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The Combined CTA1-DD/ISCOM Adjuvant Vector Promotes Priming of Mucosal and Systemic Immunity to Incorporated Antigens by Specific Targeting of B Cells

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The cholera toxin A1 (CTA1-DD)/QuilA-containing, immune-stimulating complex (ISCOM) vector is a rationally designed mucosal adjuvant that greatly potentiates humoral and cellular immune responses. It was developed to incorporate the distinctive properties of either adjuvant alone in a combination that exerted additive enhancing effects on mucosal immune responses. In this study we demonstrate that CTA1-DD and an unrelated Ag can be incorporated together into the ISCOM, resulting in greatly augmented immunogenicity of the Ag. To demonstrate its relevance for protection against infectious diseases, we tested the vector incorporating PR8 Ag from the influenza virus. After intranasal immunization we found that the immunogenicity of the PR8 proteins were significantly augmented by a mechanism that was enzyme dependent, because the presence of the enzymatically inactive CTA1R7K-DD mutant largely failed to enhance the response over that seen with ISCOMs alone. The combined vector was a highly effective enhancer of a broad range of immune responses, including specific serum Abs and balanced Th1 and Th2 CD4+ T cell priming as well as a strong mucosal IgA response. Unlike unmodified ISCOMs, Ag incorporated into the combined vector could be presented by B cells in vitro and in vivo as well as by dendritic cells; it also accumulated in B cell follicles of draining lymph nodes when given s.c. and stimulated much enhanced germinal center reactions. Strikingly, the enhanced adjuvant activity of the combined vector was absent in B cell-deficient mice, supporting the idea that B cells are important for the adjuvant effects of the combined CTA1-DD/ISCOM vector. The Journal of Immunology, 2006, 176: 3697–3706.

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3 Abbreviations used in this paper: i.n, intranasal; BM, bone marrow; CLN, cervical lymph node; CT, cholera toxin; DC, dendritic cell; GC, germinal center; LT, E. coli heat-labile toxin; ISCOM, QuilA-containing immune-stimulating complex.
by electron microscopy, and the various components were analyzed for coniugation of protein and Quilajia saponins into fractions isolated from an analytical 10–50% (w/w) sucrose gradient after centrifugation (18 h at 200,000 × g, 10°C). The contents of protein and saponins in the different fractions were determined as described, using ELISA and spectrophotometric analysis (at A214 nm), respectively. The amino acid content in each preparation was assessed. In some experiments we labeled the ISCOMs with CFSE. Briefly, 2 μl of solution, freshly prepared from a stock solution (100 mg/ml in DMSO) of the CFSE molecule, was added to a solution of cholesterol as the ISCOMs were prepared by mixing the lipids. Endotoxin contaminations were determined in the ISCOM preparations using the Limulus amebocyte lysate test (LAL Endochome; Charles River Endostase). The endotoxin levels were <110 endotoxin units/mg protein in all ISCOM preparations, whereas CTA1-OVA-DD, CTA1R7K-OVA-DD, and CT had endotoxin levels < 50 endotoxin units/mg protein.

Quality assessment of ISCOMs

We routinely analyzed the protein content in all ISCOMs using specific ELISA. When sucrose-derived fractions were used, we incubated 50 μl of each fraction in the first row of a 96-well microtiter Maxisorp plate (Nunc) at a 1/3 dilution of 50 mM carbonate buffer (pH 9.6). Three-fold dilutions in subsequent subwells were performed, and the plates were incubated at 4°C overnight. After washing, each well plate was blocked for 1 h at room temperature with PBS-Tween 20 containing 2% fat-free dried milk powder. For detection of chicken anti-PR8, a 1:500 dilution of rabbit anti-chicken polyclonal antiserum (Jackson Immunoresearch) was added (importantly, chicken Abs do not bind protein A and, thus, do not bind DD), followed by HRP-conjugated avidin (DakoCytomation). For detection of the native or mutant CTA1-OVAP-DD, we used an HRP-conjugated rabbit anti-mouse antiserum (DakoCytomation). All incubations were performed with gentle agitation for 1 h at room temperature. Plates were washed three times in PBS-Tween 20 between all steps and before the incubation in tetramethylbenzidine substrate (Svanova), and the enzymatic reaction was read at A450 nm using a spectrophotometer.

