

# *Bordetella pertussis* Adenylate Cyclase Toxin Modulates Innate and Adaptive Immune Responses: Distinct Roles for Acylation and Enzymatic Activity in Immunomodulation and Cell Death<sup>1</sup>

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Adenylate cyclase toxin (CyaA) of *Bordetella pertussis* belongs to the repeat in toxin family of pore-forming toxins, which require posttranslational acylation to lyse eukaryotic cells. CyaA modulates dendritic cell (DC) and macrophage function upon stimulation with LPS. In this study, we examined the roles of acylation and enzymatic activity in the immunomodulatory and lytic effects of CyaA. The adenylate cyclase activity of CyaA was necessary for its modulatory effects on murine innate immune cells. In contrast, acylation was not essential for the immunomodulatory function of CyaA, but was required for maximal caspase-3 activation and cytotoxic activity. The wild-type acylated toxin (A-CyaA) and nonacylated CyaA (NA-CyaA), but not CyaA with an inactive adenylate cyclase domain (iAC-CyaA), enhanced TLR-ligand-induced IL-10 and inhibited IL-12, TNF- $\alpha$ , and CCL3 production by macrophages and DC. In addition, both A-CyaA and NA-CyaA, but not iAC-CyaA, enhanced surface expression of CD80 and decreased CpG-stimulated CD40 and ICAM-1 expression on immature DC. Furthermore, both A-CyaA and NA-CyaA promoted the induction of murine IgG1 Abs, Th2, and regulatory T cells against coadministered Ags in vivo, whereas iAC-CyaA had more limited adjuvant activity. In contrast, A-CyaA and iAC-CyaA induced caspase-3 activation and cell death in macrophages, but these effects were considerably reduced or absent with NA-CyaA. Our findings demonstrate that the enzymatic activity plays a critical role in the immunomodulatory effects of CyaA, whereas acylation facilitates the induction of apoptosis and cell lysis, and as such, NA-CyaA has considerable potential as a nontoxic therapeutic molecule with potent anti-inflammatory properties. *The Journal of Immunology*, 2005, 175: 730–738.

**B**ordetella pertussis, the causative agent of whooping cough, and *Bordetella bronchiseptica*, which causes chronic respiratory disease in a variety of mammals, both express adenylate cyclase toxin (CyaA)<sup>5</sup> (1). This toxin is encoded by the *cyaA* gene and is an essential virulence factor for both *Bordetella* species. CyaA specifically binds to the  $\alpha_M\beta_2$  integrin (CD11b/CD18) on the surface of macrophages and dendritic cells (DC) (2, 3). Upon entry into target eukaryotic cells, CyaA is activated by binding calmodulin and converts ATP into cAMP, generating supraphysiological concentrations of this powerful signaling molecule. Treatment of neutrophils with CyaA leads to inhibition of chemotaxis, phagocytosis, and superoxide generation

(4). CyaA has also been shown to induce apoptosis in macrophages (5, 6).

We and others have previously shown that CyaA of *B. pertussis* is an effective vaccine adjuvant in vivo, enhancing Ab responses to coadministered Ags (7–10). Our studies also demonstrated that CyaA promotes Th2 and type 1 regulatory T (Tr1) cell responses to coadministered Ags (9). Studies with innate immune cells in vitro indicated that these adaptive immune responses were mediated via the modulation of DC and macrophage activation. Treatment of LPS-stimulated macrophages or murine bone marrow-derived DC with CyaA suppressed production of the proinflammatory cytokines and chemokines IL-12p70, TNF- $\alpha$ , and CCL3 (MIP-1 $\alpha$ ) and enhanced IL-10 production (9). Furthermore, CyaA enhanced surface expression of the costimulatory molecule CD80 on immature DC, but decreased expression of LPS-induced ICAM-1 (CD54) and CD40, indicating that CyaA selectively modulates DC activation and maturation.

The enzymatic domain of CyaA is located in the 400 NH<sub>2</sub>-terminal amino acids, while interaction of CyaA with CD11b/CD18 and with eukaryotic membranes is mediated through the 1306 residue COOH-terminal region of the protein, that has similarity with the repeat in toxin (RTX) family of bacterial pore-forming toxins (3). This family of toxins requires amide-linked fatty acylation by a toxin-specific accessory protein for function, as originally demonstrated for the *Escherichia coli* hemolysin, HlyA (11). Likewise, acylation of CyaA by its specific accessory protein, CyaC, is necessary for the toxin to lyse RBC, J774 macrophages, and Jurkat T cells (6, 12). Differences in the acylation pattern have been demonstrated to alter the hemolytic and pore-forming activity of CyaA, but not its capacity to deliver the enzymatic domain into the cell cytosol (13, 14). The channel-forming activity of CyaA

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<sup>5</sup> Abbreviations used in this paper: CyaA, adenylate cyclase toxin; RTX, repeat in toxin (protein family); DC, dendritic cell; A-CyaA, acylated CyaA; NA-CyaA, nonacylated CyaA; iAC-CyaA, CyaA with an inactive adenylate cyclase domain; Tr1, type 1 regulatory T; LDH, lactate dehydrogenase; KLH, keyhole limpet hemocyanin; CpG-ODN, oligodeoxynucleotide-containing CpG motif

exhibits a cooperative concentration-dependent curve, indicative of protein oligomerization, while intracellular cAMP accumulation can be triggered by protein monomers (3, 15). These differences have led researchers to suggest that cell lysis and intracellular cAMP accumulation occur through separate mechanisms.

The aim of this study was to examine the role of acylation and of the enzymatic activity of CyaA in its lytic, adjuvant, and immunomodulatory effects. Our findings demonstrated that an enzymatically inactive mutant of CyaA was devoid of immunomodulatory activity, but retained the ability to induce cell lysis and caspase-3 activation. In contrast, a nonacylated toxin, like the wild-type toxin, modulated DC maturation and cytokine production and acted as an adjuvant for promoting Th2 and Tr cell responses to coinjected Ags *in vivo*, but had limited ability to induce cell death and caspase-3 activation in macrophages.

## Materials and Methods

### Expression and purification of CyaA

The CyaA proteins were expressed and purified from *E. coli* XL-1 Blue carrying either plasmid pJR1 (expressing His-CyaA alone), pJR2 (expressing His-CyaA and CyaC), or pNM2 (expressing enzymatically inactive His-CyaA and CyaC). Plasmid pJR1 was constructed in the following manner. *cyaA* was subcloned as a *KpnI/BamHI* fragment from pAPB6 (pBluescript SK- containing *cyaA*) (9) into pASK-IBA6 (IBA) to generate plasmid pAPB7. A *KpnI/HinDIII* fragment comprising *cyaA* from pAPB7 was then cloned into the corresponding sites of pQE-80 (Qiagen) to generate pJR1. Plasmid pAPB9 was constructed by inserting a 3.5-kb *SstI* fragment encoding the 5' end of *cyaA* into the corresponding site in pBluescript SK-. Using pAPB9 as a template, a 0.4-kb PCR product was generated with oligonucleotides pUC Forward (CCAGTCCAGCAGCTTGTAAACG) and PAB27 (CAACCC CAATCGGATCCCGCGGGCCACGCCAATCCTTTG; introduced *BamHI* site underlined, mutated bases in italics) and a 1.7-kb product with oligonucleotides PAB17 (CAAAGGATTGGGCGTGGCCGCCGGGA TCCGATTGGGGGTTG; introduced *BamHI* site underlined, mutated bases in italics) and PAB29 (CGTAGATCTCCATGGGACTGAGC; *NcoI* site underlined). The former PCR product was digested with *XbaI/BamHI* and the latter was digested with *BamHI/NcoI* and ligated with *XbaI/NcoI*-digested pAPB9 to create pNM1. The 2.5-kb *Clal/KpnI* fragment of pNM1 was inserted into the corresponding sites of pJR2 to create pNM2. This plasmid encodes a H63A/K65A/S66G mutant of His-CyaA together with CyaC. The sequence and orientation of the cloned genes were confirmed by restriction digestion and sequencing (MWG Biotech).

