urea, pH 4.6, and then against Dulbecco's PBS containing 0.1 mM  $\text{CaCl}_2$  and 3 M urea, pH 8.0. LPS was determined to be 234 pg LPS per  $\mu\text{g}$  protein for CyaA and 187 pg LPS per  $\mu\text{g}$  protein for iAC-CyaA by the PyroGene recombinant factor C endotoxin detection assay (Cambrex, Nottingham, UK), performed as described by the manufacturer. All chemicals were from Sigma-Aldrich (Dublin, Ireland) unless stated otherwise.

### Animals

Female, specific pathogen-free C57BL/6 mice were purchased from Harlan (UK). All mice were maintained according to European Union regulations, and experiments were performed under license from the Department of Health and with approval from the Trinity College Dublin Bioresources Ethics Committee (Ireland).

### DC

Bone marrow-derived, immature DC were prepared by culturing bone marrow cells obtained from the femurs and tibiae of C57BL/6 mice in RPMI 1640

containing 10% FCS (Biosera), 100 U/ml penicillin, 1 mg/ml streptomycin, and 200  $\mu\text{M}$  L-glutamine (Sigma-Aldrich) and supplemented with conditioned media from a GM-CSF-expressing cell line (J558-GM-CSF) to give a final concentration of 40 ng/ml GM-CSF. On Day 3, fresh medium containing 40 ng/ml GM-CSF was added. On Day 6, semiadherent cells were removed using 0.02% EDTA (Sigma-Aldrich). Cells were reseeded in medium supplemented with 40 ng/ml GM-CSF. On Day 8, fresh medium containing 40 ng/ml GM-CSF was added. On Day, 10 loosely adherent cells were collected, washed, and used for assays.

DC were cultured at  $1 \times 10^6$  cells/ml with CyaA (1  $\mu\text{g}/\text{ml}$ ), iAC-CyaA (1  $\mu\text{g}/\text{ml}$ ), LPS (50 ng/ml, Alexis Pharmaceuticals, Nottingham, UK), and recombinant IFN- $\gamma$  (20 ng/ml, R&D Systems, Abingdon, UK) or combinations thereof. LPS, IFN- $\gamma$ , and CyaA were added simultaneously to cells. In some experiments, inhibitors of MEK (U0126), p38 MAPK (SB203580), NF- $\kappa$ B (Bay 11-7082), or protein kinase A [PKA; Rp-8-bromo-cAMP-phosphorothioate (Rp-8-Br-cAMPS)] were added to cells 2 h prior to the addition of CyaA, LPS, or IFN- $\gamma$ . Inhibitors were purchased from Calbiochem, Merck Biosciences (Nottingham, UK). In other experiments, 10  $\mu\text{g}/\text{ml}$  anti-IL-10 mAb (BD Biosciences, Oxford, UK) or 5  $\mu\text{g}/\text{ml}$  Actinomycin D (Sigma-Aldrich) was added to cells at the same time as CyaA, LPS, and IFN- $\gamma$ . Alternatively, DC were stimulated with 1  $\mu\text{M}$  PGE2 (Calbiochem).

### Cytokine assays

Supernatants were removed from cells after the indicated times, and concentrations of IL-10 and IL-12p70 were measured by immunoassay using pairs of antibodies purchased from R&D Systems, according to the manufacturer's instructions.

### Real-time RT-PCR

Total cellular RNA was prepared using Trizol (Invitrogen, Dublin, Ireland). Single-stranded cDNA was synthesized from 1  $\mu\text{g}$  RNA according to the Moloney murine leukemia virus RT protocol (Promega, Southampton, UK).  $\text{MgCl}_2$  and RNasin were also purchased from Promega. dNTPs were obtained from Sigma-Aldrich and random decamers from Ambion (Applied Biosystems, Warrington, UK). Real-time PCR for the detection of mRNA was performed using sense and antisense primers and a FAM-labeled MGB Taqman probe for IL-10 (Mm00439616\_m1), IL-12p35 (Mm00434165\_m1), IL-12p40 (Mm00434174\_m1), IRF-1 (Mm00515191\_m1), and IRF-8 (Mm00492567\_m1), purchased from Applied Biosystems. 18S rRNA was used as an endogenous control and for standardization. Samples were assayed on an Applied Biosystems 7300 real-time PCR machine. Relative quantification was performed according to the  $\Delta\Delta$  comparative threshold method, and untreated cells as calibrator were set to 1 [21].

### Western blotting

Cell lysates were prepared and resolved on 10% SDS-PAGE gels and transferred to a nitrocellulose membrane (Schleicher and Schuell, Dassel, Germany). Blots were incubated with anti-phospho (p)-p38, anti-I $\kappa$ B $\alpha$  (Cell Signaling Technology, Hertfordshire, UK), anti-p38, anti-p-ERK, anti-ERK, anti-IRF-1, anti-IRF-8 (Santa Cruz, Heidelberg, Germany), or anti- $\beta$ -Actin (Sigma-Aldrich) and peroxidase-conjugated secondary antibodies and detected using ECL (Amersham Biosciences, Little Chalfont, UK). Differences in expression of proteins were determined by densitometry analysis of blots using Scion Image software (Scion Corp., Frederick, MD, USA).

### cAMP quantification

DC were stimulated for 30 min with CyaA or iAC-CyaA (1  $\mu\text{g}/\text{ml}$ ). Intracellular cAMP accumulation was measured by competitive ELISA using the Amersham Biosciences Biotrak enzyme immunoassay kit. Samples were serially diluted to obtain values within the linear range of the standard curve.

### Statistics

Statistical analyses were carried out using GraphPad prism, Version 4.0 (GraphPad Software, San Diego, CA, USA). Differences between mean values were assessed by ANOVA or where only two samples were compared by two-tailed Student's *t*-test and were considered significant for *P* values <0.05.

## RESULTS

### CyaA enhances IL-10 protein and mRNA expression and suppresses LPS- and IFN- $\gamma$ -induced IL-12 expression in DC

We have previously demonstrated that CyaA can act as an adjuvant to promote antigen-specific Th2 and IL-10-secreting Treg cell responses to coadministered antigens in vivo, and this reflects its ability to modulate TLR activation of DC [6]. In this study, we have examined the mechanisms responsible for these immunomodulatory effects. Consistent with our previous reports [5, 6], we found that CyaA synergized with the TLR agonist to promote IL-10 production, while inhibiting IL-12p70 production by DC. Incubation of immature bone marrow-derived DC with CyaA alone did not induce IL-10. Treatment of DC with LPS (50 ng/ml) induced low levels of IL-10 production, and coincubation with CyaA resulted in significantly increased production of IL-10 (Fig. 1A). Incubation of DC with CyaA alone did not induce IL-12p70 induction. In contrast, treatment with LPS and IFN- $\gamma$  induced significant IL-12p70 production by DC, and this was significantly inhibited by cotreatment with CyaA (Fig. 1B).

