

# In Vitro Induction of Immunoglobulin A (IgA)- and IgM-Secreting Plasma Blasts by Cholera Toxin Depends on T-Cell Help and Is Mediated by CD154 Up-Regulation and Inhibition of Gamma Interferon Synthesis<sup>∇</sup>

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**Cholera toxin (CT) and the type II heat-labile enterotoxins (LT-IIa and LT-IIb) are potent immunological adjuvants which are hypothesized to enhance the production of antibody (Ab)-secreting cells, although their mechanisms of action are not fully understood. The treatment of splenic cells with concanavalin A (ConA) plus CT enhanced the production of immunoglobulin A (IgA) and IgM by dividing cells that expressed high levels of major histocompatibility complex class II (MHC-II), CD19, and CD138 and low levels of B220 a phenotype characteristic of plasma blasts. LT-IIa or LT-IIb moderately enhanced IgA and IgM production without enhancing plasma blast differentiation. CT up-regulated CD25, CD69, CD80, CD86, and MHC-II in isolated B cells but failed to induce proliferation or differentiation. The treatment of unfractionated splenic cells with ConA plus CT induced B-cell proliferation and differentiation, but the elimination of CD4<sup>+</sup> T cells inhibited this effect. CT treatment of ConA-activated CD4<sup>+</sup> T cells up-regulated CD134 and CD154, whereas the blockage of CD40-CD154 interactions inhibited the induction of plasma blasts and Ig synthesis. The treatment of unfractionated splenic cells with CT, LT-IIa, or LT-IIb enhanced the production of interleukin-6 (IL-6) and IL-10, whereas the production of gamma interferon was inhibited in both CD4<sup>+</sup> and CD8<sup>+</sup> T cells mostly by CT. Thus, major regulatory effects of CT on lymphocytes are likely exerted early during the induction of immune responses when B and T cells initially encounter antigen. Neither LT-IIa or LT-IIb had these effects, indicating that type II enterotoxins augment Ab responses by other mechanisms.**

The heat-labile enterotoxins (HLT) of *Vibrio cholerae* and *Escherichia coli* belong to a family of structurally related bacterial enterotoxins that induce diarrheal symptoms in humans and animals. HLT are oligomeric proteins composed of a single A polypeptide which is noncovalently bound to a pentameric array of B polypeptides (20). Two types of HLT have been distinguished on the basis of distinct immune system reactivities: the type I HLT include the *V. cholerae* enterotoxin cholera toxin (CT) and the *E. coli* enterotoxin LT-I; the type II HLT include LT-IIa and LT-IIb, two antigenically related enterotoxins produced by certain enterotoxigenic strains of *E. coli* (14, 21). The A polypeptides of type I HLT and type II HLT are highly homologous, which is reflected in their similar ADP-ribosylating activities. In contrast, the B polypeptides of the two classes of HLT exhibit significant divergence in amino acid sequence, which imparts upon the molecules the range of receptor binding specificities observed for the enterotoxins (20). The functional receptors for type I and type II HLT are

gangliosides, a family of structurally complex glycolipids which reside in the plasma membranes of eukaryotic cells (32). While the physiological roles of gangliosides are not well established, these molecules have been shown to influence events that lead to cellular activation, proliferation, and differentiation in lymphocytes and other cell types (3, 16, 33). In vitro binding assays have shown that the members of the type I and type II HLT exhibit differences in their relative binding affinities for various gangliosides. CT and LT-I bind with high affinity to ganglioside GM<sub>1</sub>. A more divergent pattern of ganglioside binding is observed for LT-IIa, which binds most avidly to ganglioside GD<sub>1b</sub> and with less avidity to gangliosides GD<sub>1a</sub> and GM<sub>1</sub>. LT-IIb binds with high affinity only to GD<sub>1a</sub> (13).

Despite their inherent enterotoxicities, CT, LT-IIa, and LT-IIb have been successfully employed as adjuvants in experimental animals to enhance mucosal and systemic antibody (Ab) responses (7, 15, 26, 30). Both type I and II enterotoxins induce significant mucosal (immunoglobulin A [IgA]) and systemic (IgG) Ab responses to admixed antigens (Ags) and promote the generation of Ag-specific memory B cells and Ig-secreting cells (Ig-SC) (15, 41). Although the capacity of CT, LT-IIa, and LT-IIb to augment Ab responses is beyond dispute, the cellular and molecular mechanisms by which these three enterotoxins stimulate B-cell responses and increase Ab production have not been fully described, particularly in regard

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to the mechanisms by which CT, LT-IIa, and LT-IIb elicit primary (IgM) Ab responses. Most studies evaluating the adjuvant effect of these enterotoxins on Ab responses were performed using mice that received both primary and booster immunizations (7, 15, 26, 30). Data obtained from these repeatedly immunized mice likely reflect the effects of CT, LT-IIa, and LT-IIb on memory B and T cells (41).

To elucidate the molecular mechanisms by which enterotoxins modulate Ab responses, we examined the effects of CT, LT-IIa, and LT-IIb on B and T cells during the early elicitation of Ab responses *in vitro*. We present evidence that CT, but not LT-IIa or LT-IIb, elicits the polyclonal activation of B cells and induces CD134 and CD154 up-regulation in mitogen-activated CD4<sup>+</sup> T cells. Interactions between B and T cells via CD154-CD40, and to a lesser extent via CD134-CD134L, as well as the inhibition of gamma interferon (IFN- $\gamma$ ) production in CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes, were shown to be involved in the CT-dependent stimulation of Ig-SC development. According to phenotypic patterns and function, part of the Ig-SC population induced by CT resembled the short-lived IgM-secreting plasma blasts found in the extrafollicular regions of the secondary lymphoid tissues. Our results strongly indicate that CT affects Ig production by regulating early cellular events in the induction of an immune response. Furthermore, the ganglioside binding affinities of the enterotoxins influence those regulatory activities (3).

