CTA1-DD adjuvant promotes strong immunity against human immunodeficiency virus type 1 envelope glycoproteins following mucosal immunization

Christopher Sundling,1,2 Karin Schön,3 Andreas Mörner,1 Mattias N. E. Forsell,2,4 Richard T. Wyatt,4 Rigmor Thorstensson,1 Gunilla B. Karlsson Hedestam1,2 and Nils Y. Lycke3

1Swedish Institute for Infectious Disease Control, 171 82 Solna, Sweden
2Department of Microbiology, Tumor and Cell Biology, Karolinska Institutet, 171 77 Stockholm, Sweden
3Mucosal Immunobiology & Vaccine Center (MIVAC), Department of Microbiology & Immunology, Institute of Biomedicine, University of Gothenburg, Gothenburg, Sweden
4Vaccine Research Center, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892, USA

Strategies to induce potent and broad antibody responses against the human immunodeficiency virus type 1 (HIV-1) envelope glycoproteins (Env) at both systemic and mucosal sites represent a central goal for HIV-1 vaccine development. Here, we show that the non-toxic CTA1-DD adjuvant promoted mucosal and systemic humoral and cell-mediated immune responses following intranasal (i.n.) immunizations with trimeric or monomeric forms of HIV-1 Env in mice and in non-human primates. Env-specific IgG subclasses in the serum of immunized mice reflected a balanced Th1/Th2 type of response. Strikingly, i.n. immunizations with Env and the CTA1-DD adjuvant induced substantial levels of mucosal anti-Env IgA in bronchial alveolar lavage and also detectable levels in vaginal secretions. By contrast, parenteral immunizations of Env formulated in Ribi did not stimulate mucosal IgA responses, while the two adjuvants induced a similar distribution of Env-specific IgG subclasses in serum. A single parenteral boost with Env in Ribi adjuvant into mice previously primed i.n. with Env and CTA1-DD, augmented the serum anti-Env IgG levels to similar magnitudes as those observed after three intraperitoneal immunizations with Env in Ribi. The augmenting potency of CTA1-DD was similar to that of LTK63 or CpG oligodeoxynucleotides (ODN). However, in contrast to CpG ODN, the effect of CTA1-DD and LTK63 appeared to be independent of MyD88 and toll-like receptor signalling. This is the first demonstration that CTA1-DD augments specific immune responses also in non-human primates, suggesting that this adjuvant could be explored further as a clinically safe mucosal vaccine adjuvant for humoral and cell-mediated immunity against HIV-1 Env.

INTRODUCTION

Because the majority of natural human immunodeficiency virus type 1 (HIV-1) transmissions occur via mucosal membranes an effective prophylactic vaccine should stimulate immune responses that are active at the portal site for viral entry (Gupta & Klasse, 2006; Mestecky et al., 2005). Vaccine-induced neutralizing antibodies (NAbs) that interfere with viral entry have been found to be the protective correlates of many other prophylactic antiviral vaccines (Parren et al., 2001; Wicker et al., 2007; Zignol et al., 2004). An HIV-1 vaccine that prevents or limits virus replication in the mucosa should reduce the risk of systemic viral dissemination (Haase, 2005; Maher et al., 2005; Mazanec et al., 1992) and may reduce the risk of human-to-human transmission (Kozlowski & Neutra, 2003; Mascola, 2003; Neutra & Kozlowski, 2006). However, the development of mucosal vaccines has been slow, owing mainly to the lack of safe and effective mucosal adjuvants. Furthermore, there is insufficient knowledge as to the best route for immunization of the genital tract: i.n., local vaginal, rectal or parenteral. Also, combinations of
regimens, such as mucosal priming followed by systemic boost, or, systemic priming followed by mucosal boost, have not been exhaustively investigated (Vajdy et al., 2004). Finally, an overriding problem to the development of a protective vaccine against HIV-1 is the design of an envelope glycoprotein (Env) immunogen that is capable of inducing broadly NAb (Burton et al., 2004, 2005; Karlsson Hedestam et al., 2008).