The N-terminal His-tagged proteins were purified from inclusion bodies by DEAE-Sepharose and Ni<sup>2+</sup>-agarose chromatography (9). LPS was removed from CyaA by dialysis first against Dulbecco's PBS (Sigma-Aldrich), 1 mM EDTA, 1 M urea, pH 4.6, and then against Dulbecco's PBS, 0.1 mM CaCl<sub>2</sub>, 2 M urea, pH 8.0. All chemicals were from Sigma-Aldrich, unless indicated otherwise. LPS was measured by a highly sensitive colorimetric *Limulus* amoebocyte lysate assay (Cape Cod Associates). The proteins were judged to be >95% pure by Coomassie-stained SDS-PAGE gels. Proteins were transferred to nitrocellulose membrane following SDS-PAGE, probed with anti-His Abs (Santa Cruz Biotechnology) and anti-CyaA Abs (gift from E. Hewlett, University of Virginia School of Medicine, Charlottesville, VA) and visualized by incubation with secondary anti-rabbit IgG HRP conjugated Abs and chemiluminescence detection. Each protein was recognized by both Abs.

### Mice

Female specific-pathogen-free BALB/c, C3H/HeN, and C3H/HeJ mice were purchased from Harlan U.K. and were used at 8–10 wk old. Mice were housed in individually ventilated cages, and all experiments were performed according to regulations of the Irish Department of Health and the European Union and with approval from the Bioresources Ethics Committee of Trinity College Dublin.

### cAMP quantification

The cell-free enzymatic activity of CyaA was measured as previously described (16). To assay intracellular cAMP accumulation, J774 macrophages were stimulated for 60 min with CyaA or CyaA-derivatives (1 μg/ml). cAMP was measured by competitive ELISA using the Amersham Biosciences Biotrak Enzymeimmunoassay kit. Samples were serially diluted to obtain values within the linear range of the concentration curve.

### Cell lysis assays

Toxin was added to J774 macrophages at the indicated concentrations with or without 10 μg/ml anti-mouse CD11b Ab (M1/70) or purified rat IgG2b, κ isotype control (both from BD Pharmingen); Abs were added 30 min before the addition of CyaA and plates incubated at 37°C for 6 h. Cell lysis was measured by the release of lactate dehydrogenase (LDH) into the culture supernatants. LDH activity in the supernatants was quantified by the CytoTox 96 assay (Promega). Percent lysis = [(OD of sample – OD of untreated cells)/(OD of 100% cell lysis – OD of untreated cells)] × 100. Alternatively, cell death was quantified using the CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega) according to the manufacturer's instructions. Briefly, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2-*H*-tetrazolium inner salt, was added to the cells after incubation for 6 h with toxin. Release of a soluble formazan product, which requires dehydrogenase enzymes found only in living cells, was measured by the absorbance at 490 nm, and this is directly proportional to the numbers of viable cells. Cell death shown represents decrease in viability compared with control (medium only), which is set at 0%. Percent cell death = 100 – (OD of sample × 100/OD of untreated cells).

### Morphological assessment of CyaA treated J774 cells

J774 cells were plated on glass coverslips one day before treatment. Cells were treated with 1–10 μg/ml CyaA or nonacylated CyaA (NA-CyaA), 1 μM cycloheximide, or medium only for 6 h. After treatment, coverslips were removed and cells were fixed with 3% paraformaldehyde in PBS, washed, and mounted on glass slides for analysis. Cells were visualized under white light using a BX51 microscope (Olympus) with attached camera, and images were acquired using AnalySIS software (Soft Imaging System).

### Caspase-3 activation assay

J774 cells were treated for 6 h with CyaA or CyaA derivatives (0.1–10 μg/ml) in medium lacking FCS. Cells were washed and lysed in 300 μl of lysis buffer (150 mM NaCl, 20 mM Tris (pH 7.5), 1% Triton X-100, 100 μM PMSF, 10 μg/ml leupeptin, 5 μg/ml aprotinin) and analyzed for protein concentration. Fifty-microliter samples were added to 50 μl 2× reaction buffer (100 mM HEPES (pH 7.4), 150 mM NaCl, 10.2% CHAPS, 4 mM DTT) and 50 μM caspase-3 substrate (Ac-DEVD-aminofluorocoumarin; Alexis) and incubated at 37°C. Fluorescence caused by liberation of free aminofluorocoumarin was monitored over 60 min (excitation, 390 nm; emission, 510 nm).

### Effect of CyaA on cytokine release by macrophages and DC

Bone marrow-derived immature DC were prepared by culturing bone marrow cells obtained from the femur and tibia of mice in RPMI 1640 medium with 8% FCS supplemented with supernatant (10%) from a GM-CSF-expressing cell line (J558-GM-CSF). On day 3, fresh medium with 10% GM-CSF cell supernatant was added to adherent cells. On day 7, cells were collected, washed, and used for assays. DC and J774 macrophages were cultured at 1 × 10<sup>6</sup> cells/ml, and CyaA was added at the indicated concentrations. Where indicated, purified anti-mouse CD11b Ab (M1/70) or purified rat IgG2b, κ isotype control were added at a concentration of 10 μg/ml, 30 min before the addition of CyaA. Polymyxin B was added to the appropriate wells at a concentration of 10 μg/ml, 2 h after stimulation with CyaA, LPS, or phosphorothioate-stabilized oligodeoxynucleotide-containing CpG motifs (CpG-ODN; 5'-TCCATGACGTTCTCTGATGCT-3'; synthesized by Sigma-Genosys) was added at the indicated concentrations. Supernatants were collected after 4 and 24 h for analysis of IL-10, IL-12 p70, TNF-α, and CCL3 concentrations by ELISA (R&D Systems DuoSet ELISA kits).

### Effects of CyaA on DC maturation

Immature DC were cultured with CyaA or derivatives (1 μg/ml) and polymyxin B (10 μg/ml), and 2 h later, CpG-ODN (10 μg/ml) was added. After 24 h, incubation cells were collected and washed in PBS with 0.05% BSA and 0.02% NaN<sub>3</sub>. Expression of surface markers was assessed using biotinylated anti-CD11c with Streptavidin-PerCP, FITC-labeled anti-CD40, PE-labeled anti-CD80, FITC-labeled anti-ICAM-1, or the appropriate isotype control. All Abs were purchased from BD Pharmingen. After incubation for 30 min at 4°C, cells were washed, immunofluorescence analysis was performed on a FACSCalibur (BD Immunocytometry Systems), and gated CD11c-positive cells were analyzed using CellQuest version 3.1 software. A total of 30,000 cells were analyzed per sample.