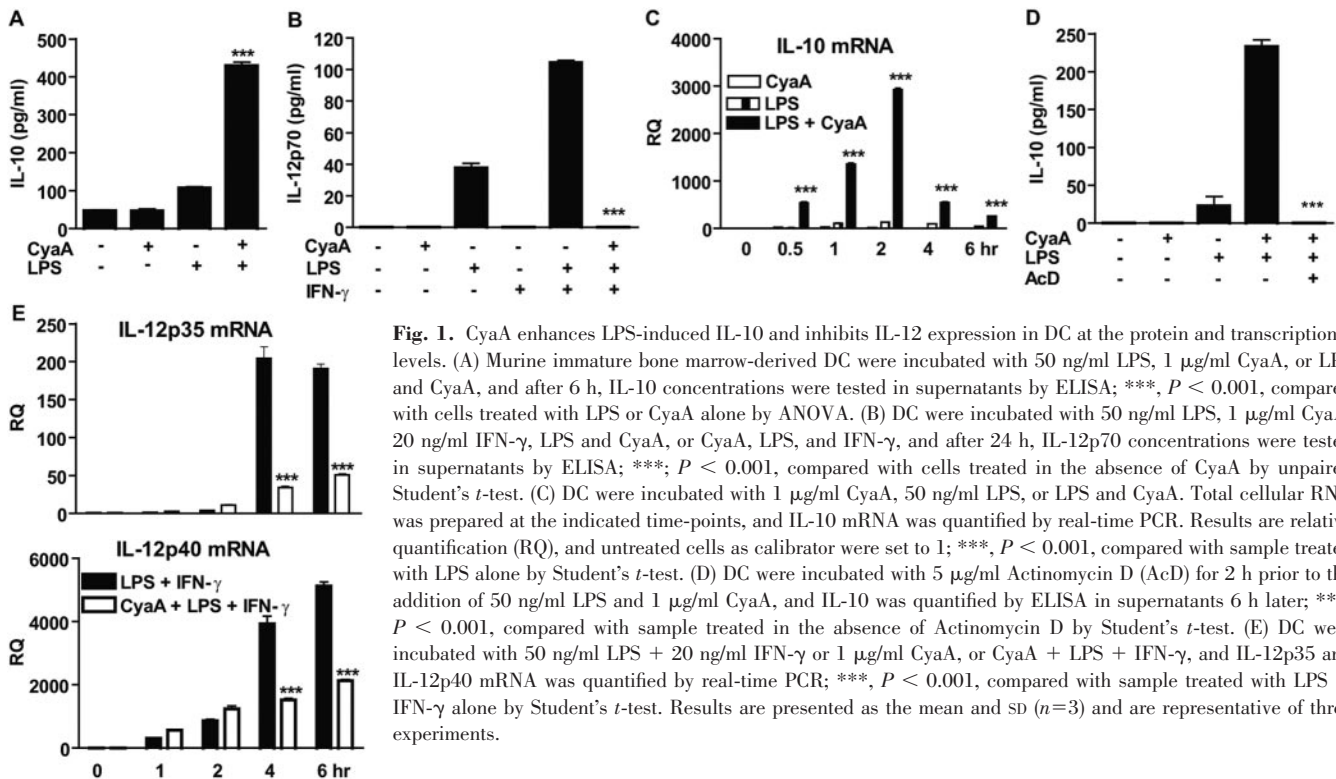
To investigate if enhancement of IL-10 and inhibition of IL-12 protein by CyaA reflected modulation of mRNA expression, we used real-time RT-PCR to quantify IL-10 and IL-12 mRNA expression. Incubation of DC with CyaA alone failed to enhance IL-10 mRNA expression (Fig. 1C). As observed at the protein level, treatment of DC with a low concentration of LPS (50 ng/ml) alone induced a small increase in IL-10 mRNA expression. However, cotreatment with CyaA resulted in significantly increased expression of IL-10 mRNA (Fig. 1C). A net increase in an mRNA species can result from inhibition of the

decay of cytoplasmic mRNA [22]. To investigate if this accounted for the increase in IL-10 mRNA expression observed, DC were treated with Actinomycin D, an inhibitor of de novo transcription. Pretreatment of cells for 2 h with Actinomycin D reversed the induction of IL-10 seen in response to LPS and CyaA (Fig. 1D). These results favor a direct transcriptional mechanism of up-regulation of IL-10 production in response to LPS and CyaA.

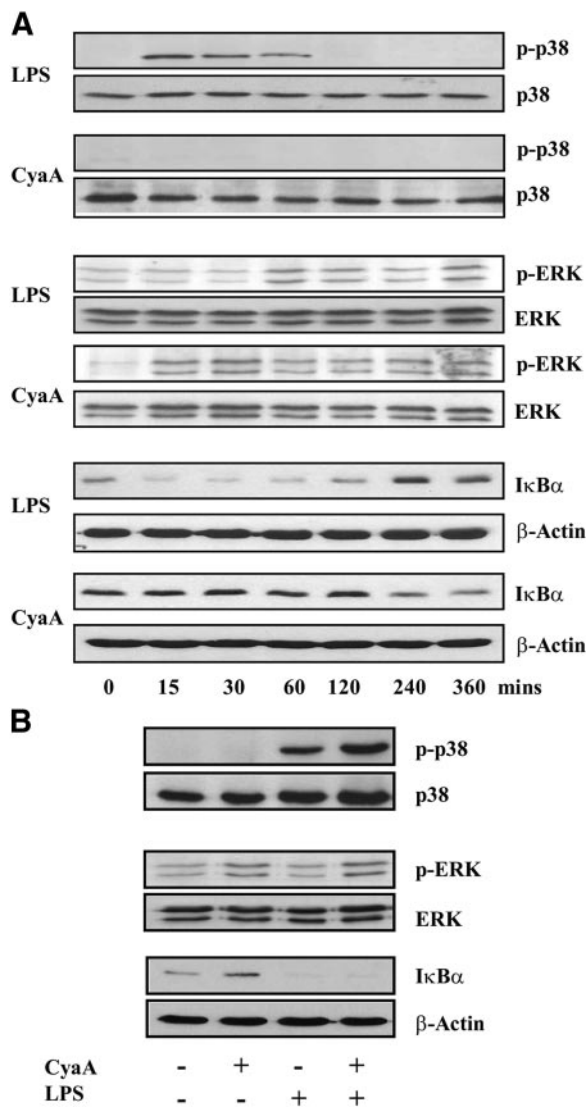
IL-12p70 is the bioactive form of IL-12 and is a heterodimer comprised of independently regulated 40 kDa (IL-12p40) and 35 kDa (IL-12p35) subunits. Quantification of IL-12p35 and IL-12p40 mRNA by real-time RT-PCR demonstrated that expression of both of these subunits was increased in DC following treatment with LPS and IFN- $\gamma$ . Cotreatment with CyaA blocked the increase in IL-12p35 and IL-12p40 mRNA expression induced with LPS and IFN- $\gamma$  (Fig. 1E).