The HIV-1 Env glycoproteins consist of trimers of non-covalently associated gp120–gp41 heterodimers, critical for viral entry, and the sole targets for NAbS. Over the past decade, it has become apparent that unmodified monomeric Env variants (gp120) fail to elicit NAbS (Albrecht, 2003; Pitisuttithum et al., 2006). Therefore, in attempts to better mimic the native viral spike, soluble gp140 trimers containing full-length gp120 covalently linked to the gp41 ectodomain were designed (Binley et al., 2000; Kim et al., 2005; Sanders et al., 2002; Srivastava et al., 2003; Yang et al., 2000, 2002). Subsequent immunogenicity studies suggested that there was an incremental advance in elicitation of NAbS using soluble trimers as opposed to monomeric gp120 (Li et al., 2006; Yang et al., 2001). Other oligomeric Env gp140 proteins, with or without a deletion of the second major variable region of gp120 (AV2), have also been evaluated for their capacity to stimulate NAbS (Barnett et al., 2001; Derby et al., 2006; Xu et al., 2006a, b). Barnett et al. (2008) recently demonstrated that combining mucosal and parenteral immunizations in non-human primates a gp140 molecule, lacking the V2 region, mediated protection against mucosal challenge with the homologous simian-human immunodeficiency virus (SHIV).

Mucosal delivery of vaccines in general has been hampered by the lack of effective and non-toxic adjuvants that function at mucosal sites and which promote both strong local mucosal IgA and serum IgG antigen-specific antibodies. Apart from the bacterial holotoxins, cholera toxin (CT) and Escherichia coli heat-labile toxin (LT), few substances have proven effective mucosal adjuvants in experimental animal models (Lavelle et al., 2004; Levine & Sztein, 2004; Lycke, 2007). These molecules are AB5 complexes with an ADP-riboosylating A1-enzyme linked to a pentamer of B-subunits, which bind to the GM1-ganglioside, present on all nucleated cells (Hol et al., 1995). Because of the toxicity of these molecules and the reported side effects after i.n. administration, most notably Bell’s palsy, it is unlikely that these holotoxins would be deemed safe enough for clinical use (Mutsch et al., 2004).

In fact, all holotoxins and their mutants with binding to the ganglioside receptors, carry a similar risk of negatively affecting the central nervous system following i.n. administration. Therefore, we have developed a non-toxic adjuvant based on the CTA1-enzymic activity, but without the promiscuous cholera B-subunit (CTB) binding to the ganglioside receptors (Agren et al., 1997). The CTA1-DD molecule has been extensively evaluated for its mucosal adjuvant activity in mice (Agren et al., 1997; Choi et al., 2002; De Filette et al., 2006; Eriksson et al., 2004). Importantly, we have previously demonstrated that i.n. administration of the CTA1-DD adjuvant does not affect nervous tissues and efficiently stimulates both systemic and mucosal antibody, and CD4 + and CD8 + T-cell responses to levels comparable to those seen with intact CT holotoxin (Eriksson et al., 2004; Simmons et al., 1999).

To date, the CTA1-DD adjuvant has proven highly effective for experimental i.n. immunizations against several infectious disease agents, including those from rotavirus, influenza virus, Helicobacter pylori and Mycobacterium tuberculosis (Akhani et al., 2006; Andersen et al., 2007; Eliasson et al., 2008; McNeal et al., 2007). In the present study, we asked if CTA1-DD would augment HIV-1 Env-specific immune responses in both mice and non-human primates and whether antibody responses at mucosal sites were elicited. We also asked if the adjuvant effect of CTA1-DD was dependent on the MyD88 signalling pathway for toll-like receptor (TLR)-stimulation and we compared the adjuvant effect of CTA1-DD with those of three other adjuvants: Ribi, LT63 and CpG oligodeoxynucleotides (ODN).

**METHODS**

**Animals.** BALB/c (H-2b) and C57BL/6 (H-2a) mice were obtained from B&K Universal AB Sollentuna, Sweden, or from Harlan Olac, whereas MyD88−/− mice on a B6-background (Adachi et al., 1998) were bred at the Experimental Biology (EBM) unit at the University of Goteborg, Sweden. All mice were sex and age matched and kept under specific-pathogen-free conditions at the EBM or at the animal facility at the Department of Microbiology, Tumor and Cell Biology at Karolinska Institutet, Sweden. Seven female cynomolgus macaques (Macaca fascicularis) of Chinese origin, 5–6 years old, were housed in the Astrid Fagraeus laboratory at the Swedish Institute for Infectious Disease Control, Stockholm, Sweden. Housing and care procedures were in compliance with the provisions and general guidelines of the Swedish Animal Welfare Agency and all procedures were approved by the Local Ethical Committee on Animal Experiments. The animals were housed in pairs in 4 m² approved cages. They were habituated to the housing conditions for more than 6 weeks before the start of the experiment, and subjected to positive reinforcement training in order to reduce the stress associated with experimental procedures.