### Adjuvanticity of NA-CyaA and acylated CyaA (A-CyaA)

Groups of 6- to 8-wk-old BALB/c mice ( $n = 5$ ) were immunized once or twice (days 0 and 21) in the hind footpads with PBS, keyhole limpet hemocyanin (KLH; 5  $\mu\text{g}$ ) alone or with A-CyaA, NA-CyaA, or CyaA protein with an inactive adenylate cyclase domain (iAC-CyaA; 0.2, 1, or 5  $\mu\text{g}$ ). Seven days after the last immunization, mice were sacrificed and serum and popliteal lymph node cell suspensions were prepared. Lymph node cells ( $1 \times 10^6/\text{ml}$ ) were cultured in cRPMI with KLH (2–50  $\mu\text{g}/\text{ml}$ ), PMA (25  $\text{ng}/\text{ml}$ ), and anti-CD3 (0.5  $\mu\text{g}/\text{ml}$ ) or cRPMI alone. Supernatants were collected 72 h later and assayed by ELISA for IL-10, -4, -5, and IFN- $\gamma$ . The medium was replaced, and 24 h later, [ $^3\text{H}$ ]thymidine (0.5  $\mu\text{Ci}/\text{well}$ ) was added. After 6 h, cells were harvested with an automated cell harvester, and incorporated thymidine was detected by scintillation counting as a measure of proliferation. Results are expressed in cpm. KLH-specific IgG, IgG1, and IgG2a serum titers were determined by ELISA as previously described (9).

### Statistical analysis

One-way ANOVA was used to test for statistical significance. Statistical significance was recorded at  $p < 0.05$ . Multiple comparisons between groups were assessed using the Tukey-Kramer post hoc test.

## Results

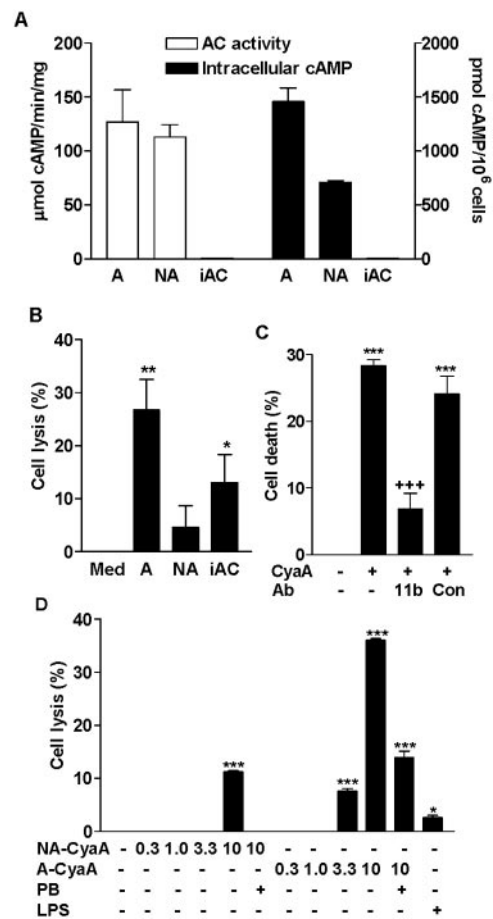
### Purification and biochemical characterization of acylated, nonacylated, and enzymatically inactive CyaA

To examine the role of acylation and adenylate cyclase activity in the adjuvant and immunomodulatory effects of CyaA, we generated and purified CyaA and CyaA derivatives lacking acylation or deficient in adenylate cyclase activity. A-CyaA was purified from *E. coli* XL-1 Blue (pJR2) expressing His-CyaA and CyaC together under the control of the IPTG-inducible promoter  $p_{tac}$ . NA-CyaA was purified from *E. coli* carrying pJR1, a similar plasmid that lacks *cyaC*. iAC-CyaA was generated by site-directed mutagenesis of the *cyaA* gene so that it would produce a mutated protein with H63A, K65A, and S66G substitutions; Lys<sup>65</sup> and His<sup>63</sup> have previously been shown to be essential for the catalytic activity of CyaA (17, 18). LPS copurifies with CyaA, as they appear to form a complex, as is the case for other RTX proteins (9, 19), and the LPS content of the two A-CyaA, NA-CyaA and iAC-CyaA preparations used was 191 and 217, 184 and 124, and 191 and 200  $\mu\text{g}$  of LPS per microgram of protein, respectively. Although the LPS content has not been addressed in many studies, these concentrations are considerably lower than those reported by others (10, 20). The presence and absence of palmitic fatty acid acylation on A-CyaA and NA-CyaA, respectively, was confirmed by inducing protein expression in *E. coli* in the presence of [ $^{14}\text{C}$ (U)]palmitic acid, and demonstrating that the A-CyaA preparation was modified by posttranslational palmitoylation by the coexpressed accessory protein CyaC, whereas CyaA expressed in the absence of CyaC (NA-CyaA) does not become palmitoylated (data not shown).

Both A-CyaA and NA-CyaA were enzymatically active, exhibiting specific activities within the spectrum of those reported from other laboratories (10, 15, 21, 22), while the iAC-CyaA was devoid of enzyme activity (Fig. 1A).

### Acylation of CyaA is required for lysis and caspase-3 activation in macrophages

We tested the role of acylation and enzymatic activity in CyaA-induced cAMP accumulation in J774 macrophages and DC, both of which express CD11b/CD18. The enzymatically active A-CyaA and NA-CyaA increased the intracellular cAMP concentration in J774 cells 1000- to 3000-fold, but iAC-CyaA did not alter cAMP concentration (Fig. 1A). A similar pattern of intracellular cAMP induction by the different toxins was observed in DC;  $2593 \pm 165$ ,  $972 \pm 350$ , and 0  $\text{pmol}$  cAMP per  $10^6$  cells for A-CyaA, NA-CyaA, and iAC-CyaA, respectively.

