

# The Adenylate Cyclase Toxin of *Bordetella pertussis* Binds to Target Cells via the $\alpha_M\beta_2$ Integrin (CD11b/CD18)

By Pierre Guermonprez,\* Nadia Khelef,† Eric Blouin,‡  
Philippe Rieu,‡ Paola Ricciardi-Castagnoli,¶ Nicole Guiso,‡  
Daniel Ladant,§ and Claude Leclerc\*

From the \*Unit of Biology of Immune Regulations, the †Unit of *Bordetella*, and the §Unit of Cellular Biochemistry, Institut Pasteur, and the ‡Unit 507, Institut National de la Santé et de la Recherche Médicale (INSERM), Department of Nephrology, Necker Hospital, Paris 75015, France; and the ¶Department of Biotechnology and Bioscience, University of Milano-Bicocca, Milano 20126, Italy

## Abstract

The adenylate cyclase toxin (CyaA) of *Bordetella pertussis* is a major virulence factor required for the early phases of lung colonization. It can invade eukaryotic cells where, upon activation by endogenous calmodulin, it catalyzes the formation of unregulated cAMP levels. CyaA intoxication leads to evident toxic effects on macrophages and neutrophils. Here, we demonstrate that CyaA uses the  $\alpha_M\beta_2$  integrin (CD11b/CD18) as a cell receptor. Indeed, the saturable binding of CyaA to the surface of various hematopoietic cell lines correlated with the presence of the  $\alpha_M\beta_2$  integrin on these cells. Moreover, binding of CyaA to various murine cell lines and human neutrophils was specifically blocked by anti-CD11b monoclonal antibodies. The increase of intracellular cAMP level and cell death triggered by CyaA intoxication was also specifically blocked by anti-CD11b monoclonal antibodies. In addition, CyaA bound efficiently and triggered intracellular cAMP increase and cell death in Chinese hamster ovary cells transfected with  $\alpha_M\beta_2$  (CD11b/CD18) but not in cells transfected with the vector alone or with the  $\alpha_X\beta_2$  (CD11c/CD18) integrin. Thus, the cellular distribution of CD11b, mostly on neutrophils, macrophages, and dendritic and natural killer cells, supports a role for CyaA in disrupting the early, innate antibacterial immune response.

Key words: *Bordetella* • adenylate cyclase • integrin • CD11b • toxin

## Introduction

*Bordetella pertussis*, the causative agent of whooping cough, secretes several toxins, including the well known pertussis toxin and the adenylate cyclase toxin, CyaA. In the murine respiratory model, CyaA is a critical virulence factor required for the early steps of lung colonization. Indeed, genetic deletion of the structural gene encoding for this toxin dramatically decreases the pathological effects of *B. pertussis* infection, reducing the number of bacteria recovered from the lung and almost abolishing the inflammatory cell recruitment and lung lesions observed after infection (1–6). Moreover, CyaA is able to induce a protective immunity against *B. pertussis* infection in the murine respiratory model (7–10).

Originally discovered by Hewlett and Wolff (11) in *B. pertussis* culture supernatants, the adenylate cyclase was later

found to be activated by the eukaryotic calmodulin (12). This striking feature quickly found a rationale when it was shown that the adenylate cyclase can enter into eukaryotic cells where, upon activation by calmodulin, it triggers a large increase in cAMP levels (13).

The CyaA toxin is encoded by the *cyaA* gene, and its expression, like that of other virulence genes of *B. pertussis*, is coordinately regulated by environmental signals through a two-component system called BvgAS. The *cyaA* gene is part of an operon that also contains the genes *cyaB*, *-D*, and *-E*, required for CyaA secretion (14).

The CyaA toxin is a bifunctional protein of 1,706 residues consisting of an NH<sub>2</sub>-terminal catalytic domain of 400 amino acids and a COOH-terminal part with 1,306 residues. This last domain is required for the binding of the toxin to the target cell membrane and subsequent delivery of the catalytic moiety into the cell cytosol (14). This COOH-terminal part also exhibits a weak hemolytic activity because of its ability to form cation-selective channels in

Address correspondence to Claude Leclerc, Unit of Biology of Immune Regulation, Institut Pasteur, 25 Rue du Dr Roux, 75724 Paris cedex 15, France. Phone: 33-1-4568-8618; Fax: 33-1-4568-8540; E-mail: cleclerc@pasteur.fr

biological membranes (15–17). It is homologous to *Escherichia coli* hemolysin (HlyA) and other members of the RTX (repeat in toxin) family of bacterial toxins. In particular, it contains a series of glycine- and aspartate-rich nonapeptide repeats that are involved in calcium binding (18, 19).

The CyaA polypeptide is synthesized as an inactive protoxin that is converted to an active toxin by posttranslational palmitoylation of two internal lysines (lysines 856 and 963). This modification requires the product of an accessory gene, *cyaC*, which is located near *cyaA* on the *B. pertussis* chromosome (20, 21).

CyaA has been shown to bind to and invade a variety of cell types, including cells lacking membrane traffic like mammalian erythrocytes (22). This suggests that the catalytic domain of CyaA is directly translocated across the plasma membranes of target cells. The internalization of the catalytic domain into the cell cytosol is calcium and temperature dependent and depends on the plasma membrane potential (22–24). However, the molecular mechanisms by which the toxin transports its NH<sub>2</sub>-terminal catalytic domain across the membrane remain largely unknown to date. Furthermore, no specific receptor has yet been identified for CyaA binding.

The physiological consequences of cellular intoxication by CyaA were characterized in vitro in phagocytes. Confer and Eaton first showed that *B. pertussis* CyaA increases the intracellular cAMP level in neutrophils or macrophages, leading to an inhibition of chemotaxis and bacterial functions such as phagocytic abilities and superoxide generation (13). These properties were later confirmed with either purified toxins or bacterial mutants genetically deleted of CyaA (25–27). On the contrary, and despite significant changes in their cAMP content, the viability of cell lines from nonhematopoietic origin appeared to be unaffected by CyaA intoxication (6, 28). Moreover, we demonstrated previously that *B. pertussis* CyaA can trigger macrophage apoptosis in vitro (29, 30) and in vivo (6). In these models, genetic deletion of the *cyaA* gene abolished macrophage apoptosis but not neutrophil death, suggesting that CyaA is mainly responsible for macrophage apoptosis and might be responsible for neutrophil apoptosis but that other factors may also be involved. Besides that, in vivo studies performed in a murine model of infection by *B. bronchiseptica*, which expresses a highly similar CyaA toxin, demonstrated that the major target of *B. bronchiseptica* CyaA-induced toxicity is a GM-CSF-dependent and cyclophosphamide-sensitive population that controls the early steps of infection (31). These criteria identified neutrophils and possibly other cells, including macrophages or dendritic cells. These populations of target cells for CyaA are the same that limit the early phases of infection, favoring the development of an adaptative immune response that controls the later phases of infection (31).