#### MATERIALS AND METHODS

**Expression and purification of recombinant enterotoxins.** Recombinant enterotoxins produced in *E. coli* were purified from periplasmic extracts by a combination of nickel affinity and gel filtration chromatography as previously reported (16).

**Mice and cells.** To produce single-cell suspensions, spleens, aseptically harvested from naive BALB/c mice of 8 to 12 weeks of age, were pressed through a cell strainer (Becton-Dickinson, San Jose, CA) into phosphate-buffered saline (PBS; GIBCO, Gaithersburg, MD). Mononuclear cells were isolated from the cell mixture by Ficoll-Hypaque (Pharmacia, Piscataway, NJ) density gradient centrifugation. Cells were washed two times with PBS to remove the Ficoll-Hypaque. The number of viable cells in the mononuclear fraction was routinely  $\geq 90\%$  of total cell counts, as determined by using a routine trypan blue (Invitrogen, Carlsbad, CA) dye exclusion protocol. All studies were performed with approval from The University at Buffalo Institutional Animal Care and Use Committee.

**Cell purification and depletion.** Single-cell suspensions from spleen were incubated with CD16/32 Ab ( $\alpha$ CD16/32; PharMingen, San Diego, CA) followed by incubation for 20 min at 6°C with  $\alpha$ CD4,  $\alpha$ CD8a, or  $\alpha$ CD19 microbeads (Miltenyi Biotec, Auburn, CA). CD4<sup>+</sup>, CD8<sup>+</sup>, or CD19<sup>+</sup> cells were magnetically selected by two successive rounds of enrichment using LS-positive selection columns (Miltenyi Biotec). To purify cells expressing CD11b or CD138, cells were labeled with  $\alpha$ CD11b-phycoerythrin (PE) or  $\alpha$ CD138-PE (PharMingen), respectively, and thereafter with  $\alpha$ PE microbeads (Miltenyi Biotec). Cells were subsequently enriched as described above. The positive fractions routinely contained  $\geq 95\%$  of CD4<sup>+</sup>, CD8<sup>+</sup>, CD11b<sup>+</sup>, or CD138<sup>+</sup> cells. To deplete cell suspensions of CD4<sup>+</sup>, CD8<sup>+</sup>, or CD138<sup>+</sup> cells, cells were labeled with  $\alpha$ CD4 or  $\alpha$ CD8a microbeads or with  $\alpha$ CD138-PE and then with  $\alpha$ PE microbeads, respectively. Cell suspensions were depleted of CD4<sup>+</sup>, CD8<sup>+</sup>, or CD138<sup>+</sup> cells by using an LD column (Miltenyi Biotec). The depleted fraction did not contain detectable amounts of CD4<sup>+</sup>, CD8<sup>+</sup>, or CD138<sup>+</sup> cells, as measured by fluorescence-activated cell sorting (FACS).

**Lymphocyte activation.** CD138 cell-depleted populations of splenic mononuclear cells ( $2 \times 10^6$ /ml/well) labeled (27) or unlabeled with carboxyfluorescein succinimidyl ester (CFSE; Molecular Probes, Eugene, OR) were suspended in complete RPMI 1640 medium (GIBCO) containing 10% fetal calf serum. Cells were added to 6-well or 24-well culture plates containing 2  $\mu$ g/ml of concanavalin A (ConA; Sigma, St. Louis, MO) or 10  $\mu$ g/ml of *E. coli* lipopolysaccharide (LPS; Calbiochem, San Diego, CA). LPS directly activates B cells via Toll-like receptor

4 (TLR-4) and induces the proliferation and differentiation of these cells in the absence of CD4<sup>+</sup> T lymphocytes, and therefore it was used to stimulate T-cell-independent B-cell responses (22). ConA effectively transduces intracellular signals via a T-cell receptor-CD3 complex and accordingly was used to imitate the Ag-specific stimulation of T cells (38). Cell cultures were treated with either CT, LT-IIa, or LT-IIb at a concentration of 1  $\mu$ g/ml and incubated at 37°C in humidified air plus 5% CO<sub>2</sub> for 4 or 7 days. In some experiments, CD4<sup>+</sup> or CD19<sup>+</sup> cells that were purified by magnet-activated cell sorting (MACS; Miltenyi Biotec), as indicated above, were pretreated on ice for 10 min with CT (1  $\mu$ g/10<sup>6</sup> cells), washed three times with PBS, and cultured with CD11b<sup>+</sup> cells (10<sup>6</sup> cells) plus ConA. Splenic CD11b<sup>+</sup> cells which express high levels of major histocompatibility complex class II (MHC-II) molecules are required for the ConA-induced activation of T lymphocytes. Thus, purified CD11b<sup>+</sup> cells were used in combination with the mitogen to stimulate CD4<sup>+</sup> T cells (2, 23). The pretreatment of purified CD8<sup>+</sup> or CD8<sup>-</sup> cell fractions with CT was performed as indicated above.