### CyaA enhancement of LPS-induced IL-10 is dependent on p38 MAPK, MEK, and NF- $\kappa$ B

The MAPKs p38 and ERK as well as NF- $\kappa$ B have been implicated in the signaling pathways for TLR agonist-activated IL-10 production in a variety of cell types [10–14]. Here, we examined the role of p38, ERK, and NF- $\kappa$ B in the synergistic induction of IL-10 by CyaA and LPS. Although CyaA alone had no effect on p38 activation, LPS induced rapid phosphorylation of p38 with maximum activation seen at 15 min and expression returning to that seen in untreated cells after 2 h (Fig. 2A). However, when CyaA was added in combination with LPS, we found that CyaA significantly enhanced activation of p-p38 MAPK (CyaA or LPS vs. LPS+CyaA-treated DC;  $P < 0.05$ ;  $n = 4$ ; representative blot in Fig. 2B, top panel), which was still detectable at 2 h (not shown). Treatment of DC with



**Fig. 1.** CyaA enhances LPS-induced IL-10 and inhibits IL-12 expression in DC at the protein and transcriptional levels. (A) Murine immature bone marrow-derived DC were incubated with 50 ng/ml LPS, 1  $\mu$ g/ml CyaA, or LPS and CyaA, and after 6 h, IL-10 concentrations were tested in supernatants by ELISA; \*\*\*,  $P < 0.001$ , compared with cells treated with LPS or CyaA alone by ANOVA. (B) DC were incubated with 50 ng/ml LPS, 1  $\mu$ g/ml CyaA, 20 ng/ml IFN- $\gamma$ , LPS and CyaA, or CyaA, LPS, and IFN- $\gamma$ , and after 24 h, IL-12p70 concentrations were tested in supernatants by ELISA; \*\*\*,  $P < 0.001$ , compared with cells treated in the absence of CyaA by unpaired Student's  $t$ -test. (C) DC were incubated with 1  $\mu$ g/ml CyaA, 50 ng/ml LPS, or LPS and CyaA. Total cellular RNA was prepared at the indicated time-points, and IL-10 mRNA was quantified by real-time PCR. Results are relative quantification (RQ), and untreated cells as calibrator were set to 1; \*\*\*,  $P < 0.001$ , compared with sample treated with LPS alone by Student's  $t$ -test. (D) DC were incubated with 5  $\mu$ g/ml Actinomycin D (AcD) for 2 h prior to the addition of 50 ng/ml LPS and 1  $\mu$ g/ml CyaA, and IL-10 was quantified by ELISA in supernatants 6 h later; \*\*\*,  $P < 0.001$ , compared with sample treated in the absence of Actinomycin D by Student's  $t$ -test. (E) DC were incubated with 50 ng/ml LPS + 20 ng/ml IFN- $\gamma$  or 1  $\mu$ g/ml CyaA, or CyaA + LPS + IFN- $\gamma$ , and IL-12p35 and IL-12p40 mRNA was quantified by real-time PCR; \*\*\*,  $P < 0.001$ , compared with sample treated with LPS + IFN- $\gamma$  alone by Student's  $t$ -test. Results are presented as the mean and SD ( $n = 3$ ) and are representative of three experiments.



**Fig. 2.** CyaA enhances LPS-induced p38 MAPK and ERK phosphorylation. (A) DC were treated with 50 ng/ml LPS or 1  $\mu$ g/ml CyaA for the indicated times. Whole cell lysates were analyzed by immunoblotting with antibodies specific for p-p38, p-ERK, and I $\kappa$ B $\alpha$ . Equal protein loading was confirmed by immunoblotting with anti-p38, ERK, and  $\beta$ -Actin. (B) DC were treated with medium only, 50 ng/ml LPS, 1  $\mu$ g/ml CyaA, or LPS and CyaA for 1 h (p38) or 15 min (ERK and I $\kappa$ B $\alpha$ ). Whole cell lysates were analyzed by immunoblotting with antibodies specific for p-p38, p-ERK, and I $\kappa$ B $\alpha$ . Equal protein loading was confirmed by immunoblotting with anti-p38, ERK, and  $\beta$ -Actin.

CyaA induced significant activation of ERK within 15 min, and this activation was sustained for up to 6 h (CyaA vs. medium-treated DC;  $P < 0.05$ – $P < 0.01$ ;  $n = 4$ ; representative blot in Fig. 2A). Activation of ERK was detected 2 h after treatment of DC with LPS (50 ng/ml). However cotreatment of DC with CyaA in combination with LPS led to significant activation of ERK at the earlier time-point of 1 h (CyaA or LPS vs. LPS+CyaA-treated DC;  $P < 0.05$ ;  $n = 4$ ; representative blot in Fig. 2B, middle panel). We also found that LPS induced rapid activation of NF- $\kappa$ B in DC, as measured by degradation of I $\kappa$ B $\alpha$ , whereas treatment with CyaA alone led to significant I $\kappa$ B $\alpha$  degradation at later time-points (4–8 h; CyaA vs. medium-treated DC;  $P < 0.01$ – $P < 0.001$ ;  $n = 4$ ; representative blot in

Fig. 2A). However, addition of CyaA did not affect I $\kappa$ B $\alpha$  degradation induced by LPS (Fig. 2B, bottom panel).

We next examined the effects of specific inhibitors of p38, MEK, and NF- $\kappa$ B on the enhancement of IL-10 production by CyaA. Pretreatment of DC with a p38 MAPK-specific inhibitor SB203580 (1  $\mu$ M) reversed p38 activation (Fig. 3A) and completely blocked the induction of IL-10 by LPS and CyaA (Fig. 3B). Furthermore, inhibition of MEK by pretreatment with the MEK-specific inhibitor U0126 (10  $\mu$ M) inhibited phosphorylation of ERK (Fig. 3A) and significantly inhibited IL-10 production induced by LPS and CyaA (Fig. 3B). Inhibition of NF- $\kappa$ B with the specific inhibitor BAY 11-7082 (10  $\mu$ M) also significantly inhibited IL-10 production induced by LPS and CyaA (Fig. 3B). These findings demonstrate that p38 MAPK, ERK and NF- $\kappa$ B are involved in the synergistic induction of IL-10 by CyaA and LPS.