Determination of immunogenicity

T cell-dependent immunity was assessed in cervical lymph nodes (CLNs) or spleens from immunized or control mice as previously described (38). Briefly, single-cell suspensions were prepared by passing the tissue through a nylon mesh. RBCs were lysed with a hypotonic ammonium chloride-Tris solution and washed in HBSS (Invitrogen Life Technologies). The cell suspensions were resuspended at a final concentration of 2 × 10^5 cells/ml and cultured in 200-μl aliquots in 96-well microtiter plates (Nunc) in Iscove’s medium (Biochrom) supplemented with 10% heat-inactivated FCS (Biochrom), 50 μM 2-ME (Sigma-Aldrich), 1 mM L-glutamine (Biochrom), and 50 μg/ml gentamicin (Sigma-Aldrich; Iscove’s complete medium) and cultured for 72 h at 37°C in 5% CO₂ either alone or with 1 μM p323 (KJ Ross-Petersen) or 1 μM p323 micellized in Triton-X100 (Promega). Proliferation was assessed after addition of 1 μCi/well [3H]thymidine (Amersham Biosciences) for the last 9 h of culturing. [3H]Thymidine uptake was determined using a beta scintillation counter (Beckman Coulter). After 96 h of culture, in vitro unstimulated and PR8 micell-restimulated cell supernatants were stored at −70°C until assayed. Cytokine responses to recall Ag in vitro were analyzed by multicytokine analysis (Luminex), which involved incubation with Ab-conjugated beads directed against mouse IL-5, IL-10, and IFN-γ (Bio-Rad) according to the manufacturer’s instructions. The assay was read on a Luminex 100 and analyzed using Bio-Plex Manager software; the concentrations of cytokines were determined against a panel of cytokine preparations of known concentrations.

Specific Ab responses in serum, genital tract secretions, or bronchial lavage were determined using ELISA as described previously (26). PR8-specific total IgG, IgG1, and IgG2a, and IgG1 concentrations in genital tract secretions were determined using polystyrene, 96-well microtiter plates (Nunc) coated with PR8 micelles (1 μg/ml). Total IgE in serum was assessed using rat anti-mouse IgE (Serotec)-coated, soft, 96-well ELISA plates (Dynatech Laboratories). After blocking with 0.1% BSA/PBS, serum samples were diluted 1/500 for specific responses or 1/20 for total IgE levels, followed by serial dilutions in 0.1% BSA/PBS in subsequent subwells. Bronchial lavage or vaginal secretions were diluted 1/20 serially diluted in CFSE (Molecular Probes) was added per million conjugated, isotype-specific, goat anti-mouse Abs at 1/500 (Southern Bio-technology Associates) or 0.125 μg/ml bixin-conjugated anti-mouse IgE (Serotec) were then added, followed by 2.1 μg/ml Extravidin peroxidase (Sigma-Aldrich). Nitrophenyl phosphate (1 mg/ml; Sigma-Aldrich) in ethanolinamine buffer (pH 9.8) or o-phenylenediamine substrates (1 mg/ml; 100 mM H₂O) in citrate-phosphate buffer (pH 4.5) containing 0.04% H₂O₂, were used, and enzymatic reactions were read in a Titer-Tek Multiscan spectrophotometer (Labsystems). IgE concentrations were calculated in micrograms per milliliter from a standard curve generated by serial dilutions of
purified IgE of known concentration (BD Pharmingen). PR8-specific log_{10} Ab titers (means ± SD) were defined as the interpolated reading giving rise to an absorbance of 0.4 above background, which consistently gave readings on the linear part of the curve.

In vivo distribution of ISCOMs

CFSE-labeled ISCOMs containing 5 μg of either CTA1-OVA-DD or OVA were injected s.c. into the footpad of BALB/c mice. Two, 4, 24, and 48 h later, the draining popliteal lymph nodes were harvested; they were either frozen for immunohistochemistry, or single-cell suspensions were prepared by forcing through a fine nylon mesh. The cells were washed in Iscove’s complete medium, followed by incubation for 5–15 min at 4°C with 1 μl of the 2.4G2-FcR-blocking Ab (BD Pharmingen) in cold PBS containing 0.1% BSA (BSA/PBS), and were added to cells aliquoted in 100 μl. The cells were labeled with 1/100 PE-conjugated anti-mouse CD19 or CD11c (BD Pharmingen) by incubation for 30 min at 4°C. After washing twice, the cells were analyzed on a FACScan flow cytometer (BD Biosciences) using CellQuest software. Cells were gated on the lymphocyte population with the gate set on either the CD19 or CD11c population, and 10,000 cells were collected, which were positive for CD19 or CD11c positive for CFSE (detected in the FL-1 channel).