Thus, in contrast to numerous in vitro studies showing that CyaA can enter a wide variety of cell lines, in vivo studies strongly suggest that CyaA targets phagocytes such as alveolar macrophages and neutrophils, representing a major arm of *B. pertussis* against the innate immune system.

Since other members of the RTX toxin family bearing cellular specificity for leukocytes have recently been shown to bind to different receptors of the  $\beta_2$  integrin family (32–34), we hypothesized that CyaA could similarly bind to one of them. The  $\beta_2$  integrin family comprises four noncovalently linked  $\alpha/\beta$  dimers sharing the same  $\beta_2$  chain but harboring different  $\alpha$  chains:  $\alpha_1\beta_2$  (CD11a/CD18, LFA-1),  $\alpha_M\beta_2$  (CD11b/CD18, CR3, Mac1),  $\alpha_X\beta_2$  (CD11c/CD18, p150/195), and the less well characterized  $\alpha_D\beta_2$  (CD11d/CD18) (35). The expression of  $\beta_2$  integrins is restricted to leukocytes. In mice, CD11a is expressed by all leukocytes; CD11b is restricted to granulocytes/neutrophils, macrophages, myeloid dendritic cells, NK cells, and subsets of CD8<sup>+</sup> T cells and B cells, whereas CD11c is restricted to dendritic cells (35, 36).

In this study, we demonstrate that CyaA binds specifically to target cells via the  $\alpha_M\beta_2$  integrin (CD11b/CD18) and that this interaction is required for the intracellular delivery of the adenylate cyclase domain into leukocytes, cAMP production, and CyaA-induced cell death.

## Materials and Methods

**Recombinant Toxins and Abs.** Protocol for CyaA production has already been described elsewhere (23). CyaA toxins were produced in an *E. coli* BLR strain harboring an expression plasmid, pCACT3, which carries the *cyaA* structural gene encoding CyaA under the lacUV5 promoter and the *cyaC* accessory gene required for activation of the protoxin. After solubilization in 8 M urea, 20 mM Hepes-Na, pH 7.5, CyaA was purified to >95% homogeneity (as judged by SDS-gel analysis; data not shown) by sequential DEAE-Sepharose and phenyl-Sepharose. In some experiments, CyaA was purified by chromatographies on calmodulin affigel. A recombinant detoxified CyaA toxin, CACTE5-Cys-OVA, harboring a unique cysteine inserted within the genetically inactivated catalytic domain was constructed by inserting an appropriate double strand oligonucleotide between the BsiwI and KpnI sites of pCACT-OVA-E5. In the resulting protein, CACTE5-Cys-OVA, the amino acid sequence ASCGSIINFEK-LGT is inserted between residues 224 and 225 of CyaA, and the LQ dipeptide is inserted between residues 188 and 189 (the latter insertion abolishes the cAMP-synthetizing activity of CyaA; reference 15). The purified detoxified protein was labeled on its unique Cys with the highly specific sulfhydryl reagent *N*-(6-[biotinamido-hexyl])-3'-(2'-pyridyldithio) propionamide (biotin-HPDP; Pierce Chemical Co.) according to the manufacturer's instructions. The biotinylated CyaA was repurified on DEAE-Sepharose to remove the unreacted biotin-HPDP reagent. Toxin concentrations were determined spectrophotometrically from the adsorption at 280 nm using a molecular extinction coefficient of  $142 \text{ M}^{-1} \times \text{cm}^{-1}$  or using the Bio-Rad Laboratories protein assay system.

Purified mAbs specific for murine CD11a (2D7, rat IgG2a,  $\kappa$ ), murine and human CD11b (M1/70, rat IgG2b,  $\kappa$ ), murine CD11c (HL3, hamster 1,  $\lambda$ ), murine CD18 (C71/16, rat IgG2a,  $\kappa$ ), and control (A95-1, or anti-CD16/32, 2.4G2, rat IgG2b,  $\kappa$ ) originated from BD PharMingen. Supernatants from anti-human CD11b (44, mouse IgG2a,  $\kappa$ ) and anti-human CD18 (TS/18, mouse IgG1,  $\kappa$ ) hybridoma were used at 1:2 dilution in blocking experiments. Supernatant from an anti-murine CD11b (5C6, rat IgG2b,  $\kappa$ ) was a gift from G. Milon (Institut Pasteur) and was

used at 1:2 final dilution in binding inhibition assays. Anti-CyaA polyclonal Abs were obtained from a rabbit immunized subcutaneously with purified CyaA. Immunoglobulins were precipitated from immune serum by 33% ammonium sulfate. After centrifugation, the pelleted proteins were resuspended in 20 mM Hepes-Na, 150 mM NaCl, pH 7.5, and extensively dialyzed against the same buffer. The Abs were then biotinylated by incubation with biotin–amidocaproate *N*-hydroxysuccinimide ester dissolved in dimethyl sulfoxide (Sigma–Aldrich) for 130 min at room temperature. 100 mM ethanolamine, pH 9.0, was added, and after 30 min of additional incubation, the mixture was extensively dialyzed at 4°C against buffer C. Biotinylated Abs were stored at –20°C.

**Cells and Culture Media.** EL4, J774A.1, LB27.4, and THP-1 were obtained from American Type Culture Collection and were cultured in RPMI 1640 supplemented with 10% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine, with or without  $5 \times 10^{-5}$  M 2-mercaptoethanol (complete medium). Fetal mouse skin dendritic cells (FSDCs;<sup>1</sup> reference 37) were cultured in complete medium, and D1 cells were cultured as described (38). Chinese hamster ovary (CHO) cells transfected for human CD11b/CD18 or CD11c/CD18 or transfected with the vector only were obtained from D. Golenbock (Boston University School of Medicine, Boston, MA) and cultured in the presence of neomycin as described previously (39). Human neutrophils were purified as described previously (40). In some experiments, they were activated by preincubation with 2 µM fMLP (*N*-formyl-met-leu-phe; Sigma–Aldrich) for 30 min at 37°C.

**CyaA Binding Assays.** All binding assays were performed in DMEM 4.5 mg/ml glucose (Life Technologies) without serum in 96-well culture plates (Costar).  $2 \times 10^5$  cells/well were incubated for 20 min (at 4 or 37°C depending on the experiments) in a 200 µl final volume. In some experiments, EDTA or EGTA (Sigma–Aldrich) was added at the indicated concentrations. In other experiments, cells were preincubated for 20 min at 4°C in the presence of blocking mAbs in 100 µl final volume. The toxin solution was added to the wells in the continuous presence of the mAbs in a total volume of 200 µl at 4°C. Plates were centrifuged at 1,500 rpm for 5 min, and supernatants were removed. Cells were incubated at 4°C for 25 min with biotinylated anti-CyaA rabbit polyclonal Abs (1:400 in DMEM; 50 µl/well) in the presence of a control (nonimmune or preimmune) rabbit serum as a saturating agent (1:50).