**Expression and purification of Env immunogens.** The gp140-F trimers and gp120 monomers were produced as described previously (Grundner et al., 2004; Yang et al., 2002). Briefly, transient transfection of gp140-F plasmids into adherent 293F cells (Invitrogen), cultured in Dulbecco’s modified Eagles medium (DMEM)/10% heat-inactivated fetal bovine serum/0.1 mM MEM non-essential amino acid solution (Invitrogen) medium, was achieved using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Every other day serum-free 293 SFM II medium (Invitrogen) was replaced in the cultures as supernatants were collected daily until day 6 post-transfection. A three-step procedure was used to purify the His6-tag-containing trimers, including lentil-lectin affinity chromatography (GE Healthcare), eluted with PBS/1 M β-mannopyranoside/0.5 M NaCl/10 mM imidazole, and captured via the His-tag by nickel-chelation chromatography (GE Healthcare) and eluted with a 300 mM imidazole-containing PBS buffer, followed...
by separation of lower molecular mass proteins by gel filtration chromatography. The gp120 monomers were produced in Freestyle 293F suspension cells by using 293Fectin transfection reagent in Opti-MEM according to the manufacturer’s instructions (Invitrogen). Supernatants from 4 days were centrifuged at 3500 g to remove cells or cell debris, filtered through a 0.22 μm filter and supplemented with Complete, EDTA-free protease inhibitor cocktail (Roche) and penicillin–streptomycin (Invitrogen) for storage at 4 °C until further purification. The gp120 monomers were purified by capturing the proteins in an IgG17b affinity column. All proteins were spun concentrated with Amicon Ultra 30 000 MWCO centrifugal Filter Devices (Millipore) to a concentration between 1 and 3 mg ml⁻¹.

**Immunizations.** Mice were immunized i.n. or intraperitoneally (i.p.) using different regimens of adjuvants and monomeric or trimeric HIV-1 Env. In brief, immunizations were performed using 10 μg Env together with 5 μg CTA1-DD with 10 day intervals between three, four or five i.n. inoculations or admixed with Ribi (MPL + TDM + CWS Adjuvant System, M6661; Sigma-Aldrich) for three i.p. injections. Additionally, two immunizations with Env and CTA1-DD i.n. were followed by a boost with Env and Ribi i.p. When indicated mice were given 5 μg per dose of LTK63 (kind gift from Novartis) or CpG ODN (CyberGene AB) with 10 μg Env i.n.; control animals were immunized three times with PBS or three, four or five times with CTA1-DD alone or Env alone. Immunizations (i.n.) were given in 10 μl PBS in each nostril and i.p. injections in 100 μl PBS or 0.9% saline (for Ribi). Serum was taken 10 days after each immunization, and spleens, bronchial alveolar lavage (BAL) and vaginal secretions were taken 10 days after the last immunization when sacrificing the mice. Monkeys were inoculated three times intramuscularly (i.m.) with 200 μg Env and Ribi in a total volume of 1 ml, divided between the left and right hind leg or i.m. with 200 μg Env and 50 μg CTA1-DD in PBS in a total volume of 100 μl divided between the nostrils. Blood samples were taken before and 2 weeks after each immunization. All immunizations and blood sampling were performed under sedation with ketamine 10 mg kg⁻¹ i.m. (Ketaminol 100 mg ml⁻¹; Intervet). The macaques were weighed and examined for swelling of lymph nodes and spleen at each immunization or sampling occasion.

**Antibody determinations.** Determinations of antibody levels in mouse serum, BAL and vaginal secretions were done by ELISA as described previously (Eliasson et al., 2008; Forsell et al., 2007; Marks et al., 2007). Briefly, ELISA plates (Nunc) were coated with 1 μg insect cell (S2)-produced YU2gp120 ml⁻¹ in 100 μl PBS at 4 °C overnight. The plates were blocked by incubation with PBS + 2 % non-fat dried milk and 5 % fetal calf serum (FCS) (Sigma-Aldrich) at room temperature and then washed three times in PBS + 0.2 % Tween 20 (Sigma-Aldrich). Serum was added in serial dilutions and incubated at room temperature before washing and incubating with goat anti-mouse total IgG-horseradish peroxidase (HRP) or IgG subclass-specific (Southern Biotech) antibody at dilution 1/5000 or goat anti-mouse IgA-HRP (Southern Biotech) at 1/500 dilution. After washing, the plates were developed using the OPD Fast kit (Sigma-Aldrich) and the optical density read at 450 nm using a Multiscan EX (MTX Lab systems). For determination of specific anti-Env responses in monkeys, Maxisorp microtitre plates (Nunc) were coated with 1 μg S2-produced gp120 ml⁻¹ in 50 mM carbonate buffer (pH 9.6), overnight at 4 °C. The serum samples were serially diluted in PBS with 20 % FCS and 0.5 % Tween 20, and added to the plates after blocking with 20 % FCS in PBS. For measurement of IgA responses, the sera were first depleted of IgG using Protein A–Sepharose CL-4B (Amersham Pharmacia Biotech). After overnight incubation at 4 °C, bound IgG or IgA was detected with HRP-conjugated anti-human IgG (BD214; DakoCytomation) or anti-monkey IgA (GAMon/IgA(Fc)/PO; Nordic Immunological Laboratories), respectively, followed by the addition of O-phenylenediamine with H₂O₂ (DakoCytomation). The plates were washed six times with PBS supplemented with 0.5 M NaCl and 0.1 % Tween 20 between all steps. Substrate reactions were stopped with 2 M H₂SO₄ and optical density read at 492 and 620 nm. In mice, log₁₀ titres were calculated as described previously (Eliasson et al., 2008) and in monkeys geometric means of end-point titres, defined as the last serum dilution giving an optical density value 2 SD above background optical density for pre-immune sera, was calculated.