**Immunophenotype.** Single-cell suspensions were stained for CD8, CD19, B220, CD25, CD40, CD54, CD69, CD80, CD86, CD134, CD154, CD138, or MHC-II by using marker-specific fluorescent antibodies (PharMingen). After incubation on ice for 10 min, cells were washed with buffer (PBS, 3% bovine serum albumin, 0.05% NaN<sub>3</sub>), incubated with 1  $\mu$ g/ml of propidium iodide (Sigma), and analyzed by FACS.  $\alpha$ CD16/32 antibodies (PharMingen) were used for blocking Fc receptors as indicated by the manufacturer. For intracellular staining, cells were fixed in 2% formaldehyde and incubated with fluorescein isothiocyanate-labeled  $\alpha$ IgA,  $\alpha$ IgG, or  $\alpha$ IgM (PharMingen) in PBS containing 2% bovine serum albumin and 0.5% saponin (Sigma). Isotype-matched fluorochrome-labeled Ab and cells that were not permeabilized with detergent were used as controls. Sample acquisition and analysis were performed using a FACS-Calibur flow cytometer (Becton-Dickinson) and the CellQuest software (Becton-Dickinson). Experiments were repeated at least twice. No fewer than 10<sup>4</sup> events were acquired for each experiment.

**Immunoglobulin analysis.** Total Ig isotype concentrations in culture supernatants were measured by enzyme-linked immunosorbent assay (ELISA). Polystyrene microtiter plates (96 well; Nunc, Roskilde, Denmark) were coated overnight at 4°C with goat anti-mouse Ig isotype-specific antibodies (Southern Biotechnology, Birmingham, AL). Serial twofold dilutions of culture supernatants were added in duplicate, and plates were incubated overnight at 4°C. Plates were washed with PBS containing 0.1% Tween 20 and incubated at room temperature for 4 h with the appropriate alkaline phosphatase-conjugated goat anti-mouse Ig isotype-specific antibodies (Southern Biotechnology). Plates were washed and developed with nitrophenyl phosphate (Amresco, Solon, OH) diluted in diethanolamine buffer (100 ml diethanolamine, 1 mM MgCl<sub>2</sub>, deionized H<sub>2</sub>O to 1 liter [pH 9.8]). Color reactions were terminated by adding 100  $\mu$ l of 2.0 M NaOH to each well. The optical density of the color reaction mixture was measured at 405 nm. Concentrations of total Ig isotypes were calculated by the interpolation of calibration curves generated by using a mouse Ig reference serum (ICN Biomedicals, Aurora, OH).

**Blocking experiments.** In experiments in which  $\alpha$ CD134L or  $\alpha$ CD154 (eBiosciences, San Diego, CA) was used to block B-cell responses, 50  $\mu$ g/ml of Ab was added to the cultures. When indicated,  $\alpha$ IFN- $\gamma$  (eBiosciences) was used at a concentration of 25  $\mu$ g/ml. In some experiments, methyl- $\alpha$ -D-mannoside (MDM; Sigma) was used to specifically inhibit the binding of ConA to splenic cells, a treatment which prevents T-lymphocyte activation. At 5 mM, MDM caused 96% inhibition of CD25 expression in CD4<sup>+</sup> T cells activated with ConA. Splenic cells were also treated with  $\alpha$ CD134L,  $\alpha$ CD154, or  $\alpha$ IFN- $\gamma$  or with MDM to monitor for cytotoxic effects of each of these reagents, of which none were noted.

**Detection of intracellular IFN- $\gamma$ .** Splenic cells were activated for 24 h with ConA plus CT, ConA plus LT-IIa, or ConA plus LT-IIb as described above. A cytokine detection kit (Becton-Dickinson Cytofix/Cytoperm) and PE-labeled  $\alpha$ IFN- $\gamma$  Ab (PharMingen) were used for the measurement of intracellular IFN- $\gamma$  in CD4<sup>+</sup> and CD8<sup>+</sup> T cells. Samples were analyzed by FACS.

**Luminex cytokine assay.** Splenic cells were activated for 4 days with ConA plus CT, ConA plus LT-IIa, or ConA plus LT-IIb as described above, and levels of interleukin-2 (IL-2), IL-4, IL-6, IL-10, and IFN- $\gamma$  secreted into culture supernatants were determined at Roswell Park Cancer Institute (Buffalo, NY) as previously reported (25).

**Statistical analysis.** Analysis of variance and the Tukey multiple-comparison tests were used for multiple comparisons. Unpaired *t* tests with Welch correction were performed to analyze differences between two groups. Statistical analyses were performed using InStat software (GraphPad, San Diego, CA). Statistical differences were considered significant at the level of *P* of  $\leq 0.05$ .

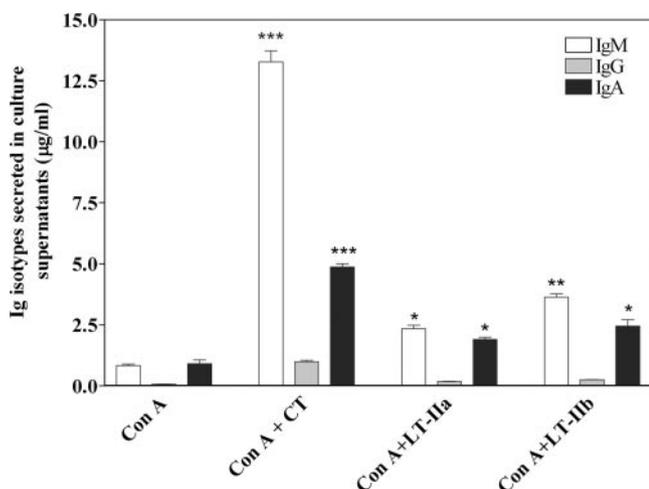


FIG. 1. CT enhances IgA and IgM secretion in ConA-stimulated splenic cells. Splenic cell suspensions were cultured with 2 µg/ml of ConA for 7 days in the presence or absence of 1 µg/ml of CT, LT-IIa, or LT-IIb. Levels of IgA, IgM, and IgG secreted into culture supernatants were examined by ELISA. Results are reported as the arithmetic means  $\pm$  standard errors of the means of results obtained from three independent experiments. \*, \*\*, and \*\*\*, significant differences at  $P$  values of  $\leq 0.05$ ,  $\leq 0.01$ , and  $\leq 0.001$ , respectively, compared to cells stimulated with ConA alone.