After centrifugation and supernatant removal, cells were stained with streptavidin–PE (BD PharMingen) at 1:300 dilution (50 µl/well). After washing, cells were analyzed by flow cytometry on a FACStar™ (Becton Dickinson) in the presence of 5 µg/ml propidium iodide. Gatings were done to exclude cell aggregates and dead cells by propidium iodide exclusion. Experimental points were fitted to a hyperbolic model  $\Delta$  mean fluorescence intensity (MFI) =  $B_{\max} \times [\text{CyaA}] / (K_d + [\text{CyaA}])$ , with  $B_{\max}$  equal to the percentage of maximal binding, using Prism software.

**cAMP Assay.** cAMP accumulation was measured by an Ag competition immunoassay (41) in which the incubation medium was composed of DMEM without serum but containing 4.5 mg/ml glucose and 20 U/ml hexokinase. Hexokinase, which catalyzes the ATP-dependent phosphorylation of glucose, was added to deplete the extracellular medium of any traces of ATP, thus

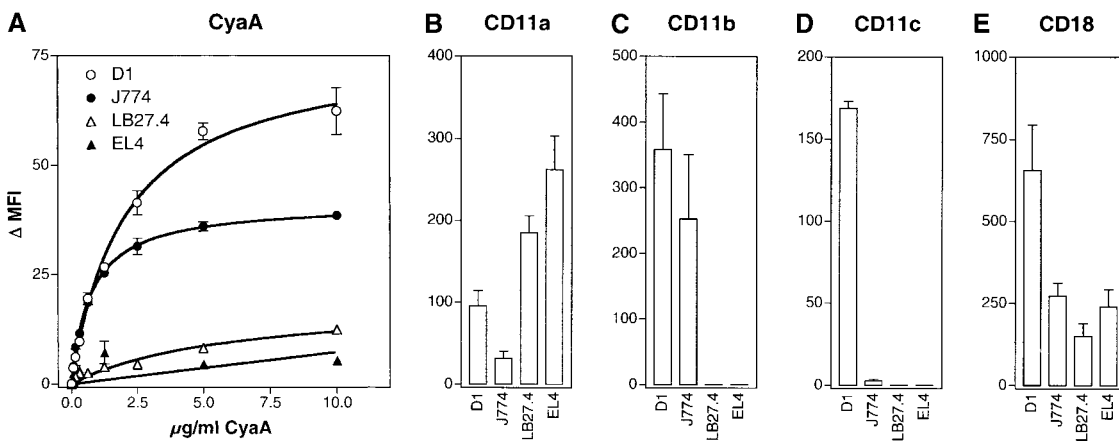
preventing the extracellular synthesis of cAMP. Therefore, the amount of cAMP measured is representative of the accumulation of strictly intracellular cAMP.  $5 \times 10^5$  cells were preincubated in 96-well plates in 100 µl/well with or without 10 µg/ml of specific mAbs at 4°C for 1 h and then incubated at 37°C for 20 min with 5 µg/ml CyaA and with 10 µg/ml of specific mAbs when present during the preincubation. For the dose–response effect of CyaA, cells were directly incubated with the toxin at 0.05, 0.5, or 5 µg/ml for 20 min at 37°C. After intoxication, cells were centrifuged at 2,500 rpm for 5 min. Samples were lysed with 100 µl of 0.1 N HCl, boiled for 5 min at 120°C, and neutralized with 100 µl of 0.125 M Tris and 0.2 M NaCl. Microtiter plates were coated with a cAMP–BSA conjugate diluted at 1:4,000 in 0.1 M Na<sub>2</sub>CO<sub>3</sub>, pH 9.5. They were washed twice in 0.1% PBS–Tween, saturated for 1 h in 2% PBS–BSA, and washed five times with 0.1% PBS–Tween. The samples and the cAMP standard (Sigma–Aldrich) were directly added to the plates coated with cAMP–BSA conjugate and serially diluted with a 1:1 mixture of 0.1 N HCl and 0.125 M Tris–0.2 M NaCl. Anti-cAMP rabbit Abs were added at 1:2,500 in 2% PBS–BSA and incubated at 37°C for 3 h. Plates were washed five times with 0.1% PBS–Tween. Anti-rabbit Abs coupled to horseradish peroxidase (Amersham Pharmacia Biotech) were added at 1:2,500 in 2% PBS–BSA, incubated at 37°C for 1 h, and revealed using the classical peroxidase reaction. Experimental points of the standard curve were fitted to a sigmoid model using Prism software.

**Toxicity Assay.** Cell death was evaluated as described previously (29, 30). In brief,  $10^5$  cells were incubated for 24 h in a 96-well plate in complete medium and washed once with serum-free medium. All cell incubations were further performed in serum-free medium. Dose–response effects were evaluated by directly applying various concentrations of CyaA to CHO cells at 37°C for 4 h. For cytotoxicity inhibition, cells were preincubated at 4°C for 1 h with or without 10 µg/ml of specific mAbs and then incubated at 37°C with 0.5 µg/ml CyaA for 2 h for J774A.1 cells or with 5 µg/ml CyaA for 4 h for CHO cells and with 10 µg/ml of specific mAbs when present during the preincubation. Cell lysis was evaluated using the Cytotox 96™ assay (Promega), which quantifies the amount of lactate dehydrogenase (LDH) released in the medium by dying cells.

## Results

**Saturable Binding of CyaA Correlates with the Presence of CD11b at the Surface of Target Cells.** To characterize CyaA cellular specificity toward a population of leukocytes, we chose four representative murine cell lines expressing various combinations of  $\beta_2$  integrins: D1, a splenic myeloid dendritic cell line; J774A.1, a tumoral macrophage; EL4, a T cell thymoma, and LB27.4, a B cell lymphoma. After a 20-min incubation with CyaA at 37°C, binding of the toxin to the cell surface was monitored by flow cytometry using biotinylated anti-CyaA Abs and streptavidin–PE. Under these conditions, we observed an efficient, dose-dependent and saturable binding of CyaA to D1 and J774A.1 cells (Fig. 1 A). The affinity of CyaA for D1 and J774A.1 cells was high since the apparent  $K_d$  were  $9.2 \pm 4.5$  and  $3.2 \pm 1.9$  nM, respectively. Binding of CyaA to EL4 and LB27.4 cells was also observed, but it was not saturable at the concentrations tested and much lower than that observed for J774 or D1.