**Cellular responses.** One week after the last immunization, spleens were removed and single-cell suspensions were prepared and resuspended at 2 × 10⁶ cells ml⁻¹ in Hanks’ buffered saline solution (Gibco). The cells were cultured in triplicates in 96-well microtitre plates (Nunc) in Iscove’s medium (Biochrom KG), supplemented with 10 % heat-inactivated FCS (Biochrom), 50 μM 2-mercaptoethanol (Sigma-Aldrich), 1 mM l-glutamine (Biochrom) and 50 μg gentamicin (Sigma-Aldrich) ml⁻¹ and cultured for 72 h at 37 °C in 5 % CO₂ either alone or with 1 μM gp120 Env protein (KJ Ross-Petersen AB) or 10 μg tetanus toxoid (TT; Statens Serum Institut) ml⁻¹. Specific cell proliferation to recall antigen or TT was assessed after 72 h of culture, by the addition of 1 μCi (37 kBq) per well [³H]thymidine (Amersham International) for the last 6 h of culture. The [³H]thymidine uptake was determined by a scintillation counter (Beckman). Data were expressed as mean c.p.m. ± SD of five mice per group in each experiment. Cytokine containing supernatants were collected after 96 h of cell culturing and stored at −70 °C until analysed. Briefly, gamma interferon (IFN-γ), interleukin (IL)-4, IL-5 and IL-10 levels in supernatants were assessed by ELISA. Microtitre plates (Dynatech Laboratories) were incubated with 2.5 μg rat anti-mouse IFN-γ (BD Biosciences) ml⁻¹ or 1–5 μg anti-mouse IL-4 (Endogen), IL-5 or IL-10 (R&D System) ml⁻¹. The sample supernatants or recombinant mouse IFN-γ, IL-4, IL-5 or IL-10 (R&D System) standards were then added to the appropriate wells. Bound cytokines were detected by sequential incubations with a polyclonal rabbit anti-IFN-γ antisera, or biotinylated mAb to mouse IL-4 (Endogen), IL-5 or IL-10 (R&D System) as described previously (Akhiian et al., 2002). The sensitivity of detection for the respective cytokine was 10 pg IFN-γ ml⁻¹ and 5 pg IL-4, IL-5 or IL-10 ml⁻¹.

**Statistical analysis.** Wilcoxon rank sum test was used for independent samples for analysis of significance in all experimental groups, except for the IFN-γ values, which were compared by the one-tailed Student’s t-test.

**RESULTS**

**CTA1-DD is a highly effective mucosal adjuvant for stimulating anti-HIV-1 Env antibodies**

Mice were immunized i.n. or i.p. with CTA1-DD adjuvant plus HIV-1 Env gp140 trimers or monomeric gp120 proteins. For comparison, we used the Ribi adjuvant i.p. together with the HIV-1 Env proteins. A summary of the immunization regimens is shown in Table 1. Control mice were immunized i.n. with CTA1-DD alone or with Env alone. We also included a group of mice immunized twice with Env and CTA1-DD i.n. and then boosted once with Env and Ribi i.p. (Fig. 1a). We detected significant amounts of serum anti-Env IgG log₁₀ titres in the mice immunized i.n. with Env and CTA1-DD while, in contrast, inoculation of CTA1-DD alone or Env alone did not induce any detectable anti-Env antibodies. Moreover, an i.p. boost of Env and Ribi in mice previously primed i.n. with CTA1-DD and Env effectively increased serum anti-Env titres, achieving 10-fold
higher serum IgG titres than after three i.n. immunizations. This response was comparable to that obtained in mice immunized three times with Env and CTA1-DD i.p. or three times with Env and Ribi i.p. Strikingly, however, whereas CTA1-DD and Ribi were equally effective as parenteral adjuvants, only CTA1-DD given i.n. stimulated Env-specific mucosal IgA responses in the BAL (Fig. 1b). Thus, CTA1-DD was an effective mucosal adjuvant for stimulating HIV-1 anti-Env serum IgG and mucosal IgA antibodies following i.n. immunizations. Of note, since we found that irrespective of whether gp140 trimers or monomeric gp120 were used for immunizations similar results were obtained and, hence, monomeric gp120 was used in all subsequent experiments in mice.