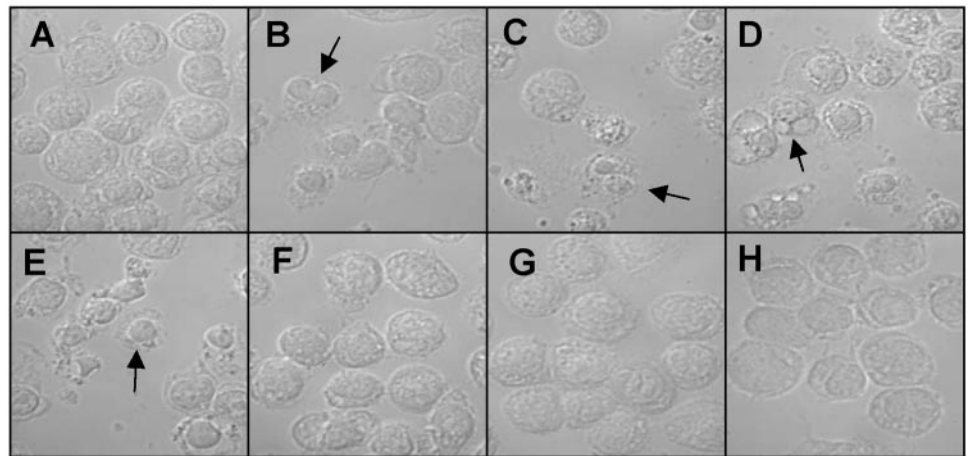


**FIGURE 1.** Induction of cell lysis by CyaA is associated with adenylate cyclase activity and intracellular cAMP elevation, but is not dependent on acylation. **A**, Cell-free adenylate cyclase activity and intracellular cAMP accumulation in J774 macrophages over 60 min in response to 1  $\mu\text{g}/\text{ml}$  A-CyaA (A), NA-CyaA (NA), or iAC-CyaA (iAC). **B**, Lysis of J774 cells, determined by LDH release assay, in response to A-CyaA, NA-CyaA, or iAC-CyaA (10  $\mu\text{g}/\text{ml}$ ) or medium only (Med). **C**, Cell death of J774 cells, determined by CellTiter 96 AQueous One Solution Cell Proliferation Assay in response to A-CyaA (10  $\mu\text{g}/\text{ml}$ ), in the absence or presence of anti-CD11b (11b) Ab or a control Ab (Con). **D**, Cell lysis of J774 cells, determined by LDH release assay, in response to increasing doses of A-CyaA and NA-CyaA (0.3–10  $\mu\text{g}/\text{ml}$ ) in the absence or presence of polymyxin B (PB) or to 200  $\mu\text{g}/\text{ml}$  LPS. Results are representative of experiments repeated at least twice. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ , vs medium control. + + +,  $p < 0.001$ , A-CyaA vs A-CyaA and anti-CD11b.

We assessed the ability of these proteins to cause lysis of macrophages using two different assays. A-CyaA and, to a lesser extent, iAC-CyaA, induced cell death in J774 macrophages; however, only minimal lysis was detected with NA-CyaA (Fig. 1B). Addition of a blocking anti-CD11b Ab, but not a control Ab to the culture significantly reduced the cell death induced by CyaA (Fig. 1C). Following incubation with polymyxin B, the low level of lysis detected with 10  $\mu\text{g}/\text{ml}$  NA-CyaA was abrogated and the lysis induced with A-CyaA was reduced (Fig. 1D). LPS alone at 200  $\mu\text{g}/\text{ml}$ , which is the maximum present in the toxin preparations, caused low but significant cell death. Thus, acylation and binding to CD11b, but not enzyme activity, is required for CyaA to lyse cells; moreover, LPS can augment this lytic effect.

The difference in cytotoxicity between A-CyaA and NA-CyaA was confirmed by examination of the morphology of CyaA-treated macrophages. The morphology of NA-CyaA treated cells was similar to that of untreated cells (Fig. 2). In contrast, J774 cells treated

**FIGURE 2.** Acylation is necessary for CyaA-induced morphological changes in macrophages. J774 macrophages were untreated (A), or treated with 3 (B), 5 (C), or 10 (D)  $\mu\text{g/ml}$  A-CyaA, 1  $\mu\text{M}$  cycloheximide (E) or 3 (F), 5 (G), or 10 (H)  $\mu\text{g/ml}$  NA-CyaA for 6 h. Altered morphology after treatment with A-CyaA and cycloheximide compared with normal morphology in untreated and NA-CyaA treated cells. Arrows indicate examples of apoptotic/necrotic cells.



with A-CyaA at concentrations of 3 or 5  $\mu\text{g/ml}$  (Fig. 2, B and C) take on apoptotic morphology, with cytoplasmic and nuclear shrinkage as well as cellular fragmentation (nuclear staining of cells with Hoechst stain showed clear chromatin condensation; data not shown). This compares with the obvious apoptotic morphology induced by cycloheximide (Fig. 2E). At higher concentrations of CyaA (Fig. 2D), a phenotype more characteristic of necrosis is apparent, with cytoplasmic and nuclear swelling visible and obvious vacuolization of the cytoplasm.

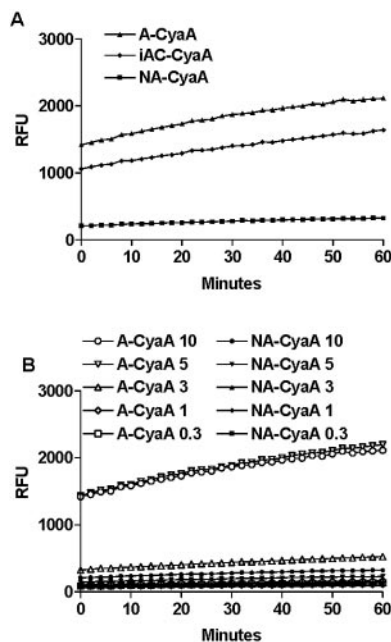
Induction of apoptosis in macrophages by CyaA has previously been reported (5, 6). A key step in apoptosis is the activation of the protease, caspase-3. A-CyaA and iAC-CyaA, but not NA-CyaA, induced caspase 3 activation (Fig. 3). A-CyaA at concentrations of 5 and 10  $\mu\text{g/ml}$  induced high levels of caspase-3 activity and 3  $\mu\text{g/ml}$  induced lower levels of activation (Fig. 3). In contrast, the caspase 3 activation curves for NA-CyaA are almost indistinguishable over all concentrations tested. These results indicate that ac-

ylation, but not enzymatic activity is required for CyaA-induced apoptosis.

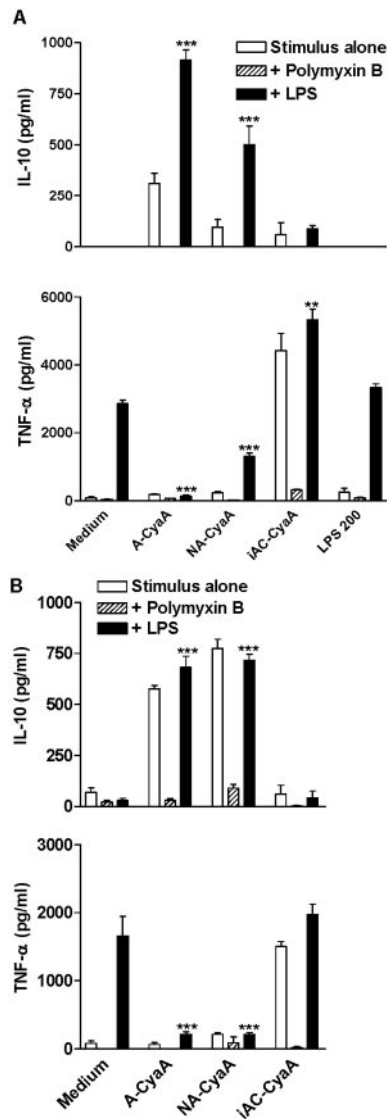
*Acylation is not required for the cAMP driven modulation of cytokine release by CyaA-treated macrophages*