Uptake of ISCOMs in vitro

Bone marrow (BM)-derived DCs or purified spleen B cells were incubated with CFSE-labeled CTA1-OVA-DD/ISCOMs or OVA/ISCOMs in RPMI 1640/10% FCS medium for 1 h at 37°C in 24-well, low adhesion plates, then washed three times, and the amount of uptake was assessed by FACS.

Immunohistochemistry

Frozen sections (6 μm) were prepared on microslides using a cryostat, fixed in 100% acetone for 10 min at room temperature, and dried before washing in PBS. The slides were then treated with 5% horse serum in PBS for 15 min in a humidified chamber. To identify germinal centers (GCs), sections were double labeled with FITC-conjugated GL-7 (BD Pharmingen) mAb and a Texas Red-conjugated anti-IgM Ab (Southern Biotechnologies), and 10% FCS (Harlan Sera Laboratories; complete medium) at 37°C in 5% CO₂. On days 0, 3, 6, and 8 of culture, the medium was supplemented with 10% supernatant from the X-63 fibroblast cell line transfected with the murine GM-CSF gene. After 10 days, nonadherent DCs were harvested by gentle washing and were typically >85% CD11c⁺, class II MHC⁺, CD40⁺, B7.1⁺, and B7.2⁺.

Assessment of Ag-presenting ability

Aliquot of 3 × 10⁶ cells to be used as APCs were plated in 12-well plates or 24-well ultra low adherence plates in 1 ml (Costar) and pulsed with Ag for 2 h at 37°C. For the last 45 min of culture, 50 μg/ml mitomycin C (Sigma-Aldrich) was added before the APCs were recovered and washed four times in RPMI 1640. After washing, APCs were plated in triplicate at 1 × 10⁶ cells/well in 96-well, flat-bottom microtiter plates (Costar) together with 2 × 10⁵ lymph node cells from DO11.10 or OT-II mice in a total volume of 200 μl. To assess T cell proliferation, 1 μCi/well [³H]TdR (West of Scotland Radioisotope Dispensary) was added for the last 16 h of culture, and cell-bound DNA was harvested onto glass-fiber filter mats (Wallac). [³H]TdT uptake was counted on a Betaplate counter (Wallac).

Statistical analysis

Data were compared using Student’s t test.

Results

Optimal conditions for incorporation of Ag and adjuvant into ISCOMs

In a previous study we showed that the CTA1-DD adjuvant could be effectively incorporated into ISCOMs and that the combined vector acquired adjuvant potency greatly surpassing either system used alone (31). In this study we explored the possibility of incorporating unrelated proteins in the same ISCOM particle using the proteins from influenza virus PR8 as a representative infectious agent together with CTA1-DD containing the MHC class II-restricted p323 peptide from OVA (CTA1-OVA-DD). In preliminary studies we established that both CTA1-OVA-DD and PR8 Ags were incorporated optimally at pH 6.0. Electron microscopic inspection and sucrose gradient analysis confirmed the successful construction of ISCOMs that carried both proteins in a balanced ratio of 1:1, with the peak distribution of each component being in fractions 5–7 of the sucrose gradient (Fig. 1). These fractions were subsequently used for the immunization studies.

Immunogenicity of combined CTA1-DD/ISCOM vector containing PR8 Ags

The immunogenicity of the combined vaccine vector was determined after i.n. immunization. Mice were immunized three times...
with PR8/CTA1-OVA-DD/ISCOMs, and the specific immune responses to recall Ag were assessed in spleen or CLN T cells 8 days after the final immunization. As we mentioned above, mice immunized with CTA1-OVA-DD/ISCOMs showed excellent priming to the OVA peptide, and these responses were much lower in mice receiving the enzymatically inactive PR8/CTA1R7K-OVA-DD/ISCOMs, or PR8 alone (l) or were given PR8 together with the CTA1-DD adjuvant. Isolated lymphocytes were stimulated 7 days after the final immunization with recall Ag, OVA323-339 or PR8 Ag in vitro. The results shown are from five individual mice per group and are representative of three identical experiments with similar results. Data are expressed as the mean cpm ± SEM (**, p < 0.01; *, p < 0.05).