**Multiple immunizations provide stronger mucosal IgA anti-HIV responses**

Next, we asked if especially the Env-specific mucosal IgA responses could be enhanced even further by multiple immunizations with CTA1-DD. We found that antibody responses following four or five repeated immunizations were greatly augmented compared with that after three i.n. immunizations with CTA1-DD and Env (Fig. 2a). Both serum IgG and BAL IgG responses were substantially enhanced by the fourth i.n. immunization with Env and CTA1-DD and serum IgG levels were further boosted by the fifth immunization (Fig. 2a, b). The serum IgG antibody titres were markedly higher than those observed after three immunizations and comparable to those obtained with Ribi adjuvant given three times i.p. (Figs 1 and 2). There were also detectable anti-Env IgG responses in the vaginal secretions of some animals after the fourth and the fifth

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<td>BALB/c (Fig. 1)</td>
<td>CTA1-DD - 3 x i.n.</td>
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<td>-</td>
<td>Env*</td>
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<td>BALB/c (Figs 2–4)</td>
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<td>Env†</td>
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<td>CTA1-DD - 3 x i.n.</td>
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<td>Env†</td>
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<td>Ribi - 3 x i.p.</td>
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<td>CTA1-DD - 3 x i.n.</td>
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<td>Cynomolgus macaques (Fig. 6)</td>
<td>Cynomolgus macaques - 3 x i.n.</td>
<td>Env†</td>
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<td></td>
<td>CTA1-DD/Ribi - 2 x i.n./1 x i.m.</td>
<td>Env‡</td>
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*Experiment performed with YU2gp120 monomers or YU2gp140 trimers of HIV-1 Env protein.
†Experiment performed with YU2gp120 monomers of HIV-1 Env protein.
‡Experiment performed with YU2gp140 trimers of HIV-1 protein.

Fig. 1. Five mice per group were immunized i.n. with HIV-1 Env and CTA1-DD or i.p. with Env and Ribi adjuvant as indicated. Env-specific serum antibodies and BAL are shown: (a) anti-Env IgG log<sub>10</sub> serum titres or (b) anti-Env IgA BAL log<sub>10</sub> titres. Immunizations (i.n.) with Env and CTA1-DD was the only protocol to stimulate anti-Env IgA in BAL (b). Parenteral immunization with Env plus CTA1-DD gave comparable serum anti-Env IgG log<sub>10</sub> titres to that of Env plus Ribi adjuvant immunized mice (a). There were no detectable anti-Env titres in either serum or BAL in mice inoculated with either Env protein or CTA1-DD alone. This is one representative experiment of three giving similar results. *, Indicates a statistical significance of P<0.05 compared with three i.n. inoculations of Env alone.
immunization (Fig. 2c, f). Most importantly, specific mucosal anti-Env IgA titres in BAL were substantially augmented by the fourth and fifth immunization with Env and CTA1-DD (Fig. 2e). There were no detectable Env-specific antibodies in serum, BAL or vaginal secretions after five inoculations of Env alone or CTA1-DD alone. Collectively, our data show that five i.n. immunizations with CTA1-DD adjuvant promoted the strongest HIV-1 anti-Env IgG and IgA antibody titres in both serum and mucosal secretions.

Distribution of serum anti-Env IgG-isotypes

The IgG-subclass distribution of specific antibodies may be used as a qualitative indicator of the immunomodulating effect of a vaccine adjuvant, in particular the induction of Th1 or Th2 dominance (Sin et al., 1999). Therefore, we analysed IgG1, IgG2a, IgG2b and IgG3 anti-Env responses in serum from i.n. (CTA1-DD and Env) and i.p. (Ribi and Env) immunized mice. We found that IgG1 serum antibodies dominated in both CTA1-DD and Ribi immunized mice (Fig. 3a). After three i.n. immunizations with Env and CTA1-DD only IgG1 anti-Env serum antibodies were detected. However, subsequent immunizations resulted in detectable IgG2a, IgG2b and IgG3 anti-Env titres (Fig. 3). Taken together both Th1- (IgG2a) and Th2-regulated (IgG1) IgG-subclasses were represented. Also, when comparing the i.n. and i.p. routes for the distribution of Env-specific IgG isotypes similar results were observed, indicating a balanced Th1 and Th2 activity in both the CTA1-DD and Ribi adjuvant systems.