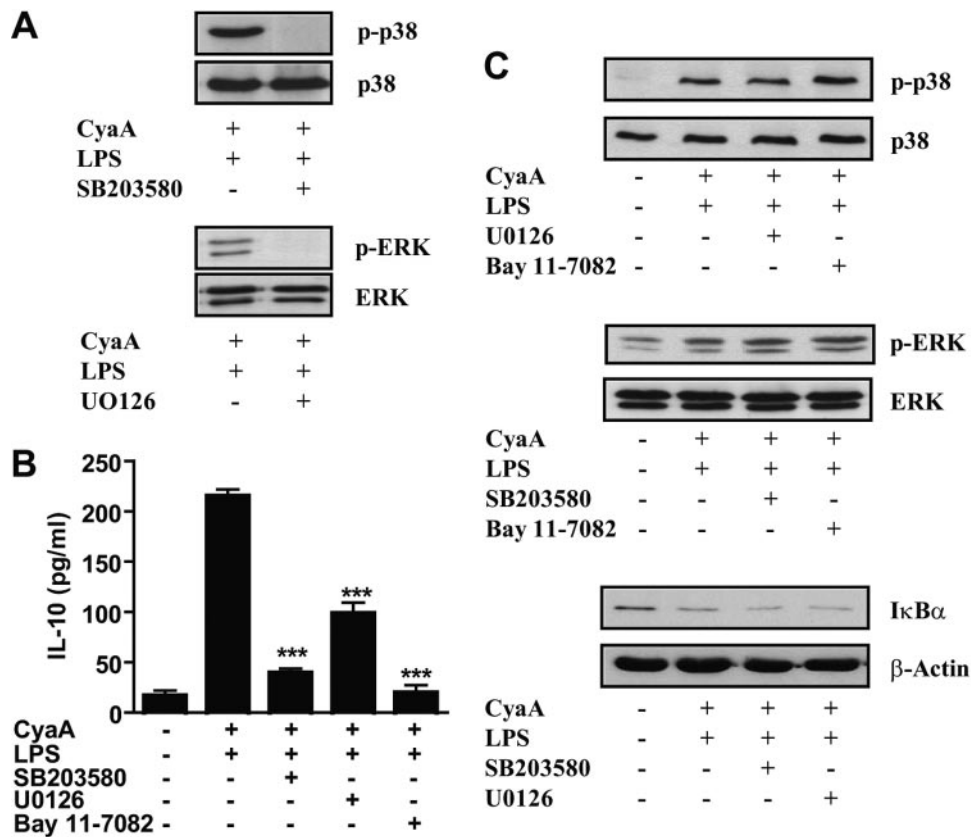
As MEK has previously been shown to be required for p38 MAPK activation downstream of Ras [23], we investigated the possibility that MEK or NF- $\kappa$ B lies upstream of p38 MAPK in the signaling pathway for IL-10 induced by LPS and CyaA. We found that inhibition of MEK or NF- $\kappa$ B by pretreatment with the corresponding inhibitors had no effect on the induction of p-p38 expression in response to CyaA and LPS (Fig. 3C, top panel). Similarly, inhibition of p38 MAPK or NF- $\kappa$ B had no effect on CyaA- and LPS-induced ERK activation (Fig. 3C, middle panel). Finally, inhibition of MEK or p38 MAPK did not prevent the degradation of I $\kappa$ B $\alpha$  following treatment with CyaA and LPS, suggesting that NF- $\kappa$ B is not a downstream target of MEK or p38 MAPK (Fig. 3C, bottom panel). These results confirm that LPS- and CyaA-induced IL-10 expression requires the activation of distinct signaling pathways involving p38, MEK, and NF- $\kappa$ B.

### Inhibition of LPS- and IFN- $\gamma$ -induced IL-12p70 is independent of MAPK activation and IL-10 induction

The induction of IL-12p70 production by human DC has been shown to be dependent on p38 MAPK [15]. NF- $\kappa$ B has also been implicated in up-regulation of IL-12p40 following CD40 ligation [16]. Furthermore, it has been reported that IL-10 induced by immunomodulatory molecules can suppress IL-12 production [24], and we found that LPS- and CyaA-induced IL-10 production was dependent on activation of ERK, p38 MAPK, and NF- $\kappa$ B. As enhancement of IL-10 production by CyaA is detectable before suppression of LPS- and IFN- $\gamma$ -induced IL-12p70, we considered the possibility that the inhibition of IL-12p70 by CyaA was mediated through IL-10 induction and was dependent on activation of MAPK or NF- $\kappa$ B. To investigate this, DC were stimulated with LPS and IFN- $\gamma$ , with and without CyaA and in the presence or absence of a neutralizing anti-IL-10 antibody. Consistent with the data shown in Figure 1, CyaA inhibited IL-12p70 production in response to LPS and IFN- $\gamma$  (Fig. 4A). This suppression was not reversed by the addition of anti-IL-10 antibody, indicating that up-regulation of IL-10 expression is not required for inhibition of IL-12p70 by CyaA.

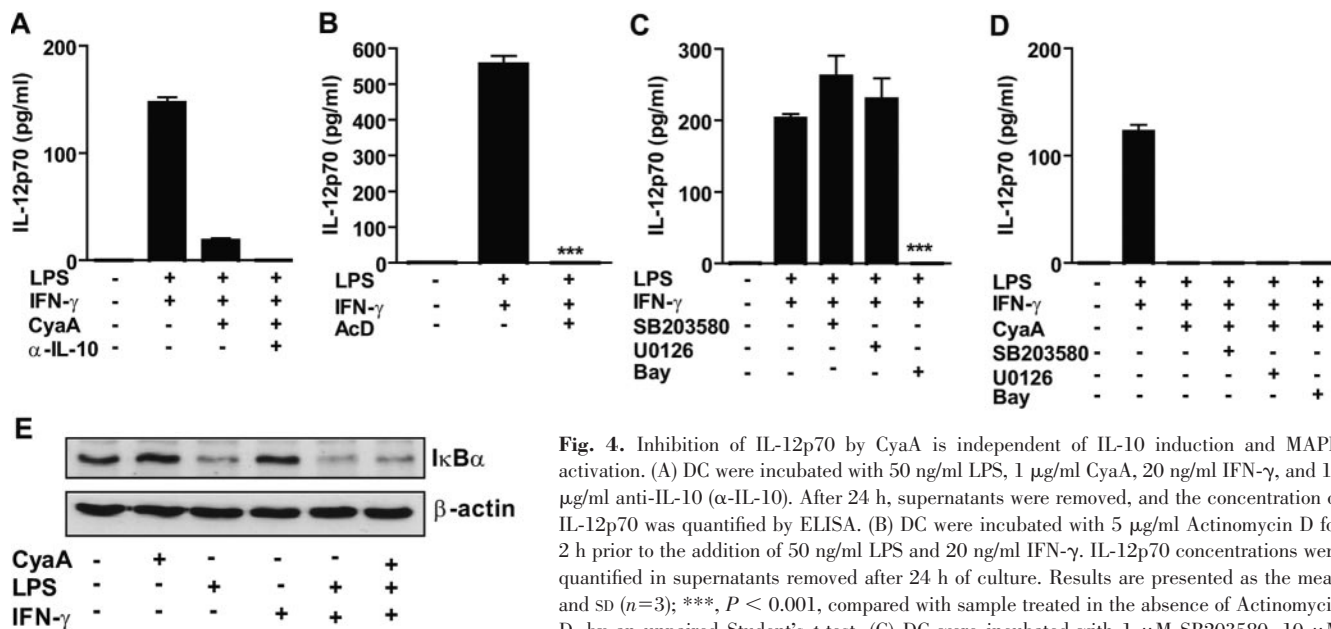
We next established that IL-12p70 production in response to LPS and IFN- $\gamma$  was a result of increased transcription. Treatment of DC with Actinomycin D (5  $\mu$ g/ml), an inhibitor of de