## RESULTS

**Induction of Ig secretion by HLT.** To evaluate the effects of HLT on Ig production, we determined the levels of IgA, IgG, and IgM in the culture supernatants of splenic cells obtained from naïve mice and stimulated with ConA for 7 days in the presence or absence of CT, LT-IIa, or LT-IIb (Fig. 1). The stimulation of splenic cells solely with ConA did not induce substantial amounts of IgA, IgG, or IgM. Low levels of IgA and IgM secretion were evident in ConA-stimulated cultures treated with either LT-IIa or LT-IIb. The treatment of splenic cells with ConA in the presence of CT, however, produced levels of IgA and IgM that were dramatically higher than the levels of IgA and IgM in the culture supernatants of cells treated only with ConA or with the additional presence of either LT-IIa or LT-IIb. Little or no IgG was detected in the supernatants of cell cultures stimulated solely with ConA or with ConA in the presence of CT, LT-IIa, or LT-IIb. These results showed that CT potentiates the selective production and secretion of IgA and IgM by ConA-stimulated splenic cells.

**Induction of Ig-SC by CT.** Although splenic cell cultures concomitantly stimulated with ConA and CT induced the appearance of Ig in the supernatant, the source of the Ig had yet to be determined. To evaluate whether CT stimulated the differentiation of B cells into Ig-SC, murine splenic cell populations were depleted of CD138<sup>+</sup> plasma cells by using MACS and the remaining cells were activated with ConA for 4 days in the presence or absence of CT, LT-IIa, or LT-IIb. The frequencies of CD138<sup>+</sup> cells expressing low levels of B220 (B220<sup>low</sup>), a phenotype typical of Ig-SC (18, 39), were determined by FACS (Fig. 2A). At day 4, a population of B220<sup>low</sup>, CD138<sup>+</sup> cells appeared in cultures treated with ConA plus CT but not in cultures treated with ConA alone, ConA plus LT-IIa

(Fig. 2A), or ConA plus LT-IIb (data not shown). From day 4 to day 7, the frequency of B220<sup>low</sup>, CD138<sup>+</sup> cells in cultures treated with ConA plus CT increased from 5% to 15%, a pattern of expression which suggested that these cells were actively proliferating (Fig. 2A). Division profiles of these putatively proliferating Ig-SC were evaluated using CFSE staining (Fig. 2B). By day 4 of culture, the loss of CFSE by the newly generated CD138<sup>+</sup> cells indicated that proliferation preceded the emergence of CD138<sup>+</sup> cells. Additionally, several phenotypic changes that accompany B-cell differentiation into Ig-SC (e.g., the down-regulation of B220 and CD40 expression) (3, 18, 39) were observed in the CD138<sup>+</sup> cell population (Fig. 2C). Levels of expression of CD19 and MHC-II molecules in the CD138<sup>+</sup> cells were also elevated to levels comparable to those in cells expressing high levels of B220. Taken together, the high levels of CD19 and MHC-II expression and the division profile of the CD138<sup>+</sup> cells strongly indicated that these cells were plasma blasts. Subsequent staining for intracellular Ig confirmed that the CD138<sup>+</sup> cells contained mainly IgA or IgM (Fig. 2D). In addition, following purification by MACS and overnight culturing, CD138<sup>+</sup> cells secreted only IgA and IgM in the culture supernatant. No Ig secretion was detectable in the culture supernatant of CD138<sup>-</sup> cells (data not shown).

The data suggest that the treatment of splenic cells with ConA plus CT induced the development of two populations of Ig-SC which, although expressing the same surface marker phenotype (B220<sup>low</sup>, CD138<sup>+</sup>, CD19<sup>+</sup>, MHC-II<sup>+</sup>, and CD40<sup>low</sup>), differed in the levels of production of predominant Ig classes (i.e., IgA and IgM).

**Direct activation of B cells by CT.** In the eventual differentiation of B cells into Ig-SC, cellular activation precedes proliferation (18, 39). To investigate whether CT induces the activation of resting B cells, the expression levels of various activation molecules were examined after the treatment of cells with CT. CD19<sup>+</sup> B cells purified from splenic populations and cultured for 48 h in the presence or absence of CT, LT-IIa, or LT-IIb were subsequently analyzed for levels of expression of CD25, CD54, CD40, CD69, CD80, CD86, and MHC-II (Fig. 3). Each of these surface markers is known to be up-regulated in B lymphocytes after antigen recognition (19, 33). Compared to untreated CD19<sup>+</sup> cells, cells stimulated with CT exhibited enhanced surface expression of CD25, CD69, CD80, CD86, and MHC-II. LT-IIa and LT-IIb did not increase the expression of these five markers in CD19<sup>+</sup> cells (data not shown). Levels of expression of CD40 and CD54 by CD19<sup>+</sup> cells were not altered by treatment with either CT, LT-IIa, or LT-IIb. The results of these experiments firmly demonstrated that CT, but not LT-IIa or LT-IIb, directly induces the activation of resting B lymphocytes.

**B-cell proliferation induced by activated CD4<sup>+</sup> T cells is enhanced by CT.** Consecutive divisions of B lymphocytes increase the probability of Ig-SC development (18). Thus, the increased generation of Ig-SC observed in cultures stimulated with ConA plus CT could be, in part, a result of the capacity of CT to induce the proliferation of B cells. To explore this possibility, splenic cells labeled with CFSE were stimulated with ConA for 4 days in the presence or absence of CT, LT-IIa, or LT-IIb. The proliferation of CD19<sup>+</sup> cells was undetectable in untreated control cultures (data not shown). Stimulation with ConA elicited a modest proliferation of CD19<sup>+</sup> cells (Fig.