Mucosal CTA1-DD adjuvant promotes priming of Env-specific T-cell responses

To characterize the capacity of CTA1-DD to augment Env-specific cellular responses, we analysed cell proliferative (Fig. 4a) and cytokine responses (Fig. 4b) in mice immunized three times i.n. with Env and CTA1-DD adjuvant or with Env alone. Splenocytes were prepared 8 days after the third

![Graphs showing immune responses](https://via.placeholder.com/150)

**Fig. 2.** Five mice per group were inoculated three, four or five times with HIV-1 Env and CTA1-DD i.n. as indicated and control mice were inoculated five times with either Env alone or CTA1-DD alone. Anti-Env IgG log$_{10}$ titres and anti-Env IgA log$_{10}$ titres in serum (a, d), BAL (b, e) or vaginal secretions (VAG) (c, f) are shown. Immunizations (i.n.) with CTA1-DD stimulated significantly enhanced mucosal IgA and IgG anti-Env antibody responses after four and five immunizations, respectively (b, e). HIV-1 Env or CTA1-DD given alone failed to stimulate anti-Env titres in either serum, BAL or VAG. This is one representative experiment of two giving similar results. *, Indicates a statistical significance of $P<0.05$ compared with three i.n. inoculations of Env alone.
immunization and cultured together with recall antigen in vitro. Cell proliferation in response to Env protein was significantly enhanced in mice immunized with Env and CTA1-DD adjuvant, while non-adjuvant Env-immunized mice failed to respond (Fig. 4). The cytokine response as measured in the culture supernatants was dominated by IFN-\(\gamma\) (Fig. 4), while IL-4 and IL-5 were not detected (data not shown). Weak IL-10 responses were also found in Env plus CTA1-DD immunized mice (data not shown). Cells cultured with an unrelated antigen, TT, did not show proliferation or IFN-\(\gamma\)-production above medium background, indicating that the response to HIV-1 Env was antigen-specific (Fig. 4a, b). These data demonstrated that the CTA1-DD adjuvant also significantly enhanced priming of HIV-1 Env-specific T-cell responses following i.n. immunizations.

**The CTA1-DD adjuvant is independent of MyD88 signalling**

Many adjuvants mediate their effect by activating the innate immune system by engaging one or several TLR pathways, whereas other adjuvants appear independent of TLR signalling (Gavin et al., 2006; Guy, 2007; Kwissa et al., 2007; Pasare & Medzhitov, 2005). We asked if the adjuvant effect of CTA1-DD was retained in mice lacking MyD88, an adaptor protein for signalling used by multiple TLRs (Barton & Medzhitov, 2002; McGettrick & O’Neill, 2007; Underhill & Ozinsky, 2002). Wild-type (WT) C57BL/6 and MyD88\(^{-/-}\) mice were immunized with Env plus CTA1-DD i.n. For comparison, mice were given i.n. immunizations with CpG ODN, an adjuvant known to depend on TLR9 and MyD88 signalling, and LTK63, a detoxified mutant of the LT holotoxin with well documented mucosal adjuvant functions (Tritto et al., 2007). Following three i.n. immunizations, we found that all three adjuvants were equally effective in stimulating Env-specific serum IgG antibodies in WT mice (Fig. 5). However, as predicted, the CpG ODN adjuvant was completely ineffective in MyD88-deficient mice, whereas both CTA1-DD and LTK63 were potent adjuvants also in MyD88\(^{-/-}\) mice (Fig. 5). Moreover, extended experiments in TLR4-deficient mice (signalling through the MyD88 and Trif-pathways) corroborated that CTA1-DD appears to exert its adjuvant effect in a TLR-independent fashion (N. Lycke, unpublished observation).