**Fig. 3.** CyaA enhancement of TLR agonist-induced IL-10 is dependent on activation of p38 MAPK, MEK, and NF- $\kappa$ B. DC were incubated with 1  $\mu$ M SB203580, 10  $\mu$ M U0126, or 2  $\mu$ M Bay 11-7082 for 2 h prior to the addition of 50 ng/ml LPS and 1  $\mu$ g/ml CyaA. (A) After 15 min, whole cell lysates were analyzed by immunoblotting with antibodies specific for p-p38 or p-ERK. Equal protein loading was confirmed by immunoblotting with anti-p38 and ERK. (B) IL-10 concentrations were quantified in supernatants removed after 6 h of culture. Results are presented as the mean and SD ( $n=3$ ); \*\*\*,  $P < 0.001$ , compared with cells treated with LPS + CyaA by unpaired Student's  $t$ -test. (C) Western blot analysis of p-p38, p-ERK, and I $\kappa$ B $\alpha$  expression in whole cell lysates from DC treated with 50 ng/ml LPS and 1  $\mu$ g/ml CyaA for 15 min, with or without 2 h pretreatment with the indicated inhibitors (10  $\mu$ M U0126, 2  $\mu$ M Bay 11-7082, 1  $\mu$ M SB203580). Equal protein loading was confirmed by immunoblotting with anti-p38, ERK, and  $\beta$ -Actin.



novo transcription, prevented the increase in IL-12p70 in response to LPS and IFN- $\gamma$ , indicating a direct transcriptional mechanism (Fig. 4B). We then examined the effect of MAPK and NF- $\kappa$ B inhibitors on LPS- and IFN- $\gamma$ -induced IL-12 and the suppression of this by CyaA. We found that pretreatment of

DC with a p38 MAPK-specific inhibitor SB203580 or a MEK-specific inhibitor U0126 did not block the induction of IL-12p70 by LPS and IFN- $\gamma$  and in fact, resulted in an increase, although not statistically significant, in IL-12p70 production (Fig. 4C). Furthermore, pretreatment of DC for 2 h with specific



**Fig. 4.** Inhibition of IL-12p70 by CyaA is independent of IL-10 induction and MAPK activation. (A) DC were incubated with 50 ng/ml LPS, 1  $\mu$ g/ml CyaA, 20 ng/ml IFN- $\gamma$ , and 10  $\mu$ g/ml anti-IL-10 ( $\alpha$ -IL-10). After 24 h, supernatants were removed, and the concentration of IL-12p70 was quantified by ELISA. (B) DC were incubated with 5  $\mu$ g/ml Actinomycin D for 2 h prior to the addition of 50 ng/ml LPS and 20 ng/ml IFN- $\gamma$ . IL-12p70 concentrations were quantified in supernatants removed after 24 h of culture. Results are presented as the mean and SD ( $n=3$ ); \*\*\*,  $P < 0.001$ , compared with sample treated in the absence of Actinomycin D, by an unpaired Student's  $t$ -test. (C) DC were incubated with 1  $\mu$ M SB203580, 10  $\mu$ M U0126, or 2  $\mu$ M Bay 11-7082 for 2 h prior to the addition of 50 ng/ml LPS and 20 ng/ml IFN- $\gamma$ . Supernatants were tested for the presence of IL-12p70 at 24 h. Results are presented as the mean and SD of the mean ( $n=3$ ); \*\*\*,  $P < 0.001$ , compared with sample treated with LPS + IFN- $\gamma$  by an unpaired Student's  $t$ -test. (D) DC were incubated with 1  $\mu$ M SB203580, 10  $\mu$ M U0126, or 2  $\mu$ M Bay 11-7082 for 2 h prior to the addition of 50 ng/ml LPS, 1  $\mu$ g/ml CyaA, and 20 ng/ml IFN- $\gamma$ . IL-12p70 concentrations were quantified in supernatants removed after 24 h of culture. Results are presented as the mean and SD of the mean ( $n=3$ ). (E) Western blot analysis of I $\kappa$ B $\alpha$  expression in whole cell lysates from DC treated with 1  $\mu$ g/ml CyaA, 50 ng/ml LPS, and 20 ng/ml IFN- $\gamma$  for 15 min.

inhibitors of p38 or MEK did not alter the ability of CyaA to suppress LPS- and IFN- $\gamma$ -induced IL-12p70 (Fig. 4D).

We next examined the role of NF- $\kappa$ B by examining I $\kappa$ B $\alpha$  degradation in response to LPS and IFN- $\gamma$  in the presence and absence of CyaA. Neither CyaA nor IFN- $\gamma$  alone had any effect on I $\kappa$ B $\alpha$  expression in DC (Fig. 4E). In contrast, LPS induced rapid degradation of I $\kappa$ B $\alpha$  with expression returning to those seen in untreated cells within 4 h (Fig. 2A). Costimulation of DC with CyaA or IFN- $\gamma$  did not affect LPS-induced I $\kappa$ B $\alpha$  degradation (Fig. 4E). Inhibition of NF- $\kappa$ B in DC by pretreatment with Bay 11-7082 (2  $\mu$ M) significantly inhibited IL-12p70 induced in response to LPS and IFN- $\gamma$  (Fig. 4C), indicating that NF- $\kappa$ B but not p38 or MEK activation is involved in IL-12p70 production. However, pretreatment of DC with the NF- $\kappa$ B inhibitor Bay 11-7082 did not alter the ability of CyaA to suppress LPS- and IFN- $\gamma$ -induced IL-12p70 (Fig. 4D). These findings demonstrate that despite the fact that p38, ERK, and NF- $\kappa$ B are required for induction of IL-10 by CyaA and LPS, modulation of these signaling pathways does not appear to be required for inhibition of IL-12p70 by CyaA and suggest that distinct pathways are involved in these two immunomodulatory activities of the toxin.

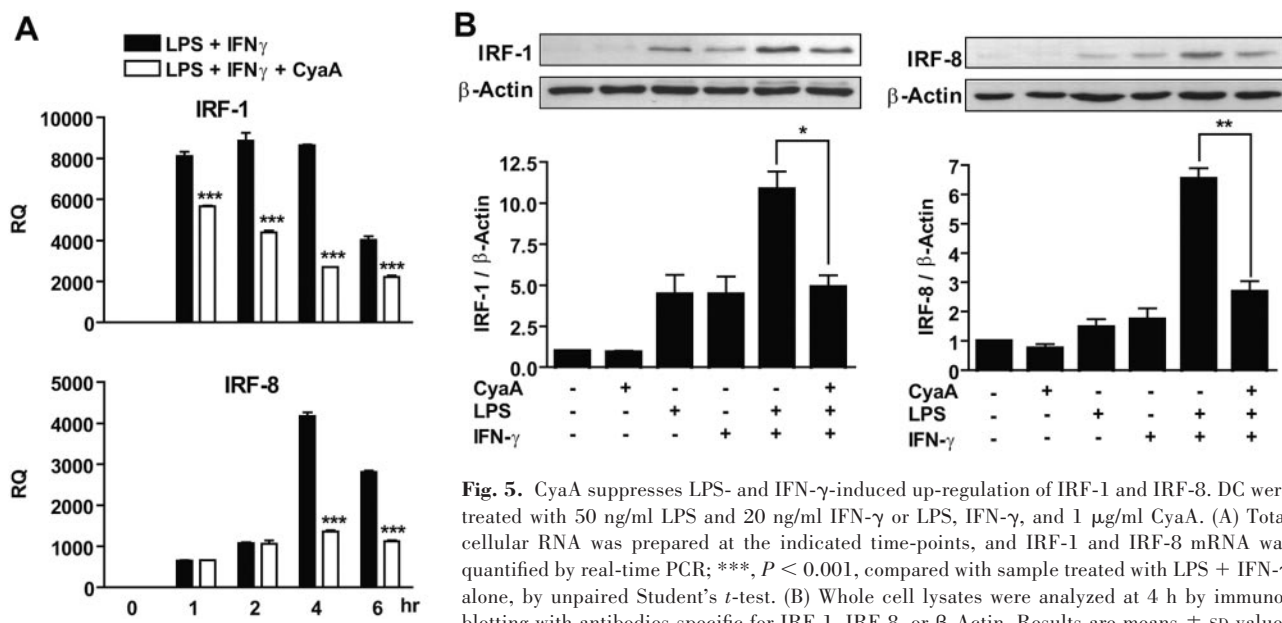
### CyaA blocks LPS- and IFN- $\gamma$ -dependent up-regulation of IRF-1 and IRF-8