We examined the influence of acylation and enzymatic activity on the ability of CyaA to modulate macrophage and DC activation. J774 macrophages or immature bone marrow-derived DC were treated with either CyaA alone (1  $\mu\text{g/ml}$ ), CyaA together with LPS (10 ng/ml) or CyaA and polymyxin B (10  $\mu\text{g/ml}$ ) to negate any effects of the low concentrations of remaining LPS in the protein preparations. The concentrations of secreted IL-10 and TNF- $\alpha$  were quantified in supernatants 4 h later. A-CyaA and NA-CyaA proteins induced IL-10 production by DC (Fig. 4A) and macrophages (Fig. 4B). Addition of polymyxin B, which chelates LPS, abrogated IL-10 production by DC and significantly reduced IL-10 production by macrophages. A concentration of 200 pg/ml LPS alone, which approximates to the concentrations present in the CyaA protein preparations used for this experiment (191 and 184 pg/ml for A-CyaA and NA-CyaA, respectively), failed to induce IL-10 production, indicating that this cytokine was secreted as a result of the synergy between LPS and CyaA in each of the protein preparations. This synergy was evident with LPS at the concentrations present in the protein preparation and was augmented in DC by addition of exogenous LPS (10 ng/ml), but was not significantly altered in macrophages. NA-CyaA and A-CyaA inhibited LPS-induced TNF- $\alpha$  secretion by macrophages and DC. In contrast, iAC-CyaA did not significantly enhance IL-10 production or inhibit TNF- $\alpha$  production (Fig. 4), indicating that adenylate cyclase activity is essential for CyaA to modulate cytokine production by macrophages. As iAC-CyaA does not inhibit TNF- $\alpha$  production, the LPS copurified with the toxins induces production of this cytokine when macrophages are incubated with the toxin alone and polymyxin B reduces this TNF- $\alpha$  to baseline concentrations. This effect is not seen with the enzymatically active toxins, because of the inhibitory activity of cAMP on TNF- $\alpha$  production.

To further investigate the role of acylation in the immunomodulatory activity of CyaA, we examined the effect of the proteins over a range of concentrations (0.1–3  $\mu\text{g/ml}$ ) on LPS-induced cytokine production (Fig. 5). At concentrations of 1  $\mu\text{g/ml}$  and above, NA-CyaA and A-CyaA exert similar effects, enhancing IL-10 and inhibiting TNF- $\alpha$  production by macrophages, while at lower concentrations, NA-CyaA had significantly less modulatory activity than A-CyaA. These data indicate that acylation is not an absolute requirement for CyaA to modulate macrophage cytokine release, but it does increase its efficiency.



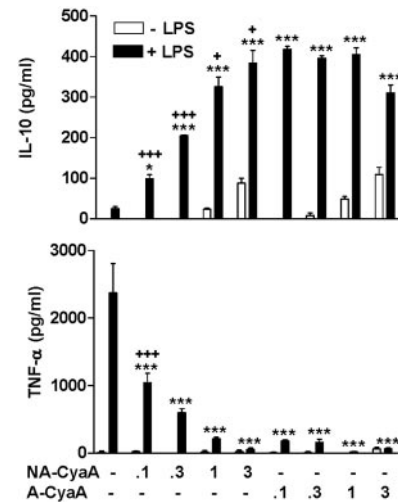
**FIGURE 3.** CyaA induce caspase-3 activation, which is dependent on acylation. A, Caspase-3 activation induced by A-CyaA, NA-CyaA, or iAC-CyaA (10  $\mu\text{g/ml}$ ) in J774 macrophages, expressed as relative fluorescence units (RFU) over time. B, Caspase-3 activation induced by A-CyaA or NA-CyaA (0.3–10  $\mu\text{g/ml}$ ) in J774 macrophages. Results are representative of three experiments.



**FIGURE 4.** The enzymatic activity, but not acylation, of CyaA is required for modulation of macrophage and DC cytokine production. Bone marrow-derived immature DC from BALB/c mice (A) or J774 macrophages (B) were incubated with medium only, A-CyaA, NA-CyaA, or iAC-CyaA (1  $\mu$ g/ml) in the presence or absence of 10  $\mu$ g/ml polymyxin B. Two hours later, 10 ng/ml LPS was added as indicated on the figure. DC were also incubated with 200 pg/ml LPS. After 4 h of incubation, supernatants were collected and IL-10 and TNF- $\alpha$  concentrations were determined by ELISA. \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ , CyaA and LPS vs LPS. Results are means  $\pm$  SD of triplicate assays and are representative of three experiments.

#### NA-CyaA modulates the effects of CpG-ODN signaling through TLR-9

To determine whether the synergistic and inhibitory effects of CyaA on IL-10 and proinflammatory cytokine production are limited to LPS, which is closely associated with the protein, or extend to other TLR ligands, we examined the effect of CyaA on cytokine production induced by a TLR-9 ligand, CpG-ODN. To eliminate any possible influence of LPS, these studies were performed with DC generated from TLR-4-defective C3H/HeJ mice. A-CyaA, NA-CyaA, and iAC-CyaA did not stimulate production of IL-10, TNF- $\alpha$ , IL-12p70, or CCL3 by C3H/HeJ DC. CpG-ODN-induced TNF- $\alpha$ , IL-12p70, and CCL3 production by C3H/HeJ DC was suppressed by A-CyaA and NA-CyaA, but not by iAC-CyaA (Fig.



**FIGURE 5.** Effect of A-CyaA and NA-CyaA toxin concentration on the modulation of macrophage cytokine production. J774 macrophages were treated with A-CyaA and NA-CyaA at the indicated concentrations (micrograms per milliliter), with and without the addition of 10 ng/ml LPS 2 h later. After a further 4-h incubation, supernatants were collected and IL-10 and TNF- $\alpha$  concentrations were determined by ELISA. \*,  $p < 0.05$ ; \*\*\*,  $p < 0.001$ , CyaA and LPS vs LPS. +,  $p < 0.05$ ; +++,  $p < 0.001$ , A-CyaA vs NA-CyaA at the same concentration. Results are means  $\pm$  SD of a representative experiment performed twice in triplicate.

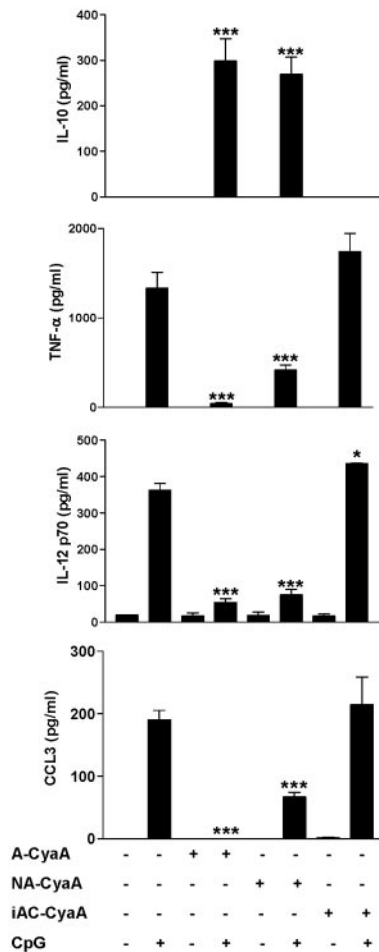
6). IL-10 production could not be detected 4 h after stimulation of DC with CpG-ODN alone; however, addition of A-CyaA or NA-CyaA, but not iAC-CyaA, stimulated significant IL-10 production. These findings demonstrate that the immunomodulatory effects of CyaA are not confined to, or dependant on, signaling of LPS through TLR-4, but can be mediated by the binding of other TLR ligands, such as CpG-ODN, to their receptors. Furthermore, these effects are dependant on enzymatic activity, but are not dependant on acylation of CyaA.