Intranasal immunization with the combined vector also stimulated strong Ag-specific Ab responses in serum, with the enzymatically active PR8/CTA1-OVA-DD/ISCOMs stimulating 10-fold higher serum anti-PR8 IgG Ab titers compared with PR8/ISCOMs alone (Fig. 3). For comparison, mice immunized with PR8 together with CTA1-DD alone gave significantly better specific IgG responses than mice immunized with only PR8 Ag, but had titers comparable to those of mice immunized with PR8/ISCOMs (Fig. 3A). Moreover, marked

**FIGURE 2.** Intranasal immunization with PR8/CTA1-OVA-DD/ISCOMs primes T cells at both systemic and mucosal sites. Priming of OVA- and PR8-specific proliferative responses in spleen (A and C) or cervical lymph nodes (B and D) by i.n. immunization was performed. Mice were immunized three times i.n. with PR8/ISCOMs, PR8/CTA1-OVA-DD/ISCOMs, the enzymatically inactive PR8/CTA1R7K-OVA-DD/ISCOMs, or PR8 alone (l) or were given PR8 together with the CTA1-DD adjuvant. Isolated lymphocytes were stimulated 7 days after the final immunization with recall Ag, OVA323-339 or PR8 Ag in vitro. The results shown are from five individual mice per group and are representative of three identical experiments with similar results. Data are expressed as the mean cpm ± SEM (**, p < 0.01; *, p < 0.05).

**FIGURE 3.** Induction of influenza-specific systemic and mucosal Ab production by immunization with PR8/CTA1-OVA-DD/ISCOMs. Serum (A), bronchial lavage (B), or vaginal secretions (C) were taken 7 days after the last of three i.n. immunizations with PR8/CTA1-OVA-DD/ISCOMs, the enzymatically inactive PR8/CTA1R7K-OVA-DD/ISCOMs, or PR8/ISCOMs alone. PR8-specific Abs were measured by ELISA and are expressed as log10 titers for individual mice; the mean is shown by the bar (**, p < 0.01; *, p < 0.05). The content of total IgA in the bronchial lavage and genital tract secretions was similar in the respective groups, indicating that the total IgA production was not differently affected by the immunizations. Thus, there was 86 ± 24, 43 ± 20, and 40 ± 16 μg/ml total IgA (in the order given above) in lavage and 1.9 ± 0.7, 1.8 ± 1.0, and 1.5 ± 0.8 μg/ml total IgA in genital tract secretions in the respective groups. This is one representative experiment of three with similar results.
bronchioalveolar and genital tract IgA responses were observed in mice immunized with the combined vector (Fig. 3), which, again, were enhanced 10-fold compared with ISCOMs alone. Interestingly, the mutant CTA1R7K-OVA-DD/ISCOM vector enhanced anti-PR8 titers 3-fold (p < 0.05) above those of mice given PR8/ISCOMs alone in both serum and secretions, suggesting that the adjuvant

FIGURE 4. The combined PR8/CTA1-OVA-DD/ISCOM vector induces a balanced Th1 and Th2 response. Mice were immunized three times i.n. with PR8/CTA1-OVA-DD/ISCOMs, the enzymatically inactive PR8/CTA1R7K-OVA-DD/ISCOMs, PR8/ISCOMs alone, or PR8 Ag plus CT, and anti-PR8 IgG1 (■) and IgG2a (□) serum titers as well as total serum IgE (nanograms per milliliter) responses were measured by ELISA 7–9 days after the final immunization (A and B). Spleen cells were restimulated for 96 h with PR8 Ag in vitro and IL-10 (C), IL-5 (D), and IFN-γ (E) concentrations in culture supernatants were determined by ELISA. The results (picograms per milliliter) are expressed as the mean ± SEM of individual samples from five mice per group (*, p < 0.05). This is one of three experiments with similar results.