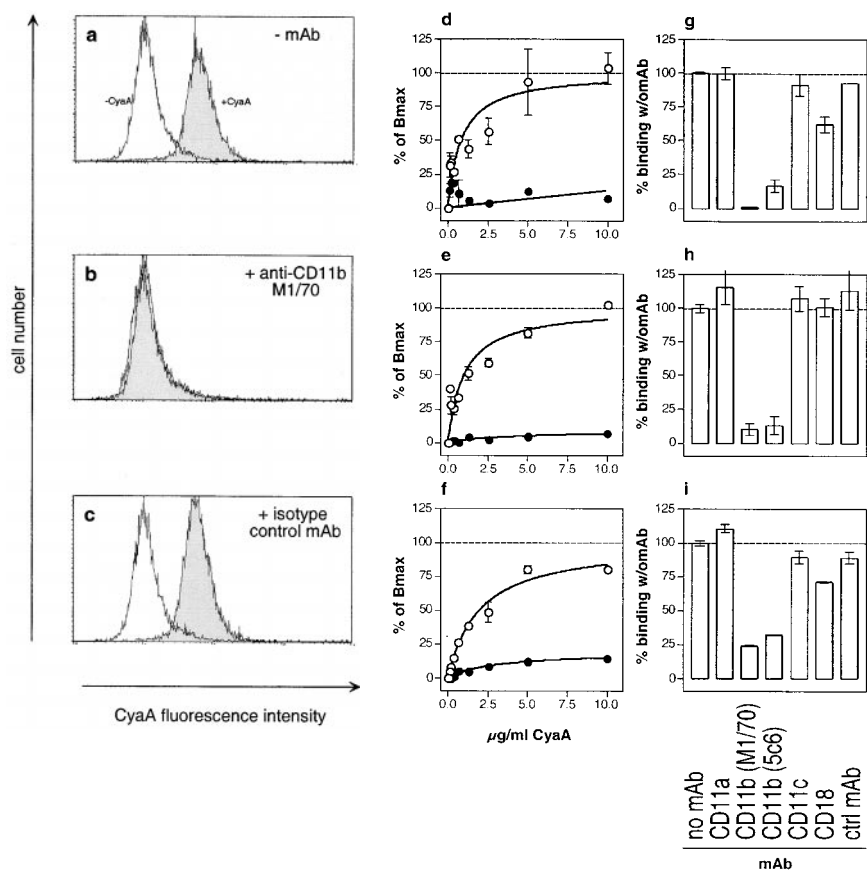
<sup>1</sup>Abbreviations used in this paper: CHO; Chinese hamster ovary; FHA, filamentous hemagglutinin; FSDC, fetal mouse skin dendritic cell; LDH, lactate dehydrogenase; MFI, mean fluorescence intensity.



**Figure 1.** Saturable binding of CyaA to different cell lines correlates with their CD11b expression. (A) CyaA binding at the surface of dendritic cells (D1), macrophages (J774A.1), B cells (LB27.4), and T cells (EL4) was performed at 37°C for 20 min. Surface-bound CyaA was detected by flow cytometry using biotinylated anti-CyaA polyclonal Abs and streptavidin-PE. Binding is expressed as  $\Delta\text{MFI} = (\text{MFI value of cells incubated with CyaA}) - (\text{MFI of cells without CyaA})$ . (B–E) Cell-surface expression of  $\beta_2$  integrins on D1, J774A.1, LB27.4, and EL4 cells. CD11a (B), CD11b (C), CD11c (D), and CD18 (E) expression was determined by flow cytometry using specific mAbs coupled to PE. Integrin expression is expressed as  $\Delta\text{MFI} = (\text{MFI value of cells stained with specific mAb}) - (\text{MFI of cells stained with an isotype control mAb})$ .

To determine if the binding of CyaA to the D1 and J774 cell lines correlates with the expression of one of the members of the  $\beta_2$  integrin family, we performed a phenotypic analysis of these cells by flow cytometry using mAbs specific for the three  $\alpha$  chains of the well-characterized  $\beta_2$  integrins (CD11a, CD11b, and CD11c) and for the common

$\beta$  chain (CD18) (Fig. 1 B–E). D1 cells were strongly positive for CD11a, CD11b, CD11c, and CD18. J774A.1 cells expressed mostly CD11b and CD18 but were also positive for CD11a. EL4 cells and LB27.4 cells expressed mostly CD11a and CD18. Taken together, these data show that the efficient and saturable binding of CyaA to murine D1



**Figure 2.** CyaA binding to murine cell lines is blocked by anti-CD11b mAb. Cells were preincubated at 4°C for 15 min with or without 20  $\mu\text{g/ml}$  of specific mAbs and then incubated at 4°C for 20 min with 5  $\mu\text{g/ml}$  CyaA and 10  $\mu\text{g/ml}$  of corresponding mAbs. Surface-bound CyaA was detected with biotinylated anti-CyaA polyclonal Abs, revealed by flow cytometry on living cells. (a–c) Fluorescence histograms of D1 dendritic cells were preincubated with medium alone (a), with M1/70 anti-CD11b mAb (b), or with an isotype-matched rat IgG2b (c) and then incubated with (gray) or without (white) CyaA in the presence or absence of mAbs. The cell number is plotted against the log of PE fluorescence. (d–f) D1 dendritic cells (d), FSDCs (e), or J774A.1 macrophages (f) were preincubated with medium alone (○) or with M1/70 anti-CD11b mAb (●), and then incubated with CyaA with or without M1/70 anti-CD11b mAb.  $B_{\text{max}}$  was determined by fitting experimental points to  $\Delta\text{MFI} = B_{\text{max}} \times [\text{CyaA}] / (K_d + [\text{CyaA}])$ . Binding of CyaA is plotted as a percentage of  $B_{\text{max}}$  plotted against CyaA concentration. (g–i) Effect of specific mAbs on the binding of a fixed dose of CyaA. D1 (g), FSDCs (h), or J774A.1 (i) cells were preincubated with or without specific mAbs (anti-CD11a, 2D7, anti-CD11b, M1/70 or 5C6, anti-CD11c, HL3, anti-CD18, C17/16, control A95-1), and incubated with CyaA at the fixed concentration of 5  $\mu\text{g/ml}$ . Values of  $\Delta\text{MFI}$  obtained for CyaA binding on cells treated with specific mAbs were normalized as a percentage of the  $\Delta\text{MFI}$  values obtained for CyaA binding without mAb.