This study has demonstrated that NF- $\kappa$ B activation is required for IL-12p70 production by DC in response to treatment with LPS and IFN- $\gamma$  (Fig. 4). In addition, we have shown that although CyaA can suppress IL-12p70 expression, it does not modulate NF- $\kappa$ B activation in response to LPS and IFN- $\gamma$  (Fig. 4E), suggesting that CyaA modulates IL-12p70 production through other signaling pathways. The IRF family, in particular, IRF-1 and IRF-8, has been implicated in IL-12 signaling [25–27]. In addition, it has recently been reported that IRF-1 is essential for IL-12-IFN- $\gamma$  signaling by enhancing IL-12R $\beta$

expression on CD4<sup>+</sup> T cells [28]. Here, we studied the effect of CyaA on expression of IRF-1 and IRF-8. We found that treatment of DC with LPS and IFN- $\gamma$  enhanced expression of IRF-1 and IRF-8 mRNA and that this was significantly reversed by cotreatment with CyaA (Fig. 5A). Furthermore, this pattern of expression was confirmed at the protein level; treatment with LPS or IFN- $\gamma$  alone led to moderate increases in expression of IRF-1 and IRF-8, but the highest expression was seen in response to cotreatment with LPS and IFN- $\gamma$  (Fig. 5B). This is consistent with the observation that costimulation with LPS and IFN- $\gamma$  is required for maximal IL-12p70 expression. Treatment of DC with CyaA significantly reduced expression of IRF-1 and IRF-8 protein induced with LPS and IFN- $\gamma$ . These findings suggest that CyaA may inhibit IL-12 production by suppressing expression of the transcription factors IRF-1 and IRF-8.

### The up-regulation of IL-10 and inhibition of IL-12p70 by CyaA are dependent on its adenylate cyclase activity, and these effects can be mimicked by PGE2

The adenylate cyclase activity of CyaA has previously been shown to be required for certain but not all of its immunomodulatory effects on innate and adaptive immunity [5, 29]. Here, we have examined the role of adenylate cyclase activity in the enhancement of IL-10 and suppression of IL-12p70 by CyaA. We used a mutant form of CyaA (iAC-CyaA) with substitutions in three amino acids (H63A, K65A, S66G), which abrogated adenylate cyclase activity [5]. Treatment of DC with CyaA but not with iAC-CyaA enhanced intracellular cAMP concentrations in DC (Fig. 6A). In addition, unlike CyaA, iAC-CyaA did not synergize with LPS in the induction of IL-10 nor did it inhibit LPS- and IFN- $\gamma$ -induced IL-12p70 production (Fig. 6B). These data suggest a crucial role for the accumulation of cAMP in the induction of IL-10 and suppression of



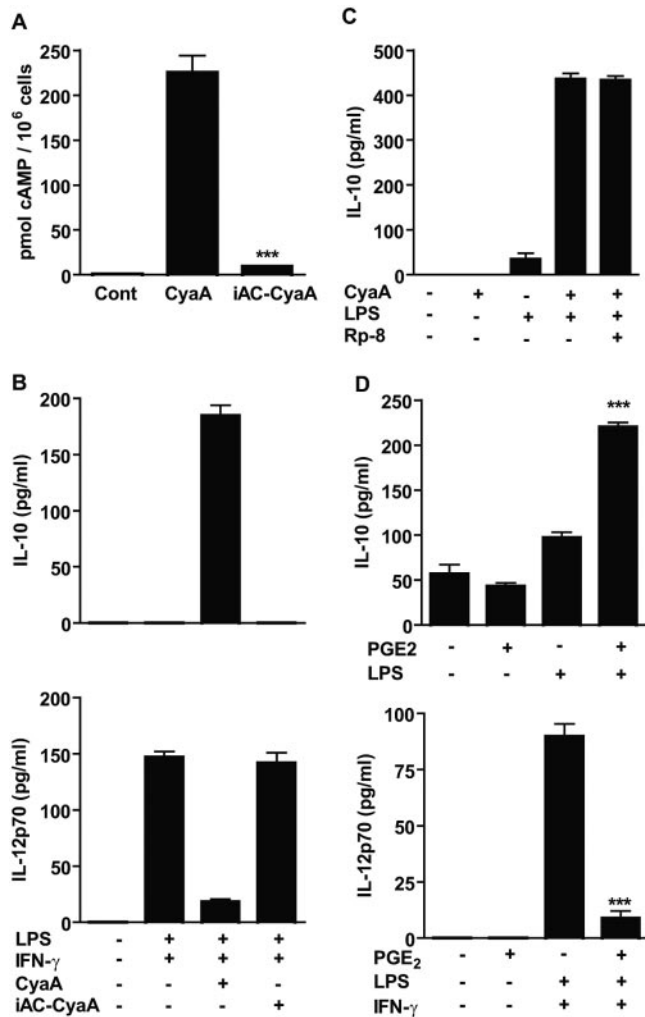
**Fig. 5.** CyaA suppresses LPS- and IFN- $\gamma$ -induced up-regulation of IRF-1 and IRF-8. DC were treated with 50 ng/ml LPS and 20 ng/ml IFN- $\gamma$  or LPS, IFN- $\gamma$ , and 1  $\mu$ g/ml CyaA. (A) Total cellular RNA was prepared at the indicated time-points, and IRF-1 and IRF-8 mRNA was quantified by real-time PCR; \*\*\*,  $P < 0.001$ , compared with sample treated with LPS + IFN- $\gamma$  alone, by unpaired Student's  $t$ -test. (B) Whole cell lysates were analyzed at 4 h by immunoblotting with antibodies specific for IRF-1, IRF-8, or  $\beta$ -Actin. Results are means  $\pm$  SD values

( $n=4$ ) from densitometry quantification of band intensities relative to the  $\beta$ -Actin loading control, and one representative blot was shown above each graph; \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ , by an unpaired Student's  $t$ -test.