FIGURE 5. The combined CTA1-DD/ISCOM vector augments GC formations. Mice were given a single i.n. immunization with PR8/CTA1-OVA-DD/ISCOMs, enzymatically inactive PR8/CTA1R7K-OVA-DD/ISCOMs, PR8/ISCOMs alone, or CT plus PR8 micelles (2 µg of each). A, Fourteen days later, frozen sections of the CLNs were prepared and double labeled with Texas Red-conjugated anti-IgM (red) and FITC-labeled GL-7 (green) to detect GC reactions (green/yellow). B, the mean area of GL-7+ cells in the B cell follicles of four CLNs per mouse from five mice in each group was calculated. C, These calculations were complemented with FACS analysis of the frequency of GL-7+ cells per total B220+ CLN cells from five mice for the respective groups, using the background labeling of GC-negative naive CLN cells as a control (/). *, p < 0.05. This experiment represents one of three identical experiments with similar results.
function of the combined vector was not exclusively dependent on the enzymatic activity (Fig. 3).

**CTA1-DD-ISCOMs combined vector induced balanced Th1- and Th2-type response**

The CT adjuvant is known to skew CD4 T cell priming toward a Th2-type response, whereas ISCOMs stimulate strong Th1 immunity, with IFN-γ and CTL activity (39–41). Therefore, we predicted that the combined vector might promote a balanced Th1 and Th2 type of response to influenza Ags. In support of this, ISCOMs promoted higher relative PR8-specific IgG2a Ab responses than those stimulated by the combined vector, whereas specific IgG2a titers were lower compared with IgG1 titers in CT-immunized mice (Fig. 4A). Also, total serum IgE levels were higher in mice immunized i.n. with CT than in those given the combined vector or ISCOMs alone (Fig. 4). In addition, spleen and CLN (data not shown) cells restimulated in vitro with PR8 Ag produced high levels of both Th1- and Th2-dependent cytokines, which were much enhanced compared with those of cells obtained from mice immunized with enzymatically inactive PR8/CTA1R7K-OVA-DD or PR8/ISCOMs (Fig. 4).

**CTA1-DD/ISCOM vector stimulates germinal center reactions**

In search of an early in vivo marker that reflected the adjuvant effect of the combined vector, we investigated the ability of CTA1-OVA-DD/ISCOMs to stimulate GC formations in the regional lymph nodes. We found strong GC reactions 14 days after a single i.n. immunization with PR8/CTA1-OVA-DD/ISCOMs, whereas the enzymatically inactive PR8/CTA1R7K-OVA-DD/ISCOMs induced smaller and fewer GC reactions in the CLNs, similar to those seen after treatment with ISCOMs alone (Fig. 5). The size and frequency of GCs stimulated by the CTA1-OVA-DD/ISCOM vector were comparable with those seen after i.n. immunization with intact CT holotoxin, which is known to induce prominent GC reactions (Fig. 5) (42–44). A flow cytometric analysis of GL7⁺ B cells from CLNs also revealed the difference between immunizations with the combined vector and CT, on the one hand, and ISCOMs alone or the mutant vector, on the other (Fig. 5C). Thus, the combined vector was as effective as CT in stimulating GC formations in CLNs in i.n. immunized mice.

**Contrary to ISCOMs, the combined vector acts on B cells**

We next examined how the enhanced immune responses found after immunization with the CTA1-OVA-DD/ISCOM vector correlated with the APC population involved in their uptake. Purified BM, DCs, or derived spleen B cells were pulsed for different time periods with CFSE-labeled CTA1-OVA-DD/ISCOMs or CFSE-labeled ISCOMs containing equimolar amounts of the OVA323–339 peptide, and the uptake was analyzed by FACS. These studies showed that although DCs took up the OVA/ISCOMs and CTA1-OVA-DD/ISCOMs with equal efficiency, B cells only took up the combined vector and not the modified ISCOMs (Fig. 6A). In parallel, BM-derived DCs presented the OVA peptide to DO11.10 OVA-specific CD4⁺ T cells in vitro with identical efficiency when pulsed with all the different ISCOM constructs, whereas purified B cells presented OVA peptide only when pulsed with the combined vectors containing CTA1-OVA-DD and could not present OVA/ISCOMs themselves (Fig. 6, B and D). The ability of B cells to present Ag in the combined vector in vitro appeared not to depend on the CTA1 enzyme, because the mutant CTA1R7K-OVA-DD/ISCOMs also triggered peptide-specific T cell proliferation. Thus, the combined CTA1-OVA-DD/ISCOM vector gains access to and can be presented by a broader repertoire of APCs than conventional ISCOMs.