#### The immunomodulatory effects are mediated through CD11b

To determine whether the cytokine modulatory effects of A-CyaA and NA-CyaA are mediated by their binding to cells via CD11b/CD18, J774 macrophages were treated with the CyaA proteins and CpG-ODN in the presence of the anti-CD11b Ab M1/70, which has previously been shown to block the binding of A-CyaA to host cells (3). Addition of the anti-CD11b Ab suppressed the ability of A-CyaA and NA-CyaA to inhibit CpG-ODN-induced production of TNF- $\alpha$  and also their ability to synergize with CpG-ODN to enhance IL-10 secretion (Fig. 7). Similar results were obtained when CpG-ODN was replaced with LPS (data not shown). Thus, modulation of macrophage cytokine release by A-CyaA and NA-CyaA involves interaction with the CD11b/CD18, suggesting specific uptake of both these proteins into innate immune cells through this cell surface receptor.

#### Influence of acylation and enzymatic activity on the modulation of DC maturation by CyaA

Maturation of DC in response to pathogen-derived molecules, such as LPS and CpG-ODN, can be detected by changes in cell surface expression of costimulatory molecules. In this study, we examined the effect of acylation and enzymatic activity of CyaA on its ability to induce maturation of immature bone marrow-derived DC or to modulate CpG-ODN-induced maturation of DC. The effects of A-CyaA and its derivatives were tested in the presence of polymyxin B to examine their effects in the absence of TLR-4 signaling. Both

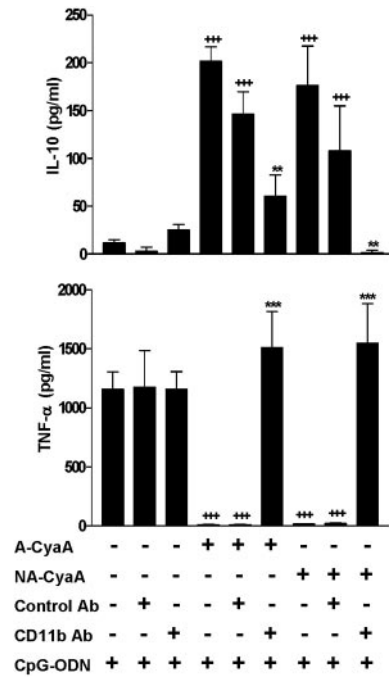


**FIGURE 6.** NA-CyaA modulates CpG-ODN-stimulated cytokine and chemokine release from DC. DC from C3H/HeJ mice were incubated with 1 μg/ml A-CyaA, NA-CyaA, or iAC-CyaA for 2 h, before the addition of 10 μg/ml CpG-ODN. Supernatants were tested for the presence of IL-10, TNF-α, and CCL3 4 h later, and for IL-12 p70 24 h later. \*, *p* < 0.05; \*\*\*, *p* < 0.001, CpG-ODN vs CyaA and CpG-ODN. Results are means ± SD of a representative experiment performed twice in triplicate.

NA-CyaA and A-CyaA enhanced DC surface expression of CD80 and suppressed endogenous ICAM-1 and CD40 expression. Furthermore, preincubation of DC with NA-CyaA and A-CyaA suppressed CpG-ODN-induced expression of CD40 and ICAM-1 (Fig. 8). In contrast, iAC-CyaA had little effect on cell surface marker expression on DC. A-CyaA and NA-CyaA also enhanced surface expression of CD80 and inhibited CpG-ODN-induced CD40 on DC from C3H/HeJ mice and modulated LPS-induced expression of CD40 and ICAM-1 on DC from BALB/c mice (data not shown). These data demonstrate that CyaA modulates DC maturation in response to TLR-4 and TLR-9 ligands and that enzymatic activity, but not acylation, is essential for these effects.

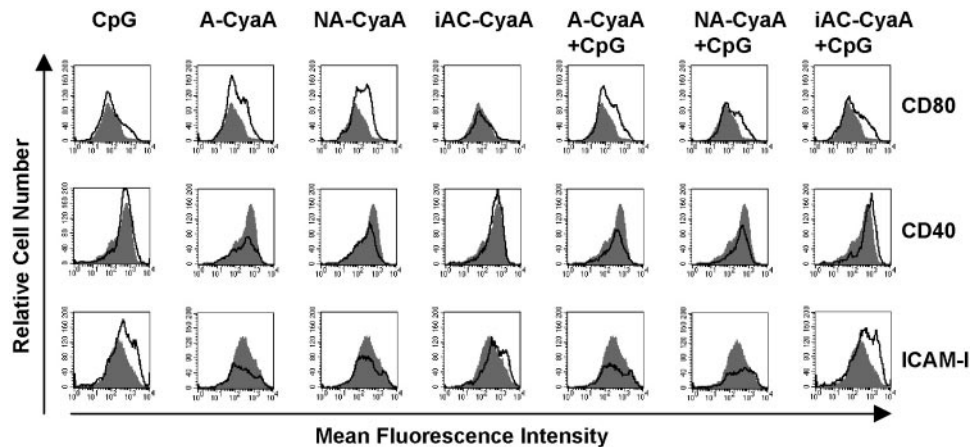
*Acylation is not required for the adjuvant activity of CyaA*

Having demonstrated that NA-CyaA and A-CyaA exert similar effects on innate cells and that iAC-CyaA lacks immunomodulatory activity in vitro, we examined the influence of acylation and enzymatic activity on the adjuvant effect of CyaA in vivo. BALB/c mice were immunized once or twice with KLH alone or with A-CyaA, NA-CyaA, or iAC-CyaA. Analysis of Ab titers revealed that NA-CyaA and A-CyaA enhance KLH-specific IgG responses to the same extent, whereas iAC-CyaA had significantly reduced



**FIGURE 7.** CyaA-induced modulation of macrophage activation is dependent on interaction with CD11b. J774 macrophages were incubated with 1 μg/ml A-CyaA or NA-CyaA for 2 h before the addition of 10 μg/ml CpG-ODN. Cells were incubated with 10 μg/ml anti-CD11b or isotype control Ab before the addition of CyaA. \*\*, *p* < 0.01; \*\*\*, *p* < 0.001, anti-CD11b vs control Ab. +++, *p* < 0.001, CpG-ODN plus toxin vs CpG alone. Results are means ± SD of a representative experiment performed twice in triplicate.