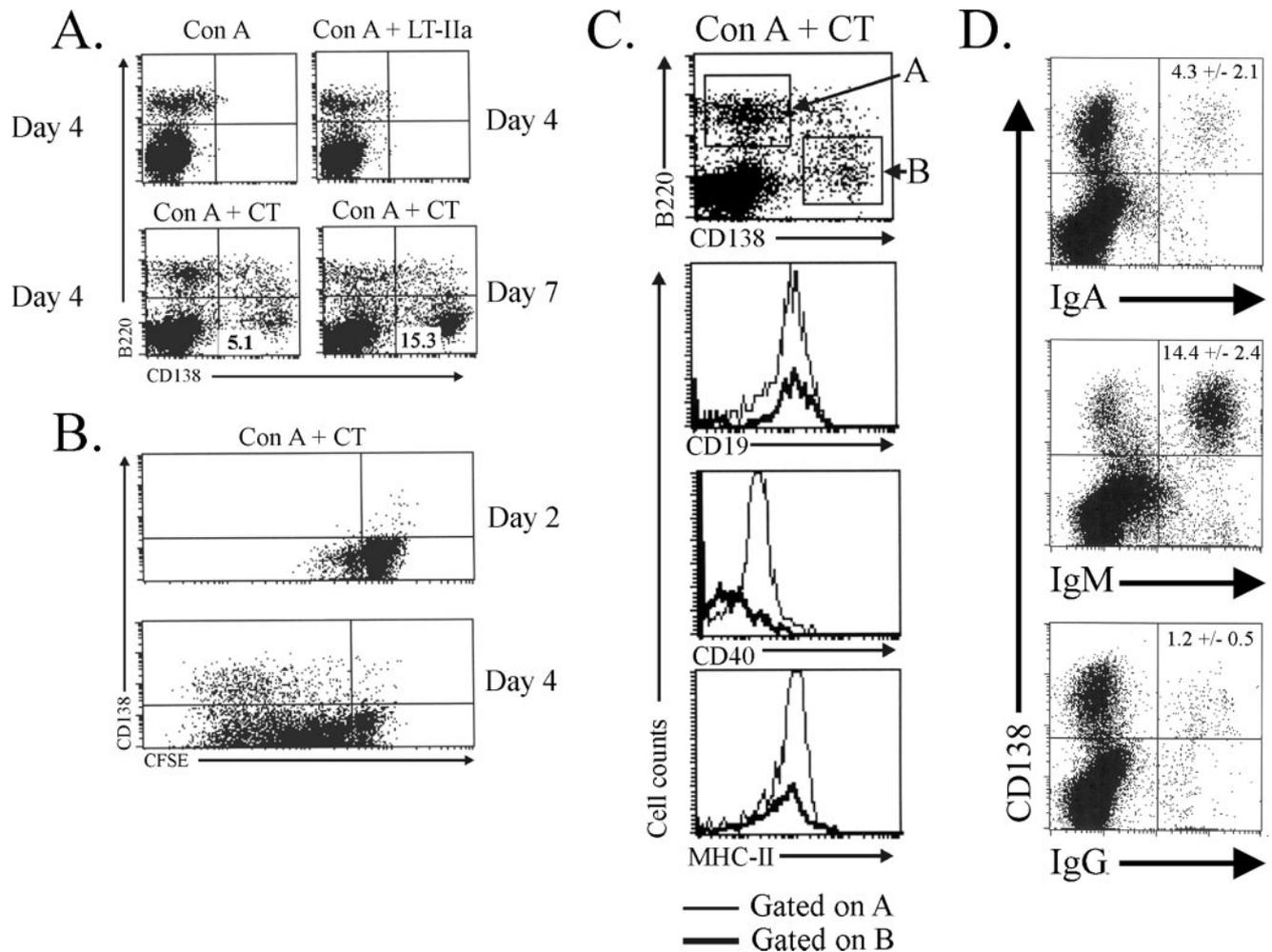


FIG. 2. CT induces a population of cells expressing a plasma blast phenotype. (A) Splenic cell suspensions depleted of CD138<sup>+</sup> cells were cultured with 2 μg/ml of ConA for 4 or 7 days in the presence or absence of 1 μg/ml of CT or LT-IIa. Cells stained for B220 and CD138 were analyzed by FACS for frequencies of viable cells expressing the two cell markers, and the frequencies are given as percentages. (B) CFSE-labeled splenic cell suspensions were cultured with 2 μg/ml of ConA for 2 or 4 days in the presence of 1 μg/ml of CT. Cells were stained with CD138, and the frequencies of dividing CD138<sup>+</sup> cells were determined by FACS. (C) Expression of CD19, CD40, and MHC-II on CD138<sup>+</sup> cells expressing high levels of B220 (gate A) and on B220<sup>low</sup>, CD138<sup>+</sup> cells (gate B) from cultures treated with ConA plus CT for 4 days. (D) Cells from cultures stimulated with ConA plus CT for 7 days were analyzed for intracellular contents of IgA, IgG, and IgM by FACS, and results are given as percentages (means ± standard deviations).

4A), which was not further increased in the presence of either LT-IIa or LT-IIb (data not shown). When cells were activated with ConA in the presence of CT, however, the frequency of dividing CD19<sup>+</sup> cells was substantially elevated in comparison to that in cultures treated solely with ConA (Fig. 4A) or with ConA plus LT-IIa or ConA plus LT-IIb (data not shown). Furthermore, CD19<sup>+</sup> cells from cultures treated with ConA plus CT experienced additional rounds of division compared to CD19<sup>+</sup> cells treated with ConA alone (Fig. 4A) or in the presence of LT-IIa or LT-IIb (data not shown).