**CTA1-DD is an effective mucosal adjuvant in non-human primates**

Finally, we investigated to what extent our findings in mice would translate into non-human primates. To this end we performed a pilot immunization trial with Env gp140 trimers and the CTA1-DD adjuvant in cynomolgus macaques (M. fascicularis). Four monkeys were inoculated twice with Env and the CTA1-DD adjuvant i.n. These animals were then boosted by a third inoculation with Env and CTA1-DD i.n. (E85 and E86) or with Env and Ribi i.m. (E83 and E84), similar to the regimens used in mice. In addition, three monkeys were inoculated three times with Env and Ribi i.m. (E79, E80 and E81). We found that CTA1-DD adjuvant stimulated Env-specific serum IgG responses (Fig. 6a, c) as well as IgA responses (Fig. 6b, d).
following two i.n. inoculations. These responses were boosted by a third immunization, either with Env and CTA1-DD i.n. or with Env and Ribi i.m. Although, the monkeys inoculated three times i.m. with Env and Ribi exhibited stronger antibody responses, we conclude that i.n. immunizations with Env and CTA1-DD effectively primed Env-specific IgG and IgA antibody responses. Importantly, no side effects were observed in monkeys that received i.n. CTA1-DD. Thus, these first preliminary results in non-human primates support the fact that the CTA1-DD adjuvant is a safe and effective mucosal vaccine adjuvant.

**DISCUSSION**

In the present study we document the adjuvant effects of CTA1-DD for mucosal and systemic immune responses following i.n. immunizations using trimeric or monomeric forms of the HIV-1 Env proteins in both mice and non-human primates. The IgG-subclass response in serum reflected a balanced Th1/Th2 type of response. Substantial mucosal anti-Env IgA antibody levels in BAL and also some in vaginal secretions were observed, especially after repeated i.n. immunizations.

The CTA1-DD adjuvant was previously shown to be non-toxic. It does not drive inflammation at the site of application and, importantly, it does not bind GM1-ganglioside receptors or accumulate in the central nervous system following i.n. administration (Eriksson et al., 2004). The latter effect was, in fact, the reason for the recent withdrawal from the market of an LT-adjuvant i.n. influenza vaccine as vaccinees displayed an increased incidence of Bell’s palsy (Mutsch et al., 2004). Because CTA1-DD lacks the CTB domain, it cannot bind to the GM1-ganglioside receptors (Agren et al., 1997). Such a risk, though, is highly likely also with mutant derivatives of CT or LT, such as i.n. vaccines adjuvanted by LTR192R or the LTK63 mutant (Dickinson & Clements, 1995; Stephenson et al., 2006). Here, we compared side by side the adjuvant potency of CTA1-DD with that of LTK63 and found that the augmenting effects on specific serum IgG-responses were comparable. A similar result has previously been reported when comparing CTA1-DD with LTR192G for mucosal immunizations with rotavirus VP6 (McNeal et al., 2007). Given, the constraints of using GM1-binding holotoxins and their mutant derivatives as vaccine adjuvants in the clinic we propose that CTA1-DD may replace these mucosal adjuvants. This may rekindle interest in i.n. administration as a primary route for safe and effective mucosal immunization.

The relative roles of serum and mucosal IgG and IgA for providing protective responses against vaginal or rectal...
transmission of HIV-1 remains poorly understood. While recent years have provided a better understanding of the early events and different cell types employed by the HIV-1 virus to infect the genital tract (reviewed by Hladik & McElrath, 2008), questions still remain as to what is the role of strong vaccine-induced mucosal immunity for protection (Haynes & Shattock, 2008). A study by Barnett et al. (2008) showed that mucosal HIV-1 Env protein immunizations can stimulate protective immune responses against a homologous vaginal SHIV challenge. These data provided proof of principle and suggested that mucosal priming followed by parenteral booster immunization may be a promising regimen to stimulate protective immunity against HIV-1. The results presented in the current study showed that mucosal administration of HIV-1 Env and CTA1-DD adjuvant effectively primed anti-Env B-cell responses in both mice and in cynomolgus monkeys. Previous studies have reported on potentially important protective roles of specific mucosal IgG and IgA against HIV-1 (Bergmeier & Lehner, 2006; Mazzoli et al., 1997, Neutra & Kozlowski, 2006; Vajdy et al., 2004; Veazey et al., 2003). However, definitive evidence that Env-specific IgA exerts a protective role still awaits to be shown. Indeed, the need for better and more standardized methods to measure Env-specific antibodies in mucosal secretions was highlighted in a recent workshop on mucosal immunity and HIV/AIDS vaccines (Girard et al., 2008). There is a general agreement that the addition of better quantitative and qualitative methods for the detection of specific antibody-secreting cells in mucosal biopsies is much warranted (Montefiori et al., 2007; Shattock et al., 2008).