IL-12p70 in murine DC. As cAMP activates PKA, we examined the role of PKA in the immunomodulatory effects of CyaA. The PKA inhibitor Rp-8-Br-cAMPS, which antagonizes the binding of cAMP to type I PKA, did not alter the synergistic effect of CyaA on LPS-induced IL-10 production (Fig. 6C) and did not affect suppression of IL-12p70 by CyaA (data not shown). PGE2 has previously been shown to stimulate cAMP production in various cell types [30, 31]. Therefore, we examined the possibility that PGE2 might mimic the effects of CyaA. We found that similar to CyaA, treatment of cells with

PGE2 alone did not induce IL-10 production; however, PGE2 synergized with a low concentration of LPS to induce IL-10 production from DC (Fig. 6D). Furthermore, PGE2 significantly suppressed LPS- and IFN- $\gamma$ -induced IL-12p70 production (Fig. 6D). These results suggest that agents that stimulate intracellular cAMP production can mimic at least some of the immunomodulatory effects of CyaA.

As we found that adenylate cyclase activity is crucial for inhibition of IL-12p70 by CyaA, we also compared the ability of CyaA and the enzymatically inactive mutant (iAC-CyaA) to suppress IRF-1 and IRF-8 expression. Although wild-type CyaA significantly inhibited expression of both transcription factors, iAC-CyaA has no effect (Fig. 7A). This suggests that accumulation of intracellular cAMP is critical for inhibition of IRF expression by CyaA. To confirm the role of cAMP in modulation of IRF expression, DC were costimulated with LPS and IFN- $\gamma$  in the presence and absence of PGE2. Similar to the effect of CyaA, PGE2 also significantly reduced expression of IRF-1 and IRF-8 in response to LPS and IFN- $\gamma$  (Fig. 7B). These findings suggest that CyaA exerts distinct modulatory effects on the TLR signaling pathways, enhancing p38- and ERK-induced IL-10 and inhibiting IRF-1- and IRF-8-induced IL-12, and that these immunomodulatory effects are dependent on cAMP induction.

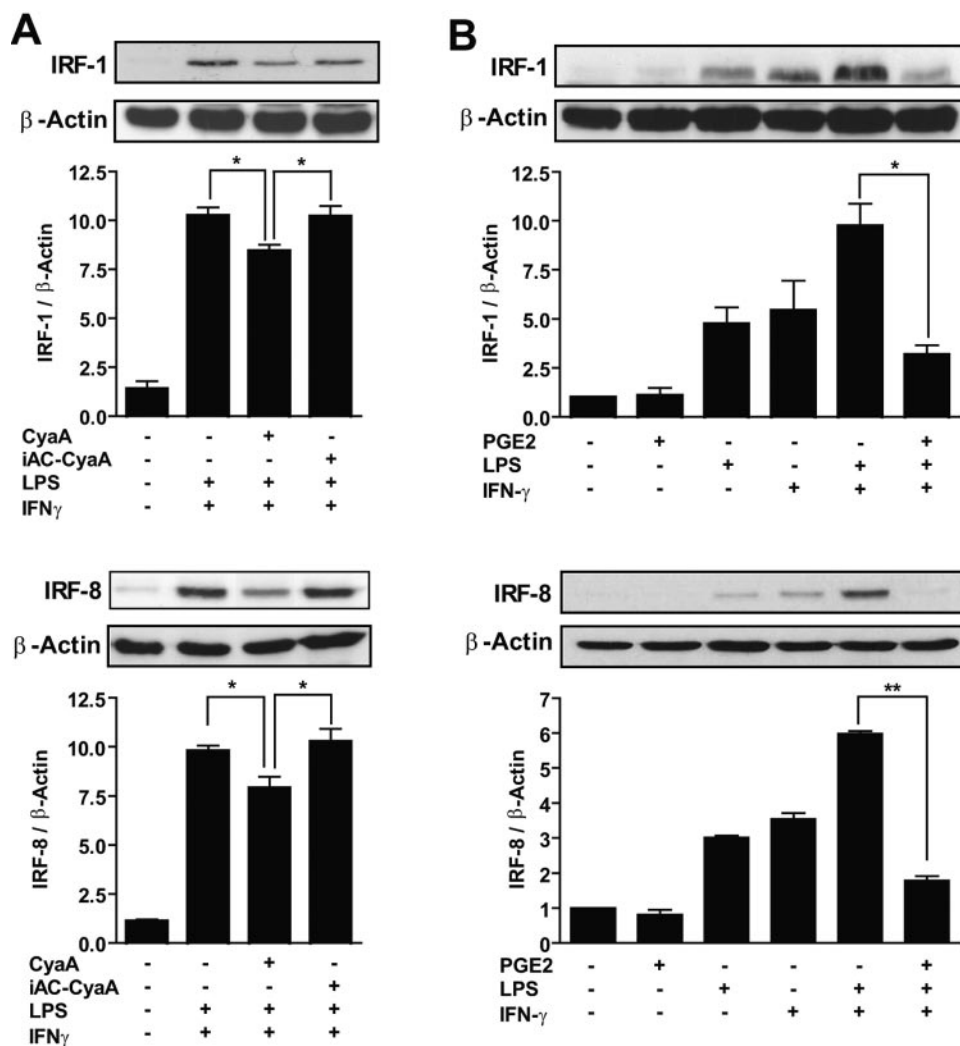


**Fig. 6.** The immunomodulatory effects of CyaA are dependent on adenylate cyclase activity and can be mimicked by PGE2, but are independent of PKA activation. (A) DC were incubated with 1  $\mu$ g/ml CyaA or 1  $\mu$ g/ml iAC-CyaA. Intracellular cAMP was measured after 30 min; \*\*\*,  $P < 0.001$ , compared with sample treated with CyaA. Cont, Control. (B) DC were incubated with 50 ng/ml LPS and 20 ng/ml IFN- $\gamma$  with or without 1  $\mu$ g/ml CyaA or iAC-CyaA. The concentrations of IL-10 and IL-12p70 were quantified by ELISA in supernatants removed after 6 and 24 h, respectively. (C) DC were incubated with medium only, 50 ng/ml LPS, 1  $\mu$ g/ml CyaA, or LPS and CyaA in the presence or absence of the PKA inhibitor Rp-8-Br-cAMPS (Rp-8; 1 mM, 2 h preincubation). IL-10 concentrations were quantified in supernatants removed after 6 h of culture. (D) DC were incubated with 50 ng/ml LPS and 20 ng/ml IFN- $\gamma$ , with or without 1  $\mu$ M PGE2 or with medium only. The concentrations of IL-10 and IL-12p70 were quantified by ELISA in supernatants removed after 6 and 24 h, respectively; \*\*\*,  $P < 0.001$ , compared with sample treated in the absence of PGE2.

## DISCUSSION

This study demonstrates that a bacterial virulence factor can exert immunosuppressive effects on the innate immune system by selectively modulating distinct TLR signaling pathways in DC, leading to enhanced anti-inflammatory and suppressed proinflammatory cytokine production. We found that CyaA from *B. pertussis* promoted phosphorylation of ERK and p38 MAPK but inhibited expression of the transcription factors IRF-1 and IRF-8. The downstream effects included enhancement of IL-10 and inhibition of IL-12 production by TLR-activated DC, a cytokine profile that programs the innate immune system to promote the induction of IL-10-secreting Treg cells at the expense of Th1 cells. These findings provide evidence of a novel approach used by a pathogen to subvert innate immunity and provide a mechanism whereby CyaA promotes bacterial colonization and persistence during infection with *B. pertussis*.