To test the hypothesis that CT-induced proliferative responses of B cells also depended upon a contribution of T lymphocytes, splenocytes depleted of CD4<sup>+</sup> cells were treated for 4 days with ConA plus CT. Neither the proliferation nor the differentiation of B cells into the B220<sup>low</sup>, CD138<sup>+</sup> phenotype (data not shown) was evident in these cultures (Fig. 4B). A similar lack of proliferation and differentiation was observed

when CD4 cell-depleted splenocyte cultures were treated for 4 days with either CT or ConA alone (Fig. 4B) or when T-cell activation by ConA was inhibited by treatment with MDM (Fig. 4C). The elimination of CD4<sup>+</sup> cells had no observable effect on the LPS-induced proliferation and differentiation of B cells into Ig-SC, indicating that B cells contained in the CD4<sup>-</sup> fraction were functionally competent and that their inability to proliferate and differentiate in response to ConA, CT, or ConA plus CT was not the result of cellular defects acquired during the magnetic elimination of CD4<sup>+</sup> cells (data not shown). Thus, the proliferation and differentiation of B cells within splenic cell cultures stimulated with ConA plus CT are dependent upon the activation of CD4<sup>+</sup> T lymphocytes.

**CT enhances the expression of CD134 and CD154 in ConA-activated CD4<sup>+</sup> T cells.** To investigate whether contact with CT renders CD4<sup>+</sup> T lymphocytes more competent to help in B-cell differentiation, the effects of CT on the expression of

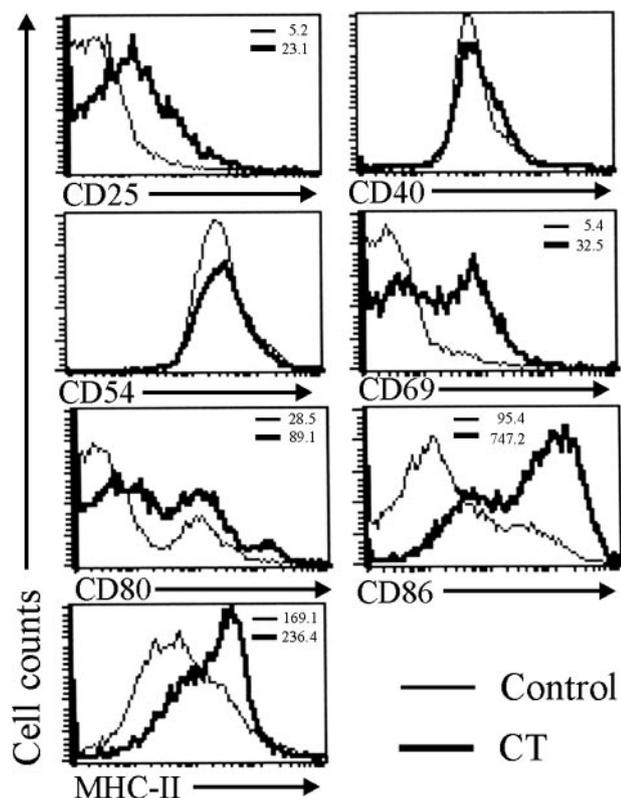


FIG. 3. Effect of CT on activation marker expression by isolated B cells. MACS-purified CD19<sup>+</sup> cells were cultured for 48 h in the presence or absence of 1  $\mu$ g/ml of CT. The expression of CD25, CD40, CD54, CD69, CD80, CD86, and MHC-II on these cells was analyzed by FACS. Numbers in each histogram represent the mean fluorescence intensities of the respective cell populations in relative units. Representative data from three independent experiments are shown.

CD134 and CD154, two activation markers which are crucial components of contact-dependent T-cell help, in CD4<sup>+</sup> T cells were examined (37, 42). Purified preparations of CD4<sup>+</sup> T cells were incubated in the presence or absence of CT, LT-IIa, or LT-IIb. After washing to remove unbound enterotoxins, cells stimulated for 6, 24, 48, or 72 h with ConA plus CD11b<sup>+</sup> cells were analyzed by FACS for levels of expression of CD134 and CD154 (Fig. 5). Treatment with ConA for 6 h in the presence of CT did not alter the expression of CD134 in CD4<sup>+</sup> T cells. Similar results were obtained in control experiments when CD4<sup>+</sup> T cells were stimulated solely with ConA. The expression of CD154 was rapidly induced on a portion of untreated CD4<sup>+</sup> T cells after 6 h of ConA activation. In contrast, the treatment of CD4<sup>+</sup> T cells for 6 h with CT in the presence of ConA slightly reduced the cells' expression of CD154. The stimulation of CD4<sup>+</sup> T cells for 24 h with ConA alone elicited the expression of CD134 by the majority of the cells, while additional treatment with CT only slightly increased the levels of CD134. Treatment with CT also increased the levels of CD134 expressed by ConA-activated CD4<sup>+</sup> T cells at 48 h. After 24 h of ConA stimulation, in comparison to untreated CD4<sup>+</sup> T cells, CD4<sup>+</sup> T cells treated with CT expressed increased levels of CD154. Importantly, enhanced levels of CD154 in CD4<sup>+</sup> T cells treated with CT were still observed at