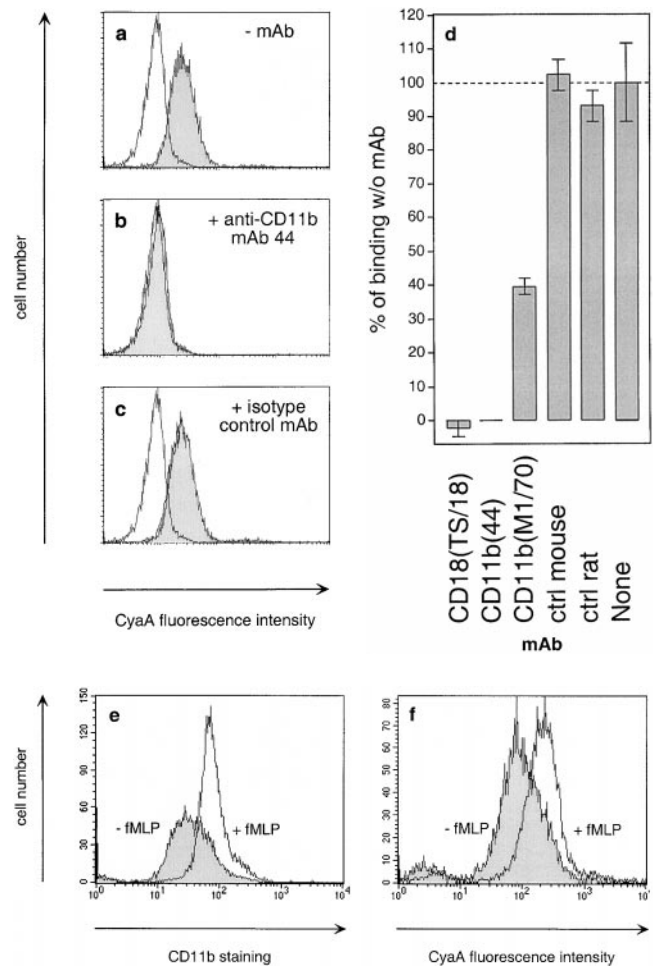
and J774A.1 cells was correlated with cell surface expression of the integrin CD11b/CD18.

**CyaA Saturable Binding Is Specifically Blocked by Anti-CD11b mAbs.** Next, we examined if CD11b/CD18 could be directly involved in the binding of CyaA to the cells expressing this integrin. As shown in fluorescence histograms in Fig. 2 a, b, and c, preincubation of D1 cells with the anti-CD11b M1/70 mAb strongly and specifically inhibited the binding of CyaA to the cell surface. Then we performed a quantitative analysis of this inhibition by calculating the percentage of MFI values in the absence or presence of mAbs at a fixed (Fig. 2 g) or varying (Fig. 2 d) concentration(s) of CyaA. Inhibition of CyaA binding to D1 cells obtained with the M1/70 anti-CD11b mAb was almost total at most CyaA concentrations tested (Fig. 2 d). This inhibition was specific since anti-CD11a, -CD11c, -CD18 mAbs, or a control mAb did not affect CyaA binding (Fig. 2 g). A second anti-CD11b mAb (clone 5c6) also inhibited binding of CyaA (Fig. 2 g). Similar results were obtained with FSDCs, an immature dendritic cell line expressing CD11b (Fig. 2, e and h), and the J774A.1 macrophage cell line (Fig. 2, f and i).

To examine whether CyaA could similarly interact with human CD11b, CyaA binding studies were performed on human neutrophils, whose high expression of CD11b is well established. Since high background fluorescence was obtained after incubation of human myeloid cells with the anti-CyaA rabbit Abs (data not shown), we set up an alternate binding assay. A detoxified form of CyaA was specifically biotinylated on a unique cysteine residue genetically introduced within its catalytic domain. Using streptavidin-PE, we were able to detect CyaA binding to neutrophils (Fig. 3). Preincubation of neutrophils with the anti-CD11b mAbs 44 or M1/70 led to a complete or partial inhibition of the binding of CyaA, respectively (Fig. 3, b and d). Unlike the anti-murine CD18 mAb C71/16 that did not block CyaA binding to murine cells, preincubation with the human anti-CD18 TS/18 mAb led to a complete inhibition of CyaA binding to human neutrophils (Fig. 3 b). Preincubation of neutrophils with fMLP increased both surface levels of CD11b (Fig. 3 e) and CyaA binding (Fig. 3 f). In agreement with these observations, CyaA was also shown to bind to the human monocytic THP-1 cell line (data not shown).

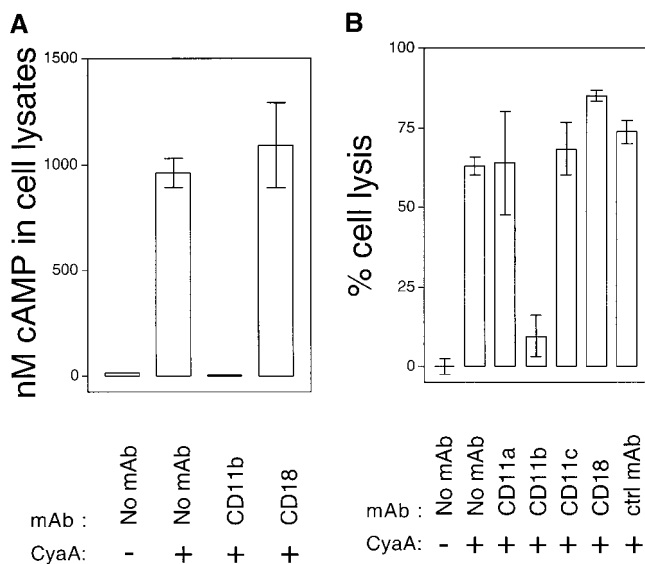
In conclusion, the CyaA binding to the surface of four myeloid cell lines from both murine and human origin (J774A.1, D1, FSDC, and THP-1) and freshly purified human neutrophils appears to be mainly mediated through the CD11b/CD18 integrin.