The CTA1-DD was recently shown to greatly augment protective immune responses against influenza A virus in a mouse model using the conserved M2e-epitope as the vaccine immunogen (Eliasson et al., 2008). This model largely reflects the protective efficacy of anti-M2e antibodies and, thus, indicates that CTA1-DD may be particularly suitable for further exploration as an adjuvant for modulating humoral immune responses (Lycke, 2007). Most adjuvants are derived from bacteria or viruses and are recognized by pattern recognition receptors (Barton & Medzhitov, 2002; Harris et al., 2006; McGettrick & O’Neill, 2007; Underhill & Ozinsky, 2002). In particular, the TLR-family of receptors are considered key elements in recognition of lipopolysaccharide (LPS), muramylpeptide, flagellin, CpG and many other molecules with adjuvant function (Harris et al., 2006). However, the bacterial holotoxins use ganglioside receptors to target cells and when administered as adjuvants they will bind also to antigen-presenting cells via these receptors (Grdic et al., 2005). Whether the holotoxins engage the TLR family of receptors for their adjuvant function in vivo has not been investigated previously. However, one study reported that the cytokine-inducing effect in macrophages–monocytes of a B-subunit of an E. coli type LT-IIb was TLR2-dependent, while this receptor was not required for cytokine production by the LT-IIb holotoxin (Liang et al., 2007). Thus, a possible role of gangliosides as co-receptors to TLR2 signalling was discussed by these authors, though the data does not support a TLR-involvement in the adjuvant effect of the LT-IIb holotoxin. We found that neither CTA1-DD nor LTK63 were dependent on the MyD88

Fig. 6. Three monkeys (E79, E80 and E81) were inoculated three times with Env and Ribi i.m. (3×Ribi), two monkeys (E83 and E84) were inoculated twice with HIV-1 Env and CTA1-DD i.n. followed by one inoculation with HIV-1 Env and Ribi i.m. (2×CTA1-DD, 1×Ribi) and two monkeys (E85 and E86) were inoculated three times with Env and CTA1-DD i.n. (3×CTA1-DD). Serum anti-Env IgG and IgA titres were assessed by ELISA and shown as geometric means or individual endpoint titres at intervals of 2 weeks after the first, second and third immunizations. (a) Geometric mean IgG titres over time. (b) Geometric mean IgA titres over time. (c) IgG end-point titres in individual animals after the third immunization. (d) IgA end-point titres in individual animals after the third immunization.
adaptor protein and subsequently did not use this pathway for exerting adjuvant effects in vivo. The MyD88−/− mice demonstrated comparable serum anti-Env IgG titres as seen in WT mice. By contrast, only WT, but not MyD88−/− mice, given the CpG ODN adjuvant developed specific anti-HIV-1 Env serum antibodies, consistent with previous findings that this adjuvant is dependent on TLR9 (Henmi et al., 2003). Moreover, the adjuvant effects of CTA1-DD and of intact CT holotoxin were also unablated in TLR4−/− mice as well as in double TLR4/MyD88-deficient mice (N. Lycke, unpublished observation). However, Ribi contains monophospholipid (MPL), a synthetic analogue of LPS that activates target cells via TLR4 and signals via both the MyD88 and Trif pathways (Baldridge et al., 2004; Vogel, 1995). As it was recently reported that the adjuvant effect of Ribi was intact in mice lacking both MyD88 and Trif, it follows that the adjuvant effect can be independent of TLR signalling, even though MPL is strictly TLR4-dependent in its other biological functions (Gavin et al., 2006). Therefore, in future experiments we will also address the TLR-independence of CTA1-DD in mice double deficient for MyD88 and Trif (Beutler, 2007).

This is the first evaluation of the adjuvant effect of CTA1-DD in non-human primates. Although, the study is quite preliminary, it indicates that CTA1-DD exerts adjuvant effects in non-human primates. Monkeys immunized two or three times with Env and CTA1-DD i.n. showed seroconversion of anti-Env IgG and IgA antibodies, albeit the responses were weaker than in animals immunized with Env and Ribi i.m. Given that mice showed no anti-Env IgG or IgA antibodies in serum when given HIV-1 Env alone and that five i.n. immunizations with Env plus CTA1-DD adjuvant significantly enhanced anti-Env antibody responses compared with three i.n. immunizations, future studies in non-human primates should use at least four subsequent HIV-1 Env plus CTA1-DD immunizations. These experiments will also address the issue of NAbS as we did not investigate this important property of anti-Env antibodies in the present study. Nonetheless, our results from the present study clearly demonstrated that CTA1-DD should be explored further as a promising mucosal vaccine adjuvant for humoral- and cell-mediated immunity against HIV-1 Env.

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