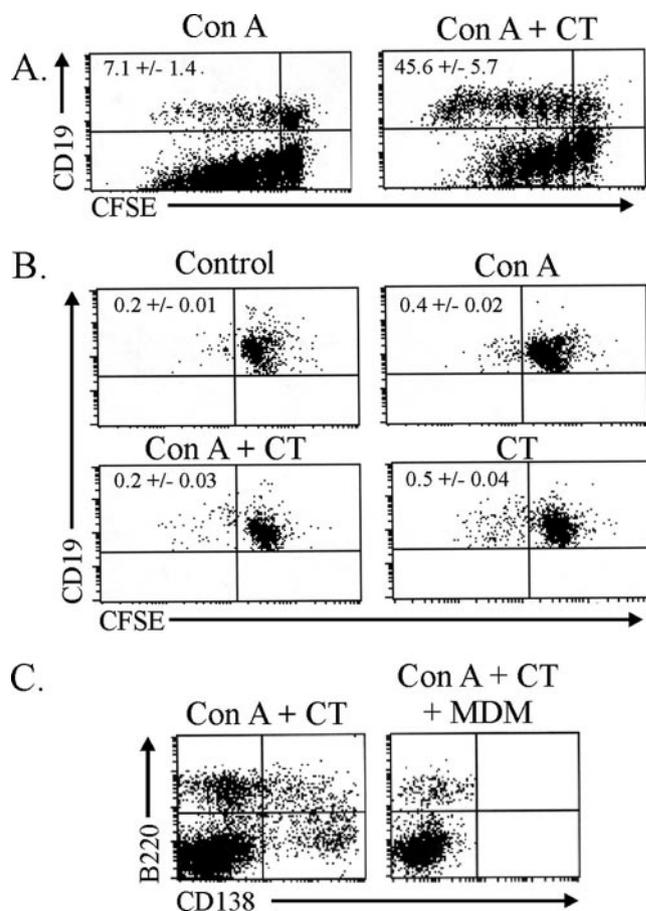


FIG. 4. Enhancement of B-cell proliferation by CT. (A) CFSE-labeled splenic cell suspensions were cultured with 2  $\mu$ g/ml of ConA in the presence or absence of 1  $\mu$ g/ml of CT. Frequencies of dividing CD19<sup>+</sup> cells were determined at day 4 of culture and are given as percentages. (B) CFSE-labeled splenic cell suspensions were depleted of CD4<sup>+</sup> cells by MACS and cultured for 4 days with either ConA, ConA plus CT, CT, or culture medium alone. Cells were stained with CD19, and the frequencies of dividing CD19<sup>+</sup> cells were analyzed by FACS. Frequencies are given as percentages (means  $\pm$  standard deviations). Plots were gated on CD19<sup>+</sup> cells. (C) Splenic cell suspensions were cultured with 2  $\mu$ g/ml ConA plus 1  $\mu$ g/ml CT for 4 days in the presence or absence of 5 mM MDM. Cells stained for B220 and CD138 were analyzed by FACS for frequencies of viable cells expressing the two cell markers.

48 h, when the levels of CD154 in untreated cells had declined. Seventy-two hours after stimulation, levels of CD134 expressed by untreated CD4<sup>+</sup> T cells were decreased relative to the 48-h levels. Treatment with CT, however, maintained higher levels of CD134 in CD4<sup>+</sup> T cells at 72 h. By 72 h, CD154 became undetectable in CT-treated or untreated CD4<sup>+</sup> T cells. The kinetics of the expression of CD134 and CD154 in CD4<sup>+</sup> T cells stimulated with ConA plus LT-IIa or LT-IIb were identical to those in CD4<sup>+</sup> T cells stimulated with ConA alone (data not shown). As expected, neither CD134 nor CD154 was detectable at any time point on CD4<sup>+</sup> T cells from unstimulated control cultures. Finally, in the absence of ConA, the treatment of purified preparations of CD4<sup>+</sup> T cells for 6, 24, 48, or 72 h with CT, LT-IIa, or LT-IIb did not induce the expression of CD25, CD69, CD134, or CD154 (data not

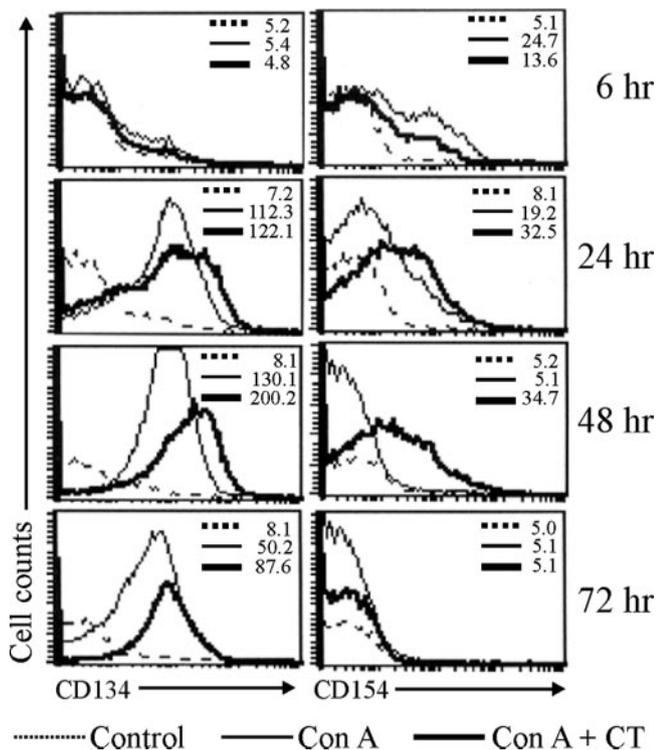


FIG. 5. CT enhances the expression of CD134 and CD154 in ConA-activated CD4<sup>+</sup> T cells. Purified preparations of CD4<sup>+</sup> T cells were incubated in the presence or absence of 1  $\mu$ g/ml CT. After washing to remove unbound enterotoxin, cells were stimulated for 6, 24, 48, or 72 h with ConA plus CD11b<sup>+</sup> cells and analyzed by FACS for levels of expression of CD134 and CD154. Plots were gated on CD4<sup>+</sup> cells. Numbers in each histogram represent the mean fluorescence intensities of the respective cell populations in relative units. Representative data from three independent experiments are shown.

shown). These results strongly indicated that CT enhances and stabilizes the expression of the activation markers CD134 and CD154 in ConA-stimulated CD4<sup>+</sup> T cells.