**CyaA-mediated cAMP Increase and Toxicity Are Specifically Blocked by an Anti-CD11b mAb.** To evaluate the physiological relevance of CD11b/CD18-dependent CyaA binding, we studied the effect of blocking mAbs on the cytotoxicity of CyaA. First we measured the amount of cAMP produced in J774A.1 cells exposed to CyaA in the presence of various mAbs. As shown in Fig. 4 A, the increase in the intracellular cAMP content induced by CyaA was totally abolished when cells were preincubated with the M1/70



**Figure 3.** CyaA binding to human neutrophils is blocked by anti-CD11b and anti-CD18 mAbs and is correlated to CD11b surface levels. (a–c) Fluorescence histograms of freshly purified neutrophils preincubated with medium alone (a), the anti-CD11b mAb 44 (b), or an isotype-matched control mouse mAb (c), then incubated with (gray) or without (white) biotinylated detoxified CyaA (5 µg/ml), and revealed by streptavidin-PE. Cell number is plotted against log of PE fluorescence. (d) Effect of specific mAbs on CyaA binding to neutrophils (anti-CD11b, 44 or M1/70, anti-CD18, TS/18, control mouse IgG2a, control rat IgG2b, A95-1). Freshly purified neutrophils were preincubated with or without specific mAbs and incubated with biotinylated detoxified CyaA. Values of  $\Delta$ MFI obtained for CyaA binding on cells treated with specific mAbs were normalized as a percentage of the  $\Delta$ MFI values obtained for CyaA binding without mAb. (e and f) Neutrophil activation triggers the increase of both CD11b surface level and CyaA binding. Fluorescence histograms of freshly purified neutrophils that were pretreated or not with 2 µM fMLP for 30 min at 37°C and then were stained with the anti-CD11b mAb (e) or 15 µg/ml biotinylated detoxified CyaA (f).

anti-CD11b mAb. The C17/16 anti-murine CD18 mAb that did not block CyaA cell binding (Fig. 2) had no effect on the intracellular cAMP content of CyaA-treated cells (Fig. 4 A). Thus, these data strongly suggest that the increase in intracellular cAMP induced by CyaA is dependent on the interaction of the toxin with CD11b. To further analyze the requirement of this molecule for the toxicity of CyaA, we evaluated the effect of mAbs specific for the different chains of the  $\beta_2$  integrin family on CyaA-mediated cell lysis. Fig. 4



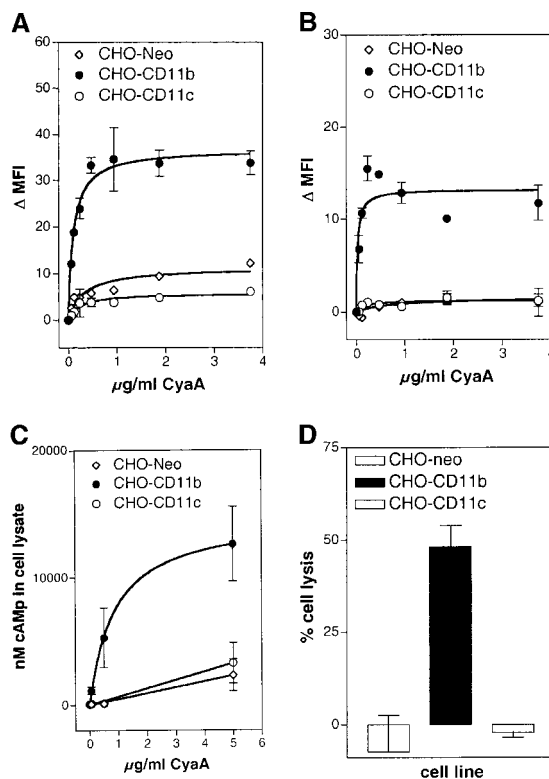
**Figure 4.** Intracellular cAMP increase and cell death mediated by CyaA are specifically blocked by an anti-CD11b mAb in J774A.1 cells. (A) Effect of specific mAbs on intracellular cAMP accumulation. J774A.1 cells were preincubated at 4°C for 1 h with or without 10 µg/ml of specific mAbs (anti-CD11b, M1/70 or anti-CD18, C17/16) and then incubated at 37°C for 20 min with 5 µg/ml CyaA and with 10 µg/ml mAbs if present during the preincubation. Intracellular cAMP contents were determined as described in Materials and Methods. (B) Effect of specific mAbs on CyaA-mediated cell death. J774A.1 cells were preincubated at 4°C for 1 h with medium alone or with 10 µg/ml of specific mAbs (anti-CD11a 2D7, anti-CD11b M1/70, anti-CD11c HL3, anti-CD18 C71/16, control 2.4G2). Then, they were incubated at 37°C for 2 h with 0.5 µg/ml CyaA and with 10 µg/ml of specific mAbs when added during the preincubation. Cell lysis was determined by LDH release using the Cytotox 96™ assay.

B shows that anti-CD11b mAb dramatically reduced the cell lysis induced by CyaA in J774A.1 (88% inhibition). The cell death induced by CyaA was unaffected by preincubation with mAbs that did not block toxin binding to cells (anti-CD11a, -CD11c, -CD18, or a control mAb).

Collectively, these data indicate that CyaA binding through CD11b is strictly required for CyaA-mediated toxicity in J774A.1 cells.

**Transfection of CHO Cells with CD11b/CD18 Confers Sensitivity to CyaA.** To confirm the role of CD11b in CyaA binding, we used CHO cells transfected with the human integrins CD11b/CD18, CD11c/CD18, or mock transfected (vector alone). As shown in Fig. 5 A, CyaA bound at 37°C to all of these three cell lines. However, CyaA binding was efficient and saturable in CHO cells expressing CD11b/CD18 but not in CD11c/CD18 or mock-transfected cells. The affinity of CyaA for CD11b/CD18 transfected cells was in the nanomolar range ( $K_d = 0.7 \pm 0.09$  nM). At 4°C, the efficiency of CyaA binding was reduced compared with the binding at 37°C (Fig. 5 B). At this temperature, the difference in CyaA-binding capability between CD11b/CD18 transfected cells and the two other cell lines was even more pronounced.

Since we found that CD11b was required for CyaA-mediated toxicity in J774A.1, we wondered if CD11b ex-



**Figure 5.** CHO cells bind CyaA and become sensitive to CyaA when transfected with CD11b, but not with CD11c. (A and B) CyaA binding at the surface of CHO transfectants. CHO cells transfected with human CD11b/CD18, human CD11c/CD18, or mock-transfected were incubated with various doses of CyaA for 20 min at 37°C (A) or 4°C (B). Surface-bound CyaA was detected with biotinylated anti-CyaA polyclonal Abs, revealed by streptavidin-PE, and detected by flow cytometry on living cells. Binding is expressed as  $\Delta$ MFI = (MFI value of cells incubated with CyaA) - (MFI of cells without CyaA). (C) Intracellular cAMP accumulation in CHO transfectants. CHO cells transfected with human CD11b/CD18, human CD11c/CD18, or mock-transfected were incubated with or without CyaA for 20 min at 37°C. Intracellular cAMP contents were determined as described in Materials and Methods. (D) Cell lysis in CHO transfectants. CHO cells transfected with human CD11b/CD18, human CD11c/CD18, or mock-transfected were incubated with 5 µg/ml CyaA for 4 h at 37°C. Cell lysis was determined by LDH release using the Cytotox 96™ assay.

pression was sufficient to confer a CyaA-sensitive phenotype to transfected CHO cells. In line with previous reports (42), CyaA induced a significant increase in intracellular cAMP in CHO cells transfected with CD11c/CD18 or in control mock-transfected cells, but only at high concentrations of toxin (5 µg/ml; Fig. 5 C). In contrast, CyaA increased the intracellular level of cAMP in CD11b/CD18 transfected CHO cells even at the lowest concentration studied (0.05 µg/ml). Moreover, the cAMP production in response to 5 µg/ml CyaA was four to five times more elevated in CD11b/CD18 transfected cells compared with CD11c/CD18 or mock-transfected cells.