It has previously been reported that signaling through TLR4 is essential for the induction of effective natural and vaccine-induced, protective immunity against *B. pertussis* [32, 33]. TLR4-mediated activation of DC by *B. pertussis* LPS results in the production of inflammatory cytokines, including IL-12, which promotes the activation of IFN- $\gamma$  secretion by NK cells and the subsequent induction of IFN- $\gamma$ -producing Th1 cells [7]. IFN- $\gamma$  plays a critical role in protective immunity; mice defective in IFN- $\gamma$  receptors develop an uncontrolled, systemic infection with *B. pertussis* [34]. However, Th1 responses are suppressed early in infection, and current evidence suggests that this reflects the induction of anti-inflammatory cytokines and Treg cells. IL-10-secreting Treg cells are induced in the respiratory tract during *B. pertussis* infection [8], and this is driven by semi-mature, regulatory DC characterized by high



**Fig. 7.** The inhibition of IRF-1 and IRF-8 by CyaA is dependent on its adenylate cyclase activity and is mimicked by PGE<sub>2</sub>. (A) DC were incubated with 50 ng/ml LPS and 20 ng/ml IFN- $\gamma$  or LPS and IFN- $\gamma$  with 1  $\mu$ g/ml CyaA or iAC-CyaA. After 4 h, whole cell lysates were analyzed by immunoblotting with antibodies specific for IRF-1, IRF-8, or  $\beta$ -Actin. (B) DC were incubated with 50 ng/ml LPS, 20 ng/ml IFN- $\gamma$ , LPS and IFN- $\gamma$ , 1  $\mu$ M PGE<sub>2</sub>, or LPS, IFN- $\gamma$ , and PGE<sub>2</sub>. After 4 h, whole cell lysates were analyzed by immunoblotting with antibodies specific for IRF-1, IRF-8, or  $\beta$ -Actin. Results are means  $\pm$  SD values ( $n=4$ ) from densitometry quantification of band intensities relative to the  $\beta$ -Actin-loading control, and one representative blot was shown above each graph; \*  $P < 0.05$ , \*\*  $P < 0.01$ , by ANOVA (A) or by an unpaired Student's  $t$ -test (B).

IL-10 and low IL-12 production. These DC are induced in response to exposure to *B. pertussis* virulence factors, including CyaA and filamentous hemagglutinin, in combination with LPS [5, 8, 35].

The enhancement of IL-10 and/or suppression of IL-12 production in response to TLR agonist activation in macrophages or DC are common immune subversion strategies used by pathogens to subvert immune responses of the host [1]. Although the precise mechanisms have in most cases not been addressed, it appears to involve direct or indirect modulation of TLR signaling pathways. It has been reported that the induction of IL-10 by LPS or CpG in macrophages or DC is mediated by activation of ERK MAPK [13, 36]. Activation of ERK has also been associated with inhibition of IL-12 production by *Leishmania* lipophosphoglycans, whereas induction of IL-12 was linked with activation of p38 MAPK, and it was suggested that ERK and p38 may differentially mediate IL-10 and IL-12 production [37]. However, we found that ERK and p38 were involved in IL-10 induction in response to CyaA and LPS. Furthermore, inhibition of NF- $\kappa$ B attenuated IL-10 induction with CyaA and LPS. These results emphasize the importance of coactivation of distinct signaling pathways in the synergistic effects of CyaA and LPS on IL-10 production. In contrast, inhibition of IL-12 by CyaA was independent of MAPK acti-

vation and IL-10 and probably mediated by inhibition of IRF-1 and IRF-8.

Our demonstration that CyaA synergy with LPS for IL-10 production involves activation of p38 as well as ERK and NF- $\kappa$ B is consistent with our finding that inhibition of p38 in TLR-activated DC enhances their ability to promote the induction of IL-10-secreting Treg cells in vivo [38]. It is also supported by the demonstration that the expression of the vaccinia virus A52R protein enhances IL-10 promoter expression through activation of p38 [39]. Although it has been reported that p38 is critical for IL-12p70 production by macrophages [40], we found that CyaA enhanced p38 and ERK and that inhibitors of these molecules did not reverse the suppression of IL-12 by CyaA and actually enhanced CpG- or LPS-induced IL-12 production by DC (ref. [38] and present study). It has also been reported that NF- $\kappa$ B [41] and IRF transcription factors are involved in IL-12 production. Mice deficient in IRF-1 [28], IRF-5 [42], and IRF-8 [43] have defective IL-12 production or IL-12 receptor expression and Th1 responses. We found that CyaA inhibited LPS- and IFN- $\gamma$ -induced IRF-1 and IRF-8 expression, suggesting that this may account for the suppression of IL-12 by CyaA. This is consistent with a study that found that IRF-1 and IRF-8 activation and IL-12 produc-



tion were enhanced in human DC infected with a mutant *B. pertussis* lacking CyaA [18].

The toxic and immunomodulatory effects of CyaA have previously been linked with enhancement of intracellular cAMP concentrations [5]. We found that an enzyme-inactive mutant of CyaA, which did not enhance cAMP concentrations, failed to induce immunomodulatory effects in DC. Furthermore, PGE<sub>2</sub>, which also enhances intracellular cAMP, mimicked the immunomodulatory effects of CyaA; PGE<sub>2</sub> enhanced LPS-induced IL-10 and suppressed IL-12 production by DC and significantly suppressed LPS- and IFN- $\gamma$ -induced expression of IRF-1 and IRF-8. It has been reported that CyaA can induce cyclooxygenase 2 expression in murine macrophages, and it was suggested that inhibition of IL-12 and TNF- $\alpha$  by CyaA may reflect an adverse effect of cAMP on NF- $\kappa$ B or MAPK activation [44]. It has also been shown that cAMP can mediate immunomodulatory effects through activation of PKA and exchange protein directly activated by cAMP [45, 46]. PGE<sub>2</sub> and a PKA-specific cAMP analog suppressed TNF- $\alpha$  production by macrophages [45]. However, we found that inhibition of the TLR agonist induced IL-12, and enhancement of IL-10 by CyaA was independent of PKA. In contrast, our findings suggest that enhancement of IL-10 is mediated by activation of MAPK, whereas inhibition of IL-12 production by CyaA in DC is more likely to involve cAMP-mediated inhibition of IRF-1 and IRF-8.

This study demonstrates that a bacterial virulence factor, CyaA from *B. pertussis*, can subvert protective innate immune responses by simultaneously promoting an anti-inflammatory cytokine and inhibiting a proinflammatory cytokine, which have critical influences on the induction of adaptive immunity. The bacterial toxin mediates its immunomodulatory effect by independently enhancing the MAPK arm of the TLR signaling pathway, while inhibiting transcription of immune response genes induced through IRF transcription factors. Our data highlight the potential importance of IRF-1 and IRF-8 in host immunity to *B. pertussis* and other bacterial pathogens.

## ACKNOWLEDGMENTS

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