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Splenic Marginal Zone Dendritic Cells Mediate the Cholera Toxin Adjuvant Effect: Dependence on the ADP-Ribosyltransferase Activity of the Holotoxin

Dubravka Grdic,* Lena Ekman,* Karin Schön,* Kristina Lindgren,* Johan Mattsson,* Karl-Eric Magnusson,† Paola Ricciardi-Castagnoli,† and Nils Lycke**

The in vivo mechanisms of action of most vaccine adjuvants are poorly understood. In this study, we present data in mice that reveal a series of critical interactions between the cholera toxin (CT) adjuvant and the dendritic cells (DC) of the splenic marginal zone (MZ) that lead to effective priming of an immune response. For the first time, we have followed adjuvant targeting of MZ DC in vivo. We used CT-conjugated OVA and found that the Ag selectively accumulated in MZ DC following i.v. injections. The uptake of Ag into DC was GM1 ganglioside receptor dependent and mediated by the B subunit of CT (CTB). The targeted MZ DC were quite unique in their phenotype: CD11c+, CD8α−, CD11b−, B220−, and expressing intermediate or low levels of MHC class II and DEC205. Whereas CTB only delivered the Ag to MZ DC, the ADP-ribosyltransferase activity of CT was required for the maturation and migration of DC to the T cell zone, where these cells distinctly up-regulated CD86, but not CD80. This interaction appeared to instruct Ag-specific CD4+ T cells to move into the B cell follicle and strongly support germinal center formations. These events may explain why CT-conjugated Ag is substantially more immunogenic than Ag admixed with soluble CT and why CTB-conjugated Ag can tolerate immune responses when given orally or at other mucosal sites. The Journal of Immunology, 2005, 175: 5192–5202.

Activation of innate immune responses is a prerequisite for an adjuvant function and a much needed component in any vaccine (1). Although we have seen exceptional expansion of our knowledge about receptor-mediated activation of innate responses over the last 5 years, we still lack a detailed understanding of how most adjuvants work in vivo (1, 2). A majority of adjuvants are microbial products that activate innate responses through pattern recognition receptors, which lead to the release of proinflammatory cytokines and up-regulate costimulatory molecules on the APC (3, 4). Although B cells and macrophages are known to act as APC, dendritic cells (DC) are considered the key APC for priming of naïve T cells (5, 6). The difficulty in targeting DC in vivo has limited our knowledge about the priming events that determine whether Ag stimulation will result in a tolerogenic or immunogenic outcome (7, 8). Immature DC that reside in tissues are known to take up Ag and, if maturation occurs, migrate to regional lymph nodes or the spleen (5). In the secondary lymphoid tissues, the DC immigrants, expressing strong costimulation, may be inherently stimulatory, but whether resident or poorly activated immigrants are tolerogenic is currently a much debated issue (7–9). In particular, we lack in vivo information about DC at specific anatomical sites, such as the marginal zone (MZ) of the spleen, the lamina propria of the mucosal membranes, or the conduit system in the peripheral lymph nodes (10–12).

Recent experiments using DC targeting in vivo have indicated that DC can be modulated to direct tolerogenic or immunogenic priming of naïve T cells depending on the degree of inflammatory signals released at the site of Ag exposure or as a result of Ag dose (7, 8, 13). A proinflammatory environment would license the naïve T cells to develop into effective helper cells for B cell immunity, whereas an anti-inflammatory environment would support the development of regulatory T cells. Relatively few studies have investigated in vivo the functions of APC exposed to adjuvants, and little is known about the maturation and migration of specific APC following exposure to immunomodulators (13, 14). Using an adoptive transfer model of TCR transgenic T cells, it was shown recently that oral feeding of protein leads to clonal expansion of naïve T cells, but in the absence of adjuvant, feeding fails to generate B cell help, while in the presence of adjuvant strong B cell help develops (15). Thus, both the tolerogenic and the productive T cell response to fed Ag involved clonal expansion, but the quality of the primed T cells was different from that of T cells in adjuvant-exposed mice. Following the priming, the tolerized T cells failed to enter B cell follicles, but upon challenge with Ag they did enter follicles, but still failed to provide adequate B cell help (15).

Cholera toxin (CT) is one of the best studied and most effective experimental adjuvants known today (16). The mechanism for its
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Materials and Methods
Preparation of conjugates
CT-OVA and CTB-OVA conjugates were prepared with modifications, as described in detail (21). Briefly, 2 mg of CT or rCTB (provided by Dr. J. Holmgren, Department of Medical Microbiology and Immunology, Göteborg, Sweden) was solubilized in dH2O, followed by addition of 8 mg/ml N-succinimidyl-(3-(2-pyridyl)-dithio)propionate (SPDP) (Pharmacia-Upjohn). Four times higher molar amounts of OVA were solubilized in 0.1 M phosphate/0.1 M NaCl buffer and coupled to SPDP, as described above. The CT/CTB-SPDP and OVA-SPDP were mixed at room temperature for 24 h, dialyzed against PBS, and concentrated on a centrifuge column. The final concentration of the conjugates was assessed by GM1 ganglioside ELISA against a standard preparation of CT or CTB (30). The relative OVA content in the conjugates was assessed by rabbit anti-OVA Abs and anti-rabbit Ig-HRP Abs using the GM1 ganglioside ELISA (30). The molar ratio of OVA to CT or CTB was always 4:1. In some experiments, we used biotinylated CTA1-DD adjuvant (35). FITC-labeled CTB (List Biological Laboratories) or OVA was used at 5 and 40 μg/dose, respectively, to detect freshly isolated MZ DC.

Animals
Eight- to 12-wk-old age-matched female BALB/c mice were purchased from M&B, and DO11.10 TCR transgenic and Rag2–/– mice were bred and maintained at the animal facility Experimentell Biomedicin at Göteborg University. Mice were kept in the pathogen-free animal facility and routinely monitored by health screening according to the Federation of European Laboratory Animal Science Association recommendations.

Immunizations
Mice were immunized with different concentrations of CT-OVA, CTB-OVA, or OVA (Sigma-Aldrich) i.v. alone or when indicated, also followed by an i.p. challenge with 200 μg of OVA. Doses given were based on the CT or CTB content, according to concentrations, determined by GMI ELISA in relation to a standard preparation, and the dose of OVA in the conjugates was 4 times that of CT or CTB, on a molar level, as indicated, with or without 2 μg of CT. Footpad injections s.c. with 5 μg of conjugates were undertaken to analyze CD11c+ cells after 24 h in the draining popliteal lymph node. Ex vivo pulsing of cultured DC for 2 h with 40 μg/ml OVA, or 5 μg/ml CT-OVA, CTB-OVA, or CTB-OVA + 1 μg/ml free CT adjuvant was performed before i.v. injection into naive BALB/c mice. Seven days later, the mice were challenged i.p. with 200 μg of OVA before sacrifice on day 14. Five to six mice were included in each group. Adop-tively transferred age-matched BALB/c mice received splenocytes i.v. from transgenic DO11.10 mice at 107 cells per mouse. After 24 h, mice were immunized with a single dose of conjugates or OVA at doses given above. When indicated, CT or CTB conjugates were preincubated for 2 h at room temperature with saturating amounts (10 nmol/ml) of blocking soluble GM1.

Serum ELISA
The mice were bled before sacrifice, and sera were assayed by ELISA. Briefly, microtiter plates (Nunc) were coated with 3 ng/ml GM1 ganglioside or 200 μg/ml OVA in PBS, as described (26). Sera were diluted in PBS. Anti-OVA or CTB Abs were followed by HRP-labeled rabbit anti-mouse Ig Abs (DakoCytomation) and visualized using o-phenylenediamine substrate (1 mg/ml)/0.04% H2O2 in citrate buffer (pH 4.5). The reactions were read in a spectrophotometer (Flow Laboratories) at 450 nm. The anti-OVA or CT/CTB serum titers were defined as the interpolated OD readings on the linear part of the curve with an absorbance of 0.4 above background and given as log10 titer means ± SE of each group.

ADP-ribosyltransferase enzymatic activity
The activity was determined using the NAD-agmatine assay, as described earlier (27). Samples of 10 μg of CT, OVA, CTB-OVA, or CT-OVA were diluted 2-fold, and the enzymatic activity was assessed. The relative activity was expressed in mean cpm of three experiments with SD <5%.

FACS analysis
Careful phenotypic analyses on isolated splenocytes, MACS-enriched CD11c+ DC, or the murine D1 cell line were undertaken (28). D1 cells were cultured in IMDM (Sigma-Aldrich) supplemented with 10% FCS and 30% R1 medium (GM-CSF-transfected NIH-3T3 fibroblast-conditioned medium). Cells were treated with OVA (40 μg), CT-OVA, or CTB-OVA (5 μg/ml) for different times (2 or 24 h), and the cell surface expression of the costimulatory molecules, CD80, CD86, and CD40 (BD Pharmingen), or binding and uptake of OVA into the targeted cells was assessed by FACS. For determination of uptake of OVA after exposure to CT-OVA of splenocytes, we treated cells with saponin for intracellular labeling with anti-OVA. The FACS analysis of OVA uptake was performed with gated CD11c+CD11b+ or CD11b+CD11c- DC and macrophages, respectively. Freshly isolated CD11c+ cells were enriched to 85–90% purity by MACS, according to the manufacturer’s instructions (Miltenyi Biotec). For FACS analysis of CT-targeted CD11c+ cells, we injected mice with a single dose of FITC-labeled CTB or OVA, at 5 or 40 μg, respectively.

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Values are given, SD detection of bound CT-OVA or CTB-OVA. OD OVA Ab, followed by HRP anti-rabbit Ig detection of bound CT-OVA or CTB-OVA. OD values are given, SD <5%. At least three experiments in each category were performed with similar results.

FIGURE 1. Superior immunogenicity in Ags conjugated to CT. A and B, Mice were given a single i.v. immunization of indicated doses of CT-OVA or CTB-OVA (A) or OVA alone or admixed with CT adjuvant at 2 μg (B), followed by a booster dose i.p. of 100 μg of OVA alone, and 10 days later analyzed for serum anti-OVA Ab responses. Titers are expressed as mean log_{10} titer ± SEM of four to five mice/group. Values of p < 0.05. C, The ADP-ribosyltransferase activity of the conjugates was determined by the NAD-agmatine assay and given as cpm with SD <5% (see Materials and Methods). D, The relative amount of conjugated OVA was calculated by GM1 ganglioside ELISA using rabbit anti-OVA Ab, followed by HRP anti-rabbit Ig detection of bound CT-OVA or CTB-OVA. OD values are given, SD <5%. At least three experiments in each category were performed with similar results.

Immunohistochemistry

BALB/c or DO11.10 mice were given an i.v. injection of CT-OVA and CTB-OVA (2.5 μg), CTA1DD biotin (10 μg), CT biotin (5 μg), heat-labile enterotoxin β subunit (LTB) (5 μg; provided by Dr. M. Levens, Department of Medical Microbiology and Immunology, Göteborg, Sweden), or mutated LTB (EtxB (G33D) (5 μg; provided by Dr. T. Hirist, Department of Pathology and Microbiology, Bristol, U.K.), and spleens were embedded in Tissue Tek (Compound Mounds), and frozen microslides were prepared using a cryostat (Zeiss). Sections were fixed in acetone, air dried, and then double labeled with: Texas Red-, PE-, FITC-, or biotin-conjugated anti-IgM (Southern Biotechnology Associates), B220, CD11c, CD11b, CD19, CD8, CD4, MHC II I-Ad, CD40, CD80, CD86, CD68 mAbs, peanut agglutinin, DEC205 mAb (Serotec), and anti-laminin (Sigma-Aldrich) at optimal dilutions and different combinations, as indicated. Rabbit anti-OVA Abs were followed by FITC-labeled anti-rabbit Ig. Biotinylated CT or CTA1DD were visualized by Texas Red-streptavidin conjugates (Vector Laboratories). Total macrophages, MZ macrophages, metallophilic macrophages or DC were detected using F4/80 (BD Pharmingen) and MOMA-2 (Serotec), ER-TR9, MOMA-1 cysteine-rich mannose receptor (CR-Fc; detected by a human fusion protein [gift from Dr. C. Mueller, Laboratoire d’Immunologie, Clinique et Cellulaire, INSERM, Paris, France]), respectively. MOMA-2 (Serotec), ER-TR9, MOMA-1 cysteine-rich mannose receptor (CR-Fc; detected by a human fusion protein [gift from Dr. C. Mueller, Laboratoire d’Immunologie, Clinique et Cellulaire, INSERM, Paris, France]), respectively, were used. For detection of all or transgenic T cells, anti-CD3 and the clonotypic KJ1-26 mAb were used, respectively. Cells in division were detected by anti-human Ki-67 (BD Pharmingen) and a control Ab. Staining of single-cell suspensions; DC were first incubated with CT- or CTB-OVA and thereafter plated on poly(L) lysine-treated coverslips and detected by rabbit anti-OVA Abs. Photography and evaluation of tissue stainings were performed using a Leica DM LB microscope.

Ag presentation assay

Different densities of freshly isolated and MACS-enriched splenic CD11c+ DC from CT-OVA, CTB-OVA, OVA, or unimmunized mice were cultured together with 10^5 DO11.10 SCID transgenic CD4+ T cells with or without 1 μM OVA-p323 peptide in 96-well cultures for 96 h. Cells were pulsed with 6 h with [3H]thymidine to assess proliferation.

Statistical analysis

We used Student’s t test for analysis of significance. *, Denotes p < 0.05.

Results

Ag conjugation to CT greatly augments immunogenicity

Following chemical conjugation of OVA to CT or CTB, we investigated the immunogenicity of adjuvant-coupled Ag with that of Ag given alone. Serum anti-OVA Ab responses in mice immunized i.v. with CT-OVA were strikingly augmented compared with those seen in CTB-OVA, OVA alone, or OVA admixed with CT adjuvant (Fig. 1). Doses (based on the content of CT or CTB) ranging from 0.5 to 5 μg CT-OVA were highly effective, while CTB-OVA required at least 10-fold higher doses to give comparable anti-OVA titers (Fig. 1A). Mice injected with OVA admixed with CT adjuvant required >100-fold higher dose of OVA than CT-conjugated OVA to give similar serum titers, and a 200-fold higher dose of OVA alone failed to stimulate anti-OVA Ab responses (Fig. 1B). Thus, CT-OVA was significantly more effective at stimulating Ab production than CTB-OVA, OVA admixed with CT, or OVA alone. Importantly, the enzymatic activity of the CT-OVA conjugate was unaltered compared with unconjugated CT, whereas CTB-OVA and OVA were completely devoid of ADP-ribosyltransferase activity (Fig. 1C). Both CT-OVA and CTB-OVA conjugates carried comparable molar concentrations of OVA, as assessed by ELISA and total protein content (Fig. 1D). Gel electrophoresis and Limulus amebocyte lysate test confirmed that the conjugates were essentially pure and that all protein preparations contained <1 pg/mg contaminating endotoxin.

FIGURE 2. OVA conjugated to CT or CTB targets DC equally well. A–C, D1 DC were incubated for 2 h in vitro with medium containing equal concentrations (5 μg/ml) of either: A, CT-OVA; B, CTB-OVA; or C, 50 μg of OVA alone. The level of bound OVA to the target cells was visualized by rabbit anti-OVA Abs, followed by FITC-labeled anti-rabbit secondary Abs (green). D, A complementing FACS analysis was performed on the treated or untreated D1 cells and illustrated by the histogram. One representative experiment of three.
Both CT and CTB target DC in vitro

To identify possible mechanisms that could explain the differential adjuvant effects of CT and CTB, we assessed whether CT and CTB conjugates delivered Ag to target cells with comparable efficiency. Therefore, DC of the D1 cell line were exposed to OVA, CT-OVA, or CTB-OVA and then analyzed for OVA content using an anti-OVA polyclonal antiserum and labeled secondary Ab (28). CT-OVA or CTB-OVA conjugates were similarly delivered to the target cells in vitro (Fig. 2). In contrast, soluble OVA given at 100-fold higher doses was ineffective (Fig. 2C). The microscopic analysis of OVA was complemented by FACS and the mean fluorescent intensity (MFI), which demonstrated equal capacity of CT and CTB conjugates to deliver Ag to the target cells (Fig. 2D). Also, freshly isolated bone marrow DC exposed to CT- or CTB-OVA conjugates confirmed this result (data not shown). Thus, CT and CTB conjugates were effective delivery vehicles for Ag loading of DC. The difference between OVA alone and the conjugated OVA was the presence of the GM1 receptor-binding element, CTB, in both conjugates.

Injected CT- and CTB-conjugated Ag colocalizes to the MZ of the spleen

Next, we investigated the distribution of Ag after i.v. injection of the conjugates in mice and focused on differences between CT and CTB as delivery vehicles for the deposition of OVA. Frozen sections of spleens were analyzed for the presence of OVA at different time points following injection, using the polyclonal anti-OVA detection system described above. Already 15 min after an i.v. injection of CT-OVA (2.5 μg/dose) we found significant numbers of OVA-containing cells in a striking band formation in the MZ area of the spleen (Fig. 3A). At 2 h, the deposition was most marked, but OVA was clearly detectable at 24 h and at reduced levels even at 48 h postinjection (data not shown). The deposition was similar following injection of CTB-OVA, as no major difference in distribution or labeling intensity was detectable compared with CT-OVA (Fig. 3B). The splenic distribution of OVA+ cells was dependent on the conjugate because no OVA could be detected following injection of even very high doses (500 μg/dose) of OVA alone (Fig. 3C). Moreover, the band-formed distribution of OVA was identical with the distribution of labeled CT (Fig. 3E) or CTB (data not shown) when given i.v. alone, suggesting that the CTB-mediated GM1 ganglioside receptor binding was responsible for the accumulation of OVA to the MZ. This was also confirmed, as nearly no OVA could be detected if conjugates were first preincubated with saturating amounts of soluble GM1 ganglioside before injection, blocking the receptor interaction with the cells of the MZ (Fig. 3D). The binding to ganglioside GM1, as a prerequisite for accumulation in MZ, was also verified using conjugates made with LTb or rLTb/G33D, a mutant of rLTb (the B subunit of E. coli heat-labile toxin), which does not bind to GM1 (data not shown). Importantly, the binding ability of CT or CTB to GM1 ganglioside receptors was not restricted to the MZ cells, but all nucleated cells avidly bound the conjugates when applied directly to the sections (Fig. 3E, inset). In contrast, injected i.v. labeled CT/CTB were localized exclusively to the MZ (Fig. 3E). Thus, the specific accumulation of OVA in the MZ of the spleen appeared to be dependent on the route of entry into the spleen rather than on a unique receptor-mediated cellular binding via CTB in the MZ. In contrast, biotin-labeled CTA1-DD, which binds Ig and does not bind GM1 ganglioside, was found to accumulate in the B cell follicle area and was not found to colocalize with the OVA-containing cells in the MZ after i.v. injections (Fig. 3F). Taken together, the impaired localization of OVA to the MZ in the GM1 ganglioside-treated CT-OVA preparation, the failure to accumulate rLTb/G33-OVA conjugates, and the lack of accumulation of labeled, nonbinding CTA1-DD to the MZ strongly support a GM1 ganglioside-mediated mechanism for the accumulation of CT-conjugated Ag to the MZ.

GM1 receptor-targeted Ag accumulates in DC of the MZ

The cells that stained brightly for presence of OVA were phenotypically characterized using markers for macrophages (anti-F4/80, MOMA-1, MOMA-2, CD11b, ER-TR9 mAbs), B cells (anti-B220 or anti-CD19 mAbs), or DCs (anti-CD11c, anti-CD8α,
antiCD11b, anti-CD4 mAbs). We found that OVA-containing cells labeled strongly with CD11c-specific Abs, but were negative for markers unique to B cells or macrophages. Confocal microscopy revealed that CD11c-positive cells were also colabeled with anti-OVA (Fig. 4A), indicating that our target population was indeed MZ DC. The OVA-containing CD11c<sup>+</sup> cells were first observed in the MZ (Fig. 4B), but over time (at 24 h), more OVA-containing cells were found to be colocalizing with the CD11c<sup>+</sup> population in the T cell zone of the spleen, as illustrated in Fig. 4, E and F. At 72 h or more after injection, no OVA was detectable in the spleens (data not shown). Noteworthily, the target population appeared to be different from the DC subtype expressing CR-Fc Ab (red) (C), and the metallophilic macrophages anti-MOMA-1 Ab (red) (D) did not bind CT-OVA (green). At 24 h, CT-OVA CD11c<sup>+</sup> MZ DC (orange, double labeled) appeared to have migrated to the T cell zone (E). Arrows indicate CD11c<sup>+</sup> with (orange) or without (red) OVA. Of note, some of the MZ DC were intensely staining with anti-OVA (green), which masked the anti-CD11c<sup>+</sup> labeling (red) (E). There was a calculated increase in absolute numbers of OVA-containing DC per µm<sup>2</sup> in the T cell zone at 24 h (F). Popliteal lymph node CD11c<sup>+</sup> cells (red) carry OVA (orange) at 24 h following s.c. injections of CT-OVA in the footpad of mice (G). These are representative illustrations of at least three separate experiments with two mice per group.

**FIGURE 4.** MZ DC are the targets for CT- or CTB-conjugated OVA. Conjugates of CT-OVA (green) or CTB-OVA (2.5 µg) accumulated in CD11c<sup>+</sup> (red) DC in the MZ of the spleen following i.v. injection (A1 and A2). Fluorescence microscopy overlay (A1) as well as confocal microscopy (A2) confirmed this picture at 2 h following i.v. injections. Anti-laminin (red or green) was used to identify the localization of OVA (blue) to CD11c<sup>+</sup> (red) cells in the MZ. CD11b<sup>+</sup> cells (green) were found outside of the border of the MZ (B). The CR-Fc expressing DC, found in the MZ (29), detected by anti-CR-Fc Ab (red) (C), and the metallophilic macrophages anti-MOMA-1 Ab (red) (D) did not bind CT-OVA (green). At 24 h, CT-OVA CD11c<sup>+</sup> MZ DC (orange, double labeled) appeared to have migrated to the T cell zone (E). Arrows indicate CD11c<sup>+</sup> with (orange) or without (red) OVA. Of note, some of the MZ DC were intensely staining with anti-OVA (green), which masked the anti-CD11c<sup>+</sup> labeling (red) (E). There was a calculated increase in absolute numbers of OVA-containing DC per µm<sup>2</sup> in the T cell zone at 24 h (F). Popliteal lymph node CD11c<sup>+</sup> cells (red) carry OVA (orange) at 24 h following s.c. injections of CT-OVA in the footpad of mice (G). These are representative illustrations of at least three separate experiments with two mice per group.
Thus, CT appeared to host an exceptional targeting ability for CD11c/H11001 DC in vivo, which was clearly GM1 ganglioside receptor mediated. To reconcile the fact that OVA accumulation occurred in DC and not in macrophages in vivo, despite similar ability to bind CT ex vivo (Fig. 3E), we analyzed whether these cell types differed in their ability to take up and accumulate OVA when presented with the CT-OVA conjugate. Previous studies had indicated that macrophages were impaired in their Ag-processing ability after CT exposure, and we speculated that this might be relevant to explain the differential accumulation of OVA to DC in vivo (35). Therefore, we incubated splenocytes from Rag2/H11002 mice with CT-OVA and analyzed by FACS the membrane and intracellular accumulation of OVA at various times. We gated on CD11cH11001/CD11b or CD11b/H11001/CD11cH11001 and found only a slight increase in MFI in CT-OVA-exposed saponin-treated DC, as compared with that seen with similarly treated macrophages (geometric mean 3.2 vs 2.9) (Fig. 5B). This difference clearly did not reflect the selective accumulation of OVA to MZ DC that we observed in vivo.

DC exposed to CT-, but not CTB-Ag conjugates undergo maturation and effectively stimulate immune responses following injection

Because the CT-conjugated OVA was significantly more immunogenic than CTB-conjugated OVA, irrespective of a similar ability to deliver Ag to the target cells, we analyzed whether the conjugates differed with regard to their immunomodulating effects on DC. To this end, we used the D1 cells again and asked whether CT- or CTB-exposed DC exhibited differences in the expression of maturational markers. The D1 cell line represents immature growth factor-dependent mouse splenic DC, which fully mature in response to bacteria or inflammatory cytokines, reflecting that D1 cells mimic the maturational process of DC in vivo (28). Indeed, we found striking differences, with CT conjugates strongly promoting DC maturation, as shown by up-regulation of CD80, CD86, CD40, and MHC class II (Fig. 6). CTB had some minor

Table I. Characterization of the CT-OVA-targeted CD11c+ DC population in the MZ*

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<tr>
<th>Marker (mAb)</th>
<th>DC Subtype</th>
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<tbody>
<tr>
<td>CD11c (HL3)</td>
<td>++++</td>
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<tr>
<td>MHCII</td>
<td>++</td>
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<tr>
<td>DEC205 (NLDC-145)</td>
<td>+</td>
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<tr>
<td>CD11b</td>
<td>—</td>
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<td>F4/80</td>
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<td>MOMA-2</td>
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<tr>
<td>ER-TR9</td>
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<td>CR-Fc</td>
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* Mice received i.v. injections with 5 μg of CT-OVA conjugate and sacrificed 2 h later. Spleens were removed and frozen sections were processed for anti-OVA staining (green) and labeling with subset-specific Abs (red) prior to microscopic analysis, as described in Materials and Methods. The compiled data from several experiments are shown, and the labeling pattern was also confirmed by FACS analysis of freshly isolated CD11c+ cells at 2 h after CTB-FITC injection (see Fig. 5).

FIGURE 5. CT-OVA conjugates accumulate distinctly in the MZ DC. An extended analysis of the targeting ability of CT to MZ DC was performed. At 2 h following i.v. injections of CT-OVA, a transverse section of the spleen showed ample accumulation of OVA (green) to the MZ with anti-IgM Texas Red (red) labeling of the B cell follicles (A, top left panel). A complementing FACS analysis of MACS-enriched CD11c+ cells (bottom left panel) demonstrated that ~20–25% of all CD11c+ cells per spleen were targeted by CTB-FITC injected i.v., whereas 50 μg of OVA-FITC given i.v. could not be detected in CD11c+ cells (bottom right panel). Of note, CD11c-negative populations (left panel) did not carry CTB-FITC given i.v. B, Whether macrophages and DC differed with regard to their ability to accumulate OVA was addressed following exposure of Rag2H11001 splenocytes for 2 and 24 h (data not shown) with CT-OVA. Uptake of OVA by CD11cH11001/CD11b+ DC or CD11b+/CD11c+ macrophages (middle panel) at 2 h following in vitro pulsing with CT-OVA showed comparably ability to accumulate membrane (no saponin) and intracellular (saponin) OVA in DC (upper panels) and macrophages (lower panels) with geometric MFI 3.2 (figures given in the histograms) in the DC vs 2.9 in the macrophage populations.
these cells were negative for CD11b

the conjugates, FACS analysis revealed that only D1 cells exposed to CT-OVA (solid line) up-regulated the expression of CD80, CD86, CD40, and MHC class II, while CTB-exposed cells (dotted line) expressed similar levels of CD80, CD86, and CD40 to those of OVA- or medium-cultured cells alone (thin line). CTB had a weak stimulating effect on MHC class II expression. This is a representative experiment of five separate experiments.

effect on MHC class II expression, but not on the other markers of DC maturation (Fig. 6). Moreover, as the CT-OVA-exposed cells were injected into mice, they effectively stimulated anti-OVA immunity, while CTB-OVA-exposed DC were inefficient (Table II). CTB-OVA-pulsed DC were no better than OVA alone-treated DC, and only the addition of intact CT to CTB-OVA-treated DC restored the OVA-priming ability of the conjugate, indicating that the ADP-ribosylating property of CT was required for augmenting DC maturation and function (Table II). Importantly, however, the injected D1 cells did not accumulate in the MZ, and neither did freshly isolated splenic DC loaded ex vivo with CT-OVA conjugate (data not shown).

CT-Ag conjugates dramatically promote the maturation of DC in vivo

The strong promoting effect of CT on DC maturation was seen also in vivo following injection of the conjugates. We found that 24 h after injection of the CT-OVA conjugates, the T cell zone was loaded with CD11c+ cells that brightly stained with anti-CD86 mAb (Fig. 7, A and D). This was in contrast to mice injected with CTB-OVA conjugates (Fig. 7B) or OVA alone (data not shown), which demonstrated few CD86-expressing cells in the T cell zone. Most CD86+ cells were located to the MZ of the spleen in CTB-OVA- or OVA-injected mice. Thus, CT-OVA immunization resulted in redistribution of CD11c+ cells, from the MZ to the T cell area, but these cells were negative for CD11b+ , which instead was found to be increased on cells outside of the MZ (Fig. 7E). In addition, FACS analysis of isolated spleen DC 24 h after injection revealed that CT-OVA dramatically augmented the level of CD86 expression, whereas CTB-OVA did not alter the expression level compared with that seen after injection of OVA alone, confirming our microscopic findings (Fig. 7C). Interestingly, CD80 was not up-regulated on the CD11c+ cells in the T cell zone following injection of CT conjugates; rather, the CD80+ cells were found outside of the T cell and MZ (Fig. 7F). To investigate whether the CD86+ DC from CT-OVA-immunized mice also mediated an augmented ability to present OVA peptides to T cells, we injected mice with OVA alone or with the different conjugates and isolated DC by MACS to >80% purity at 20 h following the injections. OVA peptide (p323)-specific DO11.10 CD4+ T cells were cultured in vitro with the differently in vivo treated DC. We found that only DC from CT-OVA-injected mice stimulated significant T cell proliferation, whereas DC from CTB-OVA- or OVA-injected mice were poor stimulators of T cell proliferation not significantly different from DC from untreated mice (Fig. 7G). Saturating amounts of peptide (1 μM) gave similar T cell proliferation in all cultures, demonstrating the presence of equal numbers of DO11.10 T cells in the cultures. Thus, CT-OVA effectively delivers Ag to DC and strongly promotes DC maturation, greatly potentiating an effective Ag presentation to T cells in vivo.

CT-Ag conjugates stimulate expansion and migration of specific T cells into the B cell follicle, resulting in augmented GC reactions

We exploited the DO11.10 TCR transgenic mouse model further and performed a more detailed analysis of the consequences of CT-directed immunomodulation (15). We found that the splenic T cell zones were filled with CD86+CD11c+ cells 24 h after injection of CT-OVA conjugates in DO11.10 mice (Fig. 7, H and I). This was not seen with CTB-OVA-injected mice (data not shown). Furthermore, after adoptive transfer of DO11.10 T cells into BALB/c mice, we measured the expansion and migration of specific T cells in the spleen. The difference between CT- and CTB-OVA conjugates on DC maturation and function, observed earlier, was clearly seen also in the adoptive transfer model. The KJ1-26+ T cells were accumulating in the T cell zone of the spleen of the CT-OVA-injected mice, whereas the CTB conjugate had minimal effects on KJ1-26+ T cell numbers (Fig. 8, A and B) as compared with OVA-immunized or untreated mice. The KJ1-26+ T cells labeled with anti-Ki67 mAb indicated that they were undergoing expansion in situ in the T cell zone (Fig. 8C, upper panel). The increase in peptide-specific T cells was most pronounced at 96 h, whereas after the KJ1-26+ T cell numbers were reduced in the T cell zone, but increased in the B cell follicles. After 6 days, significant numbers of KJ1-26+ T cells were seen in the B cell follicles in CT-OVA-immunized mice (Fig. 8D). By contrast, CTB-OVA-immunized mice exhibited only few KJ1-26+ T cells in the B cell follicle (Fig. 8E). Concomitant with this, GC were formed in the CT-OVA-immunized mice, and after 12 days large GC were observed in these mice, while CTB-OVA or nonimmunized mice had no or few and small GC (Fig. 8, G and H). These results suggest that the mechanism for CT adjuvanticity involves targeting of MZ DC, leading to activation, maturation, and migration of DC to the T cell zone. This is followed by expansion of specific T cells and

Table II. OVA-specific Ab responses in mice after transfer of DC, ex vivo exposed to CT-OVA or CTB-OVA conjugates*  

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<thead>
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<th>No.</th>
<th>Anti-OVA Ig</th>
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<tr>
<td>Medium</td>
<td>&lt;2.0</td>
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<tr>
<td>OVA</td>
<td>2.1 ± 0.05</td>
</tr>
<tr>
<td>CTB-OVA</td>
<td>2.5 ± 0.3</td>
</tr>
<tr>
<td>CT-OVA</td>
<td>3.8 ± 0.3*</td>
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<tr>
<td>CTB-OVA + CT</td>
<td>4.1 ± 0.5</td>
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* DC were treated ex vivo for 2 h with 5 μg/ml CT-OVA, CTB-OVA, CTB-OVA + CT (1 μg/ml), OVA (50 μg) alone, or cell culture medium, and thoroughly washed in medium prior to transfer to naive mice. The animals were subsequently challenged i.p. with OVA alone and sacrificed 7 days later. Serum was collected from individual mice, and serum anti-OVA Abs were determined. The relative concentration of total anti-OVA Abs was expressed as mean log10 titers ± SE of four to five mice in each group. The anti-OVA Ab response was significantly higher in CT-OVA compared with CTB-OVA-treated DC-injected mice (*, p < 0.05). One representative experiment of three is shown.
movement of T cells into the B cell follicle, finally resulting in greatly augmented GC reactions. Thus, the key adjuvant events appear to be the targeted delivery of Ag and the ADP-ribosyltransferase activity of the CTA1 acting on the MZ DC.

**Discussion**

Although many studies have addressed the mechanism for the adjuvant function of the bacterial ADP-ribosylating enterotoxins CT and heat-labile enterotoxin, this is the first report to demonstrate that CT acts as a targeting and immunomodulating vector for GM1 ganglioside-expressing DC in the MZ of the spleen. Previous studies have suggested that DC may be one of the major cellular targets for the CT adjuvant, but none have monitored the targeted DC closely and followed the in vivo priming process in detail, as was done in the present study (13, 17, 18, 20). Our approach was to follow the accumulation of Ag in targeted cells and to detect alterations in APC function induced by the ADP-ribosyltransferase activity of the CT adjuvant. This strategy was possible after we chemically coupled OVA to CT or CTB, which share the ability to bind to the GM1 ganglioside receptor. Notably, CT differs from CTB only in that the CTA1 enzyme, known to be critical for the adjuvant function, is missing in the latter (16). With this approach, we demonstrated that CT as well as CTB deliver large amounts of Ag to the MZ DC following i.v. injections, and that the accumulation of Ag was dependent on the binding to the GM1-ganglioside receptor. The targeted DC appeared to undergo maturation and

**FIGURE 7.** CT promotes CD86 expression and migration of DC to the T cell zone. CT- and CTB-OVA conjugates or OVA alone were injected i.v., and 24 h later the spleens were removed and sectioned for analysis. Fluorescence microscopy showed FITC-labeled anti-OVA (green) and PE-labeled anti-CD86 (red) to colocalize (orange) in the T cell zone of CT-OVA (A), but not CTB-OVA (B)-injected mice. Of note, the pattern of CD86-expressing cells in the MZ was seen also in untreated mice (B). The dramatic effect of CT on CD86 expression was confirmed by FACS analysis on CD11c^+^-gated cells (C). A close-up in the splenic T cell zone confirmed colabeling (orange/yellow) of CD11c-FITC (green) and CD86-expressing (red) cells (D). No cells in the T cell zone expressed CD11b (green); these cells accumulated outside of the MZ (E). Interestingly, although CT up-regulated CD80 ex vivo, we found no, or few, cells expressing CD80 (red) in the MZ or T cell zone of CT-OVA (green)-treated mice (F). We freshly isolated and MACS enriched splenic DC from CT-OVA (red line), CTB-OVA (blue line), OVA (green line), or unimmunized mice (light green line) and cultured them together with MACS-enriched Do11.10 CD4 T cells, and the proliferative responses were assessed and expressed as the mean cpm ± SD of triplicate cultures (G). As a control, all cultures stimulated comparable T cell proliferation when saturating amounts (1 μM) of OVA peptide (p323) were added to the cultures: CT-OVA 263.757 cpm vs CTB-OVA 244.370 cpm (data not shown). I, Similar experiments in OVA-TCR transgenic Do11.10 mice confirmed the massive recruitment of CD86-expressing cells (red) at 24 h into the T cell zone (FITC-labeled anti-CD3) (green) compared with controls (H). Inset, Shows accumulation of activated CD11c^+^ CD86-expressing DC (yellow) in the T cell area of the spleen following immunization (I). Experiments were performed with two mice per group, and the illustrations are representative of at least three experiments giving similar results.
migration to the T cell zone in response to the CTA1 enzyme. Using the DO11.10 transgenic T cells, we found that targeting of CT-OVA, but not CTB-OVA, to MZ DC resulted in effective priming and expansion of naive T cells concomitant with high expression of CD86 on CD11c+ cells in the T cell zone. The encounter with DC exposed to CT adjuvant seemed to instruct T cells to migrate to the B cell follicle and to promote the development of large GC reactions. Thus, for the first time, a whole sequence of events could be followed after administration of CT adjuvant by monitoring the in vivo deposition of Ag and the accumulation of Ag to the MZ DC.

The present study provides an explanation as to how it is possible that CT can act as a powerful adjuvant despite that it can bind to all GM1 ganglioside-carrying, i.e., all nucleated, cells in the body. In fact, the OVA accumulated exclusively in MZ DC when CT-OVA was injected, arguing for a selective effect on DC, sparing B cells and macrophages in the MZ. However, assessing the density of GM1 receptors on MZ cells, through direct application of labeled CTB onto spleen tissue sections, we failed to reveal any difference in receptor density between DC, macrophages, or B cells. Given that blood flowing into the sinuses of the spleen filters out through the MZ and the red pulp before emptying into the venous sinuses, most Ags are likely to be trapped in the MZ (10, 31). Depending on the type of Ag, soluble or particulate, MZ cells may be differently engaged. Macrophages have been shown to play an important role in trapping of Ags especially for capturing of polysaccharide Ags (32). Also, MZ B cells have been implicated in uptake of blood-borne Ags (33). In the light of the present findings, it is likely that soluble Ags that are taken up by DC would benefit the strongest from the use of the CT adjuvant, whereas other adjuvant mechanisms may be more effective at enhancing responses toward other types of Ags. Our work and that of others support this notion (34). We have demonstrated that whereas the adjuvant effect of CT is dependent on CD40 signaling, i.e., more active at enhancing T-dependent type of responses, the CTA1-DD adjuvant, which is targeted to B cells, also greatly augments T-independent type of responses (27). The differences in deposition of the two adjuvants in relation to the MZ documented in the present study would agree with this finding.

Because CT interacts via the GM1 ganglioside receptor, large quantities of Ag accumulated in the MZ DC. This allowed for relatively small amounts of Ag (<1 μg) to stimulate significant immune responses. At least a 100-fold higher dose of OVA, when simply admixed with CT, was required to give a comparable response to that of the CT-OVA conjugate. By comparison, the CTA1-DD molecule, which binds to B cells via Ig, did not accumulate in the MZ DC after i.v. injection, suggesting that GM1 receptor-mediated uptake is essential for effective loading of soluble Ag into the MZ DC. Nolte et al. (11) have shown that the deposition of Ag in the splenic white pulp and MZ is dependent on a conduit system and restricted by molecular size. However, we do not believe that the CT-mediated targeting of OVA to the MZ DC was merely an effect of molecular size, although the CTA1-DD is

FIGURE 8. CT promotes primed T cells to enter B cell follicles, resulting in prominent GC formations. Adoptively transferred KJ1-26+ DO11.10 T cells in syngeneic BALB/c mice were followed at different times after injection i.v. of CT-OVA or CTB-OVA. The mice were sacrificed after 96 h, and sections of spleen were labeled with anti-KJ1-26 mAb (green) and anti-B220 (red). We observed a substantial increase in OVA-specific KJ1-26+ T cell (green) numbers in CT-OVA (A), but not in CTB-OVA (B)-injected mice. The KJ1-26+ T cells (red) in the T cell zones of CT-OVA-injected mice appeared to undergo cell division in situ because the FITC-labeled anti-Ki-67 mAb (green) marker colocalized to the T cells (C); an isotype control mAb (green) is shown in the lower panel. At 6 days after injection of CT-OVA, Ag-specific T cells (green) were found to move into the B cell follicle (red) (D). By contrast, significantly weaker migration of KJ1-26+ T cells into the B cell follicle was observed after injection of CTB-OVA (E). The calculated number of KJ1-26+ T cells/mm² present in the B cell follicle at 96 h following injection of CT-OVA or CTB-OVA was determined (F). Apparent already after 6 days, but more prominent after 12 days following CT-OVA (G), but not with CTB-OVA (H) treatments, we observed large GC (green) using FITC-labeled peanut agglutinin (green) and PE-labeled anti-B220 (red) to identify the B cell follicles. These experiments are representative of at least three giving similar results.
37 kDa and the CT-OVA conjugate is much larger. Why macrophages and B cells in the MZ did not accumulate CT-OVA cannot be explained by the results from the present study. Clearly, both subsets carry GM1 ganglioside receptors and appear to bind CTB equally well ex vivo compared with DC (21). One may speculate that CT, in fact, triggers enhanced accumulation of Ag in DC over time because of facilitated uptake and reduced processing of Ag compared with that occurring in macrophages. Matousek et al. (35) showed that macrophages exposed to CT, but not CTB, reduced their processing activity. However, at variance with our in vivo finding, we failed to show a difference between macrophages and DC with regard to intracellular accumulation of OVA in vitro following CT-OVA exposure. Therefore, in future experiments, we will use various deletional models to eliminate DC or macrophages in vivo, hoping to better explain the selective accumulation of Ag to MZ DC. It should be emphasized that ~20–25% of all DC in the spleen were targeted, clearly demonstrating the powerful targeting ability of GM1-binding CT-linked Ag for the delivery to the MZ DC. However, in addition, s.c. injections of CT-OVA in the footpad targeted DC that migrated to the popliteal lymph node.

Immature DC in the blood can efficiently capture and transport Ag to the spleen (36). However, we believe that CT was specifically taken up by MZ DC because we detected OVA already at 15 min after CT-OVA injections. At 24 h, DC migrated from the MZ into the T cell zone, which was dependent on the CTA1-enzymatic activity, as CTB conjugates failed to cause this migration of CD11c^+CD86-expressing DC into the T cell zones. The targeted MHCI^+DEC205^+CD11b^+CD8α^+ MZ DC appeared to be quite unique in that they were CD11b^− and CD8α^−. A recent publication reported on splenic MZ CD11b^+CD8α^+ DC, which internalized circulating apoptotic cells and acquired CD8α during their later mobilization to T cell areas (10). Although we did not detect CD11b or CD8α on the cell surface of our OVA-high DC in the MZ, it is possible that these cells may express these markers at later time points. Interestingly, CD11b^+ cells accumulated outside of the T cell zone and the MZ subsequent to CT-OVA injections, which may be mechanistically important as it argues against the involvement of macrophages in the adjuvant effect of CT.

Previous reports on CT and its ability to stimulate cytokine, chemokine, and chemokine receptor production by DC have shown that these cells express CXCR4 and CCR7, i.e., CT may promote colocalization of DC with naive T cells (17). The effect on the chemokines and chemokine receptor expression may help explain the difference in migrating ability between CT-OVA- and CTB-OVA-targeted MZ DC. In this context, a lack of an effect of CT-OVA on chemokine and chemokine receptor expression in B cells or macrophages in the MZ also would answer the question as to why only DC appear to carry CT-conjugated Ag to the T cell zone. Recent findings in CD38-deficient mice have indicated that this ADP-ribosylating ectoenzyme may be critical for the regulation of adjuvant responsiveness in DC, as lack of CD38 negatively affected T cell-priming efficiency and humoral immunity (37). Thus, CT could provide selectivity by replacing CD38 activity in DC, and thereby, affect chemokine receptor signaling through CCR7 or CXCR4 and the ability to migrate to, e.g., the T cell zone in peripheral lymph nodes or spleen. This notion agrees well with the fact that CT-OVA, devoid of enzymatic activity, delivered Ag to the MZ DC, but failed to affect DC maturation and migration. A notable finding was that delivery of CTB-OVA did not affect the differentiation stage of the targeted DC, i.e., no expression of CD86 or migration of DC to the T cell zone and eventually only few T cells were licensed to migrate into the B cell follicle. The latter observation is in agreement with T cells tolerized by oral Ag, which fail to enter into the B cell follicle and provide B cell help (15). In fact, CTB-OVA given orally has been proven one of the most potent ways of induction of T cell tolerance (24). Taken together, these findings argue for the importance of CTA1-dependent maturation signals provided by the enzymatically intact holotoxin and acting on the targeted MZ DC. A hallmark of the effect of CT adjuvant was the development of large GC, expansion sites for the specific B cell response, and important for the generation of memory, Ig class-switching, and somatic hypermutations (38). A similar drive on GC formation is found with the CTA1 enzyme alone, as can be seen when the CTA1-DD adjuvant is used to replace the holotoxin (27). Thus, the enlarged GC appear to be a common denominator for the CTA1-dependent adjuvant mechanism (27, 38). Previously, it has been documented that CD40 is important for the development of GC (39). Recent investigations by Gray and coworkers (40) showing that DC control the migration of T cells to B cell follicles, a mechanism dependent on CD40/ OX40L interactions, appear to apply particularly well to the effect of CT-OVA conjugates. CD40 or OX40L expression on MZ DC may be critical for the adjuvant effect of CT. Ongoing studies are, therefore, addressing the effects of CT on OX40L and CD40 expression in MZ DC following immunizations.

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Disclosures

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References