We also evaluated the role of CD11b/CD18 in CyaA-mediated cell death. As shown in Fig. 5 D, >50% of CHO transfected with CD11b/CD18 were killed after a 4-h incubation with 5 µg/ml of CyaA, whereas CD11c/CD18 or mock-transfected cells were not affected by this treatment.

Altogether, these results clearly established that expression of human CD11b/CD18 integrin is sufficient for efficient binding of CyaA and subsequent toxicity in CHO cells.

**Role of Divalent Cations in CyaA Binding to Cells.** Since the interaction of integrins with their various ligands is known to be promoted by divalent cations (43), we tested the binding of CyaA to CD11b<sup>+</sup> cells in the presence of either Mg<sup>2+</sup> alone, Ca<sup>2+</sup> alone, or both Ca<sup>2+</sup> and Mg<sup>2+</sup> (Fig. 6 A). Our results clearly demonstrate that Ca<sup>2+</sup> is strictly required for CyaA binding to murine D1 and J774 cells and to human neutrophils. Strikingly, unlike other CD11b ligands, CyaA binding does not require Mg<sup>2+</sup>. In agreement with this observation, divalent cation depletion (in the presence of EDTA) or selective Ca<sup>2+</sup> depletion (in the presence of EGTA) abrogated CyaA binding in the DMEM medium containing both Ca<sup>2+</sup> and Mg<sup>2+</sup> (Fig. 6 B). Thus, Ca<sup>2+</sup> is necessary and sufficient to allow the interaction of CyaA with CD11b<sup>+</sup> cells.

## Discussion

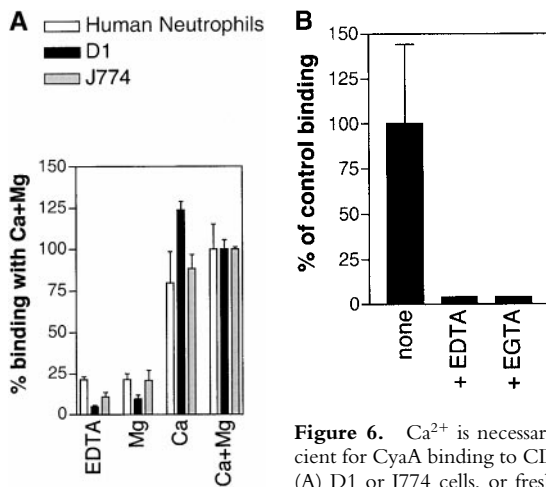
Unlike other toxins, CyaA has been considered for a long time as independent of any receptor binding. This was based on the observations that CyaA can intoxicate a wide variety of cell lines from various origins (14) and binds to Jurkat cells and sheep erythrocytes in a nonsaturable fashion (44). These observations established that nonspecific adsorption of CyaA to lipid membranes leads to some translocation of the catalytic domain into the cytosol. However, they did not rule out the existence of a specific receptor. Indeed, *in vivo* studies showed that during murine respira-

tory infection with *Bordetella* species, CyaA specifically destroyed leukocytes (especially macrophages) without dramatically damaging epithelial cells (6, 31). In this study, we hypothesized that this specificity might be linked to the recognition of a receptor specifically expressed by certain leukocytes. We showed, using myeloid cell lines, that the binding and the toxic properties of CyaA are dependent on its interaction with the integrin CD11b/CD18. Efficient and saturable binding of CyaA correlates with the expression of CD11b and is fully and specifically blocked by anti-CD11b mAbs. Moreover, expression of CD11b/CD18 in CHO cells dramatically enhances the binding of CyaA and its cytotoxicity. Our results are the first evidence supporting the interaction of CyaA with a cell-surface molecule specifically expressed on leukocytes. The nearly complete inhibition of CyaA binding by anti-CD11b mAbs and anti-CD18 for human cells suggests that CD11b/CD18 is the main receptor for CyaA in the cell lines tested. The lack of efficient binding to CD11c/CD18 transfectants, or CD11a/CD18 expressing cells such as EL4 or LB27.4, also suggests that CD11b/CD18 is the only integrin of the  $\beta 2$  family involved in the binding of CyaA to the target cells. However, the weak binding of CyaA to LB27.4 cells may suggest an ancillary role for CD11d (45) or another surface ligand.

This study clearly shows that the interaction of CyaA with CD11b<sup>+</sup> cells is strictly dependent on Ca<sup>2+</sup>, as could be expected since CyaA is a calcium-binding protein (18). Ca<sup>2+</sup> ions bind to the RTX repeat motifs with a weak affinity and trigger a conformational change that allows the toxin to bind to target cell membranes and to translocate its catalytic domain into the cytosol (13, 18, 22). More surprisingly, our study also established that the binding of CyaA to CD11b<sup>+</sup> cells is independent of Mg<sup>2+</sup>. To our knowledge, most known ligands (fibrinogen, iC3b, intercellular adhesion molecule 1) of CD11b/CD18 bind to the I domain of CD11b in a Mg<sup>2+</sup> (or Mn<sup>2+</sup>) dependent fashion (43). Structural and functional analyses have shown that the integrin I domain contains a Mg<sup>2+</sup>/Mn<sup>2+</sup> coordination site at its surface (MIDAS) that is critical for ligand binding (46–48). Therefore, CyaA might be the first example of a novel class of Mg<sup>2+</sup> independent CD11b ligand. These results also suggest that CyaA binding to CD11b/CD18 might occur independently of the CD11b I domain. Indeed, regions outside the I domain have been shown to contribute partially to the binding of some proteinaceous ligands like factor X (49). Moreover, CD11b/CD18 displays other binding sites like the well-characterized lectin-like binding site involved in the binding of  $\beta$ -glucans (50) and possibly in the lateral association of CD11b/CD18 with Fc $\gamma$ RIII (51).

Experiments are currently underway to map the regions of interaction between CyaA and CD11b/CD18. At present, we can only exclude the possibility of CyaA binding via an arginine-glycine-aspartate motif since CyaA does not contain such a sequence and an arginine-glycine-aspartate peptide does not inhibit CyaA binding to CD11b<sup>+</sup> cells (data not shown).