**Differential distribution of CTA1-DD/ISCOMs and ISCOMs in draining lymph nodes**

To confirm these properties of the combined vector in vivo, mice were injected s.c. into the footpad with CFSE-labeled ISCOMs or CFSE-labeled CTA1-OVA-DD/ISCOMs, and their distribution in the draining popliteal lymph nodes was examined at various time points. Two hours after injection, both OVA/ISCOMs and CTA1-OVA-DD/ISCOMs could be found in the deep cortical regions of the lymph node, consistent with access via the afferent lymphatics and subcapsular sinuses (Fig. 7). Thereafter, the CTA1-OVA-DD/ISCOMs began to concentrate in the B cell follicles, whereas the ISCOMs never appeared in follicles and were progressively lost from the lymph node, with only small amounts remaining in the
deep cortex after 24 h. These results were also confirmed by FACS analysis of isolated cells from the draining lymph node, which showed that CFSE-labeled CTA1-OVA-DD/ISCOMs could be detected in CD19<sup>+</sup> B cells, whereas both the combined vector and the normal ISCOMs were found in DCs (data not shown).

**CTA1-DD/ISCOMs target B cells as APCs in vivo**

To address the role of B cells in the immunogenicity of the combined vector, we purified B cells from the popliteal lymph nodes of mice immunized with OVA/ISCOMs or CTA1-OVA-DD/ISCOMs and examined their ability to present OVA to TCR-transgenic OT-II CD4<sup>+</sup>/H<sub>11001</sub>T cells in vitro. This showed that B cells from mice injected with the combined CTA1-OVA-DD/ISCOM vector, but not those injected with OVA/ISCOMs alone, could present Ag to the specific T cells (Fig. 8A).

Finally, we determined whether B cells were needed for the immunogenicity of the combined CTA1-OVA-DD/ISCOMs in vivo by immunizing μMT, B cell-deficient mice i.n. with PR8/CTA1-OVA-DD/ISCOMs or PR8/ISCOMs. When restimulated and cultured with PR8 Ag in vitro, spleen cells from wild-type and μMT mice showed equivalent responses after immunization with PR8/ISCOMs. However, in striking contrast to wild-type mice, μMT mice demonstrated no enhancement of T cell priming induced by the combined CTA1-OVA-DD/ISCOM vector (Fig. 8B). These findings also support the idea that B cells play a central role in vivo in the immunoenhancing effect of the combined CTA1-OVA-DD/ISCOMS vector.

**Discussion**

In this study we have confirmed and extended our previous findings that a potent mucosal vaccine vector can be constructed by combining the distinctive adjuvants CTA1-DD and ISCOMs. We show that the combined vector can be exploited to host additional Ags, such as influenza surface proteins, and the resulting formulation not only induces strong mucosal and systemic immune responses to all incorporated Ags, but also retains the enzymatic function of CTA1, which is required for its biological function. Our results also indicate that the explanation for the enhanced
adjuvant function of ISCOMs containing CTA1-DD compared with conventional ISCOMs is that the CTA1-DD component allows additional targeting to B cells as APCs.