adjuvant activity (Fig. 9). A single dose of 1 or 5 μg/ml of A-CyaA or NA-CyaA augmented KLH-specific IgG, whereas 0.2 μg of these preparations or 0.2 or 1 μg of iAC-CyaA had no adjuvant activity (Fig. 9A). The adjuvant effect is unlikely to be due to residual LPS in the toxin preparations as enhancement of Ab response to KLH was also observed in TLR-4-defective C3H-HeJ mice (data not shown). Although iAC-CyaA did significantly enhance Ab responses to KLH after two immunizations, the IgG titers were significantly lower than that observed with the wild-type toxin (Fig. 9B). We also assessed Ag-specific cytokine production by popliteal lymph node cells after a single immunization with KLH and CyaA or NA-CyaA. KLH-specific cytokine production was low or undetectable in lymph node cells from mice immunized with KLH alone (Fig. 10). However, lymph node cells from these mice did proliferate and secrete IL-4, IL-5, IL-10, and IFN-γ in response to PMA and anti-CD3. In contrast, Ag-specific proliferation (data not shown) and IL-4, IL-5, and IL-10 production (Fig. 10) were detected in lymph node cells from mice immunized with KLH in the presence of A-CyaA or NA-CyaA. IFN-γ was produced by lymph node cells from mice immunized with KLH in the presence of A-CyaA or NA-CyaA; however, the concentrations were low when compared with that observed in mice immunized with KLH in the presence of CpG-ODN (Ref. 9 and our unpublished observations). This cytokine profile is consistent with our previous demonstration that CyaA preferentially enhances Th2 and Tr1 responses (9) and with the observation that IgG1 was the dominant subclass of Ab induced with KLH in the presence of A-CyaA or NA-CyaA (data not shown). Therefore, we conclude that lack of acylation does not compromise the adjuvant properties of CyaA or its ability to direct the induction of T cell and Ab responses in vivo.



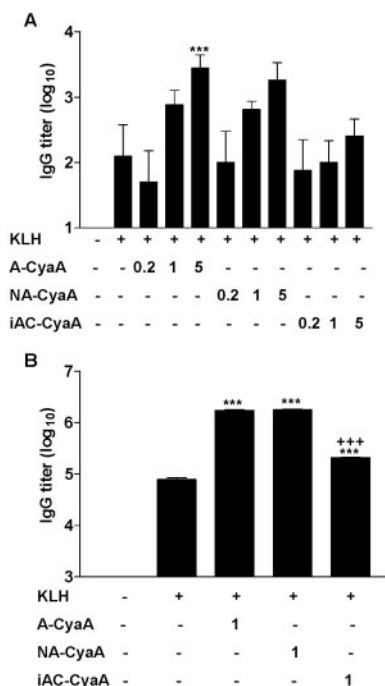
**FIGURE 8.** Enzyme activity, but not acylation, is required for modulation of DC maturation by CyaA. DC from BALB/c mice were cultured for 24 h with 1  $\mu\text{g/ml}$  NA-CyaA, A-CyaA, or iAC-CyaA alone or in the presence of CpG-ODN (10  $\mu\text{g/ml}$ ) or with CpG-ODN or medium only. Polymyxin B (10  $\mu\text{g/ml}$ ) was added to all cultures to rule out any contribution of residual LPS. Cells were harvested, washed, and labeled with anti-CD11c and anti-CD80, anti-CD40, anti-ICAM-1, or the appropriate isotype control Ab. Results of immunofluorescence analysis are shown for treated (black lines) compared with untreated (gray histograms) DC and are representative of six experiments.

## Discussion

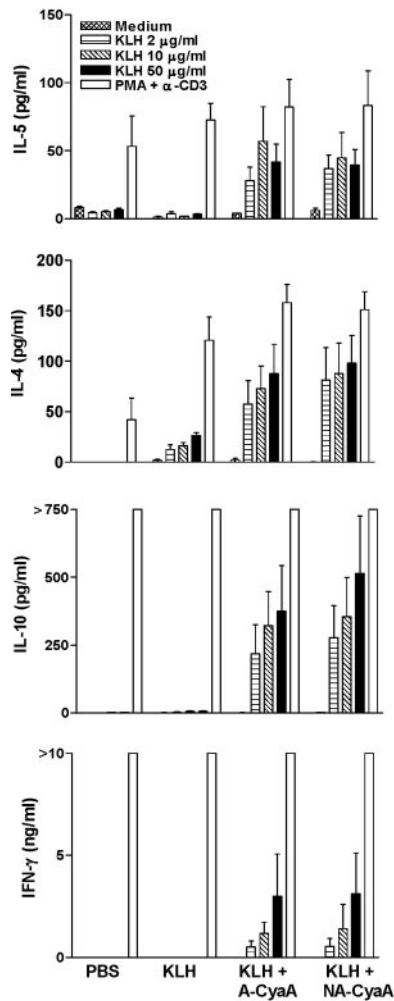
In this study, we have shown that posttranslational modification of CyaA by acylation is required for optimal caspase-3 activation and cell death in macrophages, but is not essential for CyaA to modulate innate immune cell activation in vitro or to act as an adjuvant in vivo. NA-CyaA, like A-CyaA, enhanced macrophage and DC production of the anti-inflammatory cytokine IL-10 and inhibited the production of the proinflammatory cytokines TNF- $\alpha$  and IL-12

and the chemokine CCL3 in response to TLR ligands. In contrast, a mutant toxin with substitutions in the adenylate cyclase active site failed to modulate DC or macrophage activation, indicating that enzymatic activity and consequent cAMP accumulation were required for the immunomodulatory effects of CyaA. Significantly, and in contrast to previous reports on other cell types (2, 12), we found that, while the absence of acylation reduced, it did not prevent CyaA-induced cAMP accumulation in macrophages and DC. These findings demonstrate for the first time that acylation is not essential for the immunomodulatory effects of CyaA and that these effects are principally mediated by adenylate cyclase-induced intracellular cAMP.

Lysis of eukaryotic cells by RTX toxins is dependent on posttranslational acylation (11). Likewise, acylation of the RTX domain of CyaA is considered to be necessary for its hemolytic and cytotoxic activity (2, 6, 12). Previous studies have suggested that acylation is required for efficient adjuvanticity of CyaA, while the increase in intracellular cAMP levels generated by the adenylate cyclase domain is responsible for its immunomodulatory effects (5, 6, 23). Our data demonstrate that acylation is not essential for CyaA to induce cAMP accumulation in macrophages or to modulate activation of macrophages and DC. Like A-CyaA, NA-CyaA synergized with LPS to enhance secretion of IL-10, while inhibiting TNF- $\alpha$  and IL-12 p70 production. LPS alone at the concentration present in the toxin preparations did not induce IL-10 production. Likewise, the toxins alone did not induce IL-10 in DC from TLR-4-defective mice or in macrophages or DC from normal mice in the presence of polymyxin B. However, when combined, the toxin and TLR ligand synergized to promote IL-10 production. The modulatory effects of A-CyaA and NA-CyaA on innate cell cytokine production were not confined to the TLR-4 ligand, LPS, which forms a complex with CyaA (19), but were also observed with the TLR-9 ligand, CpG-ODN. This suggests that a physical association between the two modulators (as in the case of CyaA and LPS) is not essential for the synergy, but possibly binding to two distinct receptors is involved. This hypothesis is supported by previous studies showing synergy between TLR and other signaling pathways (24, 25). NA-CyaA and A-CyaA also had similar effects on DC maturation, up-regulating CD80 expression, while down-regulating CD40 and ICAM-1 expression. Furthermore, NA-CyaA, like A-CyaA, was an effective adjuvant in vivo when