In line with previous studies (for a review, see reference 14), we observed a detectable binding of CyaA to all cell



**Figure 6.** Ca<sup>2+</sup> is necessary and sufficient for CyaA binding to CD11b<sup>+</sup> cells.

(A) D1 or J774 cells, or freshly purified neutrophils were washed twice with Tris-buffered saline and then incubated with Tris-buffered saline supplemented with either 5 mM EDTA (EDTA), 2 mM Mg<sup>2+</sup> and 100  $\mu$ M EGTA (Mg), 2 mM Ca<sup>2+</sup> (Ca), or 2 mM Ca<sup>2+</sup> and 2 mM Mg<sup>2+</sup> (Ca + Mg). After the addition of 10  $\mu$ g/ml biotinylated detoxified CyaA, all samples were incubated for 20 min at 4°C. Bound CyaA was detected by streptavidin-PE as described above, and results are expressed as a percentage of CyaA binding in the presence of 2 mM Ca<sup>2+</sup> and 2 mM Mg<sup>2+</sup>. (B) D1 cells were incubated in the presence of DMEM alone, DMEM and 5 mM EDTA, or DMEM and 5 mM EGTA, and stained with biotinylated detoxified CyaA as above.

lines tested. Furthermore, at high concentrations CyaA triggered a small but detectable cAMP increase in mock-transfected CHO cells that is not associated with cell lysis. Thus, although at high concentration CyaA can bind and enter into a wide variety of cell lines, at low concentrations only CD11b-expressing cells will be intoxicated by CyaA.

Previous studies, performed mainly on erythrocytes (devoid of CD11b) led to a two-step model for translocation of CyaA across membranes (22). First CyaA binds to the membrane via its COOH-terminal moiety without any receptor involvement, then the NH<sub>2</sub>-terminal catalytic domain translocates across the plasma membrane to the cytosol (22), coincidentally with a conformational change triggered by Ca<sup>2+</sup> binding to the RTX repeats located in the COOH-terminal domain of CyaA (18). The interaction between CyaA and CD11b/CD18 could favor the membrane insertion of CyaA, or, alternatively, CyaA may bind first to lipid membrane, and then CD11b may participate in stabilizing its interaction with membranes, allowing a more efficient intoxication.

The ability to translocate directly across the plasma membrane is a peculiarity of CyaA, which is considered to be the only toxin known to reach the cytoplasm without going through vesicular trafficking. Indeed, after binding to a specific receptor at the cell surface, other toxins acting on cytosolic targets are taken up together with their receptor by endocytic pathways. Some toxins like the *Bacillus anthracis* lethal factor or the diphtheria toxin translocate into the cytosol from acidic vesicles of the endocytic pathway. Others like the Shiga toxin, the cholera toxin, or the *Pseudomonas* exotoxin A are routed via a retrograde endocytic pathway to the endoplasmic reticulum from which they reach the cytosol (52). Unlike CD11a/CD18, which does not undergo endocytosis efficiently and may retain RTX toxins at the membrane of the cell, CD11b/CD18 can be internalized by endocytosis (53, 54). This calls into question the physiological relevance of the current model of direct translocation of CyaA from the plasma membrane to the cytosol. Current experiments are in progress to determine whether CyaA is taken up by endocytosis together with CD11b and to characterize the precise location of the membrane translocation events.

Binding of CyaA to a member of the  $\beta$ 2 integrin family is reminiscent of the behavior of other RTX toxins which were recently found to interact with these molecules (32–34). The *E. coli* HlyA, which shares a strong homology with CyaA, forms cationic pores at the plasma membrane. HlyA exhibits a specificity for leukocytes but only at a low concentration (55). This relative specificity was shown to be mediated by its interaction with the integrin CD11a/CD18 (32). Besides that, *Actinobacillus actinomycetemcomitans* and *Pasteurella haemolytica* leukotoxins (LtxA and LktA, respectively) are RTX toxins specific for human and bovine leukocytes, respectively, but also interact with CD11a/CD18 (32–34). Despite its strong homology with HlyA, CyaA recognizes another  $\beta$ <sub>2</sub> integrin (CD11b/CD18) whose cellular distribution is more restricted. Indeed, CD11b is expressed mostly on macrophages, neutrophils,

and dendritic cells, but not on the majority of T and B cells, whereas CD11a is expressed on all leukocytes including T and B lymphocytes.

The restricted specificity is in agreement with the effect of CyaA in the early phases of infection on bactericidal phagocytes such as macrophages (6, 29) and neutrophils (31). Such specific targeting of CyaA toxicity to CD11b-expressing cells may represent an escape mechanism against the early steps of the innate immune response. Indeed, this could allow the specific elimination of these cells (6, 29, 30) and/or eventually the inhibition of their oxidative microbicidal functions in the case of sublethal intoxication (13, 25, 26). The killing of lung dendritic cells, which are known to express CD11b, may also have indirect effects on the development of protective adaptive responses. Finally, the specificity of CyaA for CD11b<sup>+</sup> cells reinforces the hypothesis that the effect of CyaA on immune effector cells participates in the inhibition of antibacterial responses to bystander pathogens during *B. pertussis* infection as originally proposed by Confer and Eaton (13).

The filamentous hemagglutinin (FHA) of *B. pertussis* has also been shown previously to interact with CD11b/CD18 (56) leading to the internalization of a subset of bacteria during the course of infection (57). Moreover, FHA also interacts with a complex containing the leukocyte response integrin and the integrin-associated protein CD47 that is known to upregulate the expression of CD11b/CD18 (58). It is striking that such different factors, both in terms of structure and function, target the same molecule on leukocytes. One could imagine that they may cooperate to efficiently deplete the CD11b/CD18-expressing leukocytes. FHA may, for example, act first to bind to cells and facilitate the interaction of CyaA with this receptor. Such a hypothesis was suggested previously in a study showing that both CyaA and FHA are necessary to inhibit monocyte function (59). Therefore, the interplay between FHA and CyaA remains to be determined.

This selective binding of CyaA to CD11b-expressing cells might be exploited to specifically target this particular subset of cells. Detoxified mutants of CyaA might be used to deliver pharmacologically active molecules to CD11b<sup>+</sup> cells, without noticeably affecting other cell types. In fact, we recently developed such an approach by genetic insertion into detoxified CyaA of viral (60) or tumoral peptides (61). Once delivered into the cytosol of APCs and after proteasome-dependent intracellular processing, the inserted epitopes are presented by MHC class I molecules to CD8<sup>+</sup> T cells (62) and in vivo, stimulate protective CTL responses against viral challenge (60) or tumor graft (61). The present results highlight the strong potential of delivery vectors based on such CyaA detoxified mutants.

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