Previous studies have shown that many protein Ags, including those prepared from influenza PR8, are highly immunogenic when incorporated into ISCOMs (39–44) as is the CTA1-DD construct containing the OVA323–339 peptide (31). We demonstrate that CTA1-DD/ISCOMs can be modified to incorporate PR8 Ags, and the resulting vector induces mucosal and systemic immune responses when given i.n. Not only was the combined vector highly immunogenic, but it was also, because, as batch-to-batch quality control responses to the integrated OVA peptide reflected, a stable, reproducible, and effective vaccine vector for strongly enhanced immune responses (31). This occurs using very low doses of Ag, with as little as 2 μg of PR8 protein and the equivalent of 150 ng of OVA peptide per dose being immunogenic by the nasal or parenteral routes (31). In addition, the combined CTA1-DD/ISCOM vector induces a balanced Th1 and Th2 response, which comprises IFN-γ and IL-5 production as well as T cell-mediated immune responses, such as delayed-type hypersensitivity and CTL activity and serum IgG and local IgA Abs (31), with no evidence of priming of IgE production. These properties distinguish the combined vector from its individual components, first by enabling much smaller doses of Ag to be used. In addition, the use of CT is dependent on the presence of enzymatically active toxin, because ISCOMs containing the R7K mutant of CTA1-DD were barely more immunogenic than ISCOMs with PR8 alone. This underlines the critical role of enzyme function in the activity of CT-related adjuvants (47). Nevertheless, ISCOMs containing enzymatically inactive CTA1R7K-DD retained some enhanced adjuvant function, such as specific Ab production in vivo and presentation of Ag by B cells in vitro. Together, these results indicate the potentially beneficial effects of targeting B cells as additional APCs. In vitro studies have shown that ISCOM particles themselves are taken up and presented preferentially by DCs (27, 28, 48), a finding we have extended in this study by showing that ISCOMs accumulate in DCs in vitro and in the DC-rich, T cell-dependent areas of lymph nodes in vivo. Interestingly, the pattern of uptake in vivo was consistent with localization in the fibroreticular conduits of T cell areas, which others have shown to be important sites of accumulation of Ag-loaded DCs and initial interactions between DCs and T cells (49–52). In contrast, ISCOMs containing CTA1-DD were taken up very efficiently by B cells as well as by DCs in vitro and were presented by both APCs to CD4+ T cells, presumably reflecting the ability of the DD portion to bind to B cells selectively via their surface Ig. Furthermore, s.c. injected CTA1-DD/ISCOMs had a unique ability to accumulate in B cell follicles of lymph nodes, where they were retained for at least 24 h, by which time conventional ISCOMs were virtually undetectable in the lymph node. Most importantly, the augmented adjuvant properties of CTA1-DD/ISCOMs were largely absent in B cell-deficient, μMT mice. For these reasons, we propose that this novel combined vector is so effective because it targets both DCs and B cells in vivo.

Unfortunately, attempts to document the same effects of the combined vector on B cells in the nasal-associated lymphoid tissues or CLNs after i.n. immunizations failed because no CFSE-labeled DCs and B cells were detected. We have previously experienced this problem when using labeled CTA1-DD or CT given i.n. (22, 53). Despite this, we have no reason to believe that DCs or B cells were not targeted in the nasal-associated lymphoid tissues and CLNs by the combined vector, especially because distinct GCs developed in the CLNs, as shown in the present study. Also, μMT mice failed to exhibit augmented responses after i.n. immunization with the combined vector, suggesting a relative dependence on B cells for the effect.
The ISCOM particle provides a stable formulation that interacts efficiently with and activates DCs (27, 28, 48), whereas CTA1-DD allows preferential delivery into B cells. These B cells are likely to be activated as a result of both the binding of sIg and the presence of pharmacologically active CTA1 enzyme. Activated B cells have been shown to be highly efficient APCs in other systems (52, 54) and together with the efficient localization of CTA1-DD/ISCOMs in B cell follicles, these properties presumably encourage cognate interactions between Ag-specific T and B cells in evolving germinal centers. This idea is supported by the fact that GC formations were greatly enhanced after administration of CTA1-DD/ISCOMs compared with ISCOMs alone. As well as enhancing primary immune responses, as we show in this study, recent work demonstrates that such interactions are of crucial importance in sustaining memory T and B cell responses (55–57). This would be a major factor in the success of any vaccine vector, and we are currently studying the effects of B cell-targeted ISCOMs on the induction of immunological memory.

A particular asset to the combined vector was its strong augmenting effect on mucosal IgA responses. Contrary to ISCOMs themselves, which are fairly poor inducers of mucosal IgA (58), combination with the CTA1-DD adjuvant rendered ISCOMs much better IgA-stimulating properties. We consistently observed >10-fold stronger mucosal IgA responses in CTA1-OVA-DD/ISCOMs compared with i.n. ISCOM-immunized mice. Importantly, regardless of the total IgA production at the mucosal sites, the specific responses were truly augmented and were not a reflection of polyclonal stimulation of IgA production. In vaccine formulations, such strong mucosal IgA immunity may be critical for protection against many infectious diseases.

In conclusion, we have shown that a combined vector comprising CTA1-DD incorporated into ISCOMs has considerable potential as a vaccine for mucosal immunization with a variety of protein Ags. By targeting the CTA1 adjuvant to both DCs and B cells as APCs, it allows powerful immune responses to be induced using low doses of Ags and points the way to a new generation of rationally designed vaccines.

Disclosures
The authors have no financial conflict of interest.

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