**FIGURE 9.** Enzymatic activity, but not acylation, influences the adjuvant effect of CyaA. BALB/c mice were immunized once (A) or twice (B) at 0 and 21 days in the footpad with PBS, KLH (5  $\mu\text{g}$ ) alone, or with NA-CyaA, A-CyaA, or iAC-CyaA (0.2–5  $\mu\text{g}$ ). Serum was prepared 7 days later and tested for KLH-specific IgG by ELISA (expressed as end point titers). Results are means  $\pm$  SD for five mice with samples tested in triplicate. \*\*\*,  $p < 0.001$ , vs KLH alone; +++,  $p < 0.001$ , vs KLH plus A-CyaA. Results are representative of four experiments, two after a single, and two after a booster dose.



**FIGURE 10.** NA-CyaA is as effective an adjuvant as A-CyaA, enhancing Th2/Tr1 type response to coadministered Ags in vivo. Mice were immunized in the footpad with PBS, KLH (5  $\mu$ g) alone, or with NA-CyaA, or A-CyaA (1  $\mu$ g). Popliteal lymph node suspensions were prepared after 7 days and KLH-specific cytokine release determined. Results are means  $\pm$  SD for five mice with samples tested in triplicate, and are representative of three experiments.

coinjecting with a foreign Ag, stimulating the production of Ag-specific IgG1 Abs and IL-4, IL-5, and IL-10 production by T cells.

Unlike acylation, enzyme activity was required for maximal adjuvant and immunomodulatory activity of CyaA. A CyaA mutant lacking functional adenylate cyclase activity was unable to cause an increase of intracellular cAMP concentrations, did not modulate DC maturation and was unable to suppress LPS-induced TNF- $\alpha$  release or to enhance IL-10 production by innate immune cells. This suggests that increased intracellular cAMP concentrations are involved in modulation of innate immune cell activation by CyaA. This conclusion is consistent with the observations that increases in intracellular cAMP concentration by stimuli such as cholera toxin or forskolin (23, 24), lead to suppression of proinflammatory cytokines and chemokines, such as IL-12 and CXCL10 and enhancement of IL-10 and CCL17.

RTX toxins bind to leukocytes through  $\beta_2$  integrins, and CD11b/CD18 has been identified as the receptor for CyaA (2, 3). To bind RBC, which lack  $\beta_2$  integrins, CyaA and other RTX proteins require acylation (12). However, the role of acylation in the binding of RTX toxins to purified  $\beta_2$  integrins has not been reported. We have demonstrated that acylation is not essential for

CyaA to induce cAMP accumulation in innate immune cells expressing CD11b/CD18. Although cAMP induction was reduced with the nonacylated toxin, which may be due to reduced stability of interaction with CD11b (2) or less effective penetration of the cell membrane compared with acylated native toxin (12, 13), it did induce significantly increased levels of intracellular cAMP in macrophages and DC. Previous studies had suggested that acylation of CyaA was essential for the induction of increased cAMP levels in eukaryotic cells. However, these studies had not used innate immune cells but rather RBC and Jurkat T cells, each of which express low or no CD11b/CD18, and Chinese hamster ovary cells transfected with CD11b (2, 12). Previous studies have demonstrated that acylation is important for binding of CyaA to RBC, but that interaction with CD11b-expressing cells is via residues 1166–1281 (2). In addition, CyaA binding to CD11b-expressing cells is saturable, while binding to RBC is not (3, 26). These data suggest that CyaA may interact with RBC and CD11b-expressing cells by two different mechanisms. In the present study, we have demonstrated that acylation is not essential for CyaA-induced cAMP accumulation and immunomodulation in macrophages and that anti-CD11b Abs block the modulatory effects of A-CyaA and NA-CyaA. Together, these data indicate that posttranslational modification is not essential for interaction of CyaA with its  $\beta_2$  integrin receptor CD11b/CD18 on the host cell surface. Interaction of CyaA with CD11b/CD18, which is primarily expressed on macrophage and myeloid DC, may be a strategy evolved by the bacteria to target and disrupt cytokine signaling pathways in key cells of the innate immune system.

CyaA oligomerizes in the cell membrane to form pores and thus cause membrane disintegration. Our data demonstrate that in cells expressing the CyaA receptor CD11b/CD18, such as DC and macrophages, acylation is important for cytotoxicity. CyaA induces cell death in macrophages, as indicated by the DNA fragmentation seen in CyaA-treated cells in vitro (6). CyaA-deficient *B. pertussis* does not induce lysis of J774 macrophages, has reduced capacity to cause apoptosis of alveolar macrophages in vivo, and is considerably less virulent in mice (5, 27). We observed cell death in macrophages incubated with A-CyaA or iAC-CyaA. In contrast, cell lysis was only observed with the highest dose of NA-CyaA examined and this effect was abolished, as was a proportion of the lytic activity of A-CyaA, by coinubation with polymyxin B. LPS and LPS-induced TNF- $\alpha$  have been associated with apoptosis (28) and, therefore, may have contributed to the cell death observed with NA-CyaA and CyaA. This may explain the higher levels of cell lysis reported in previous studies, where steps were not taken to remove LPS during the purification of CyaA. We also provide evidence that CyaA induces activation of caspase-3, a key effector molecule in apoptosis. Caspase-3 activation was observed in macrophages treated with the wild-type toxin and with the enzymatically inactive mutant, but not with NA-CyaA. Overall our findings suggest that acylation, but not adenylate cyclase activity, facilitates CyaA-induced cell death.

Our study provides new insights into the role of acylation of RTX domains in the immunomodulatory and proapoptotic activities of bacterial toxins and has addressed the contribution of LPS, present at high concentrations in many recombinant CyaA preparations, to its immunological activity. The nonacylated toxin retains the ability to enhance cAMP, albeit at a slightly lower efficiency than the wild-type toxin. Elevation of intracellular cAMP appears to be a critical factor in the immunomodulatory activity of CyaA, but is not essential for its ability to cause cell lysis or caspase-3 activation. Like the wild-type toxin, this nonacylated



derivative specifically targets CD11b/CD18-expressing macrophages and DC, inhibiting the release of proinflammatory cytokines and enhancing IL-10 production and activating DC into a semimature phenotype that directs the induction of Th2 and IL-10-producing Tr1 cells. Thus, NA-CyaA is capable of promoting both innate and adaptive IL-10 production, without causing cell death, and as a consequence has considerable potential as an adjuvant and immunotherapeutic agent for the prevention and/or treatment of inflammatory and Th1-mediated autoimmune diseases.

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## Disclosures

K. H. G. Mills is a cofounder, director, and shareholder in Opona Therapeutics, which is involved in the development of anti-inflammatory therapeutics.

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