**Blockage of CD154 inhibits Ig-SC development and Ig secretion.** The engagement of the B-cell surface molecules CD40 and CD134L by their T-cell counter receptors (CD154 and CD134, respectively) has a central role in the activation, expansion, and differentiation of B cells participating in T-cell-dependent responses (37, 42). Since treatment with CT enhances the levels of CD134 and CD154 expression in ConA-activated CD4<sup>+</sup> T cells, we hypothesized that increased levels of these molecules in CD4<sup>+</sup> T lymphocytes facilitate CD134L-CD134 and/or CD40-CD154 interactions between B and T cells, thus enhancing subsequent B-cell responses. To investigate whether such improved interactions were directly responsible for the effects of CT on plasma cell induction and Ig secretion, monoclonal antibodies to CD134L or CD154 were used to block the respective receptor-ligand interactions. Populations of cells isolated from spleens of naïve mice and depleted of CD138<sup>+</sup> cells by MACS were activated with ConA plus CT for 7 days in the presence of  $\alpha$ CD154,  $\alpha$ CD134L, or isotype control antibodies, and the frequencies of cells expressing the B220<sup>low</sup>, CD138<sup>+</sup> phenotype were determined by FACS (Fig. 6A). Levels of IgA, IgG, and IgM in the superna-

tants from these cultures were also measured as a direct assessment of Ig-SC activity (Fig. 6B). Treatment with  $\alpha$ CD154 substantially reduced the frequencies of B220<sup>low</sup>, CD138<sup>+</sup> cells in comparison to those in cultures treated with control Ab, and the reduced frequencies correlated with a significant reduction in the levels of IgA, IgG, and IgM secreted into the supernatant (Fig. 6A and B). Similar results, but of lesser magnitude, were observed when  $\alpha$ CD134L was used to block CD134-CD134L interactions, i.e., both the frequencies of B220<sup>low</sup>, CD138<sup>+</sup> cells were reduced and the levels of IgA and IgM secretion were diminished (Fig. 6A and B). In contrast, levels of IgG were not affected by  $\alpha$ CD134L treatment. Combined treatment with  $\alpha$ CD154 and  $\alpha$ CD134L did not further reduce the frequency of B220<sup>low</sup>, CD138<sup>+</sup> cells or the levels of IgA, IgG, or IgM secreted into the supernatant relative to treatment with  $\alpha$ CD154 alone (Fig. 6A and B).

Finally, to evaluate the effect of  $\alpha$ CD154 on B-cell proliferation, splenic cells labeled with CFSE were stimulated with ConA plus CT in the presence of  $\alpha$ CD154 or control Ab (Fig. 6C). At day 4 following stimulation, the frequencies of dividing CD19<sup>+</sup> cells in cultures treated with  $\alpha$ CD154, as determined by the evaluation of CFSE content, were substantially lower than the frequencies of dividing CD19<sup>+</sup> cells in cultures treated with control Ab. These data show that CD154 up-regulation is responsible for the enhanced proliferation of B cells that precedes the development of Ig-SC.

Collectively, these results strongly indicated that enhanced CD40-CD154 interactions are mainly responsible for the augmented B-cell responses observed in cultures of splenic cells stimulated with ConA plus CT.

**Modulation of cytokine production by CT.** Cytokines act in various combinations at various stages of the complex B-cell activation sequence and can exert either stimulatory or inhibitory effects on B-cell responses (34). To determine whether CT modulates cytokine responses required for the elicitation of Ig responses, the levels of various cytokines produced by splenic cells were examined after treatment with ConA plus CT. Supernatants from cultures of splenic cells cultured for 4 days in the presence or absence of CT, LT-IIa, or LT-IIb were subsequently analyzed for levels of IL-2, IL-4, IL-6, IL-10, and IFN- $\gamma$  by using a Luminex cytokine assay (Fig. 7). Levels of IL-2 were substantially reduced in cultures treated with CT (data not shown). Treatment with LT-IIa or LT-IIb, however, did not affect IL-2 levels (data not shown). Levels of IL-4 were very low or undetectable in culture supernatants of cells stimulated in the presence of CT, LT-IIa, or LT-IIb or in untreated control cultures (data not shown). In contrast, IL-6 and IL-10 were both detectable at significantly higher concentrations in culture supernatants of cells treated with either CT, LT-IIa, or LT-IIb; CT and LT-IIa induced the highest levels of IL-6 and IL-10. Although the levels of IFN- $\gamma$  were reduced by treatment with LT-IIa, levels of IFN- $\gamma$  were extremely reduced in CT-treated cultures. These results indicated that CT, in particular, modulates IFN- $\gamma$  responses in cultures of splenic cells stimulated with ConA.

**Inhibition of IFN- $\gamma$  production by CT is essential to induce Ig-SC development and Ig production.** IFN- $\gamma$  exerts potent inhibitory effects on B-cell responses (31, 36). Thus, the inhibition of IFN- $\gamma$  production by CT may be an important mechanism by which the enterotoxin enhances Ab responses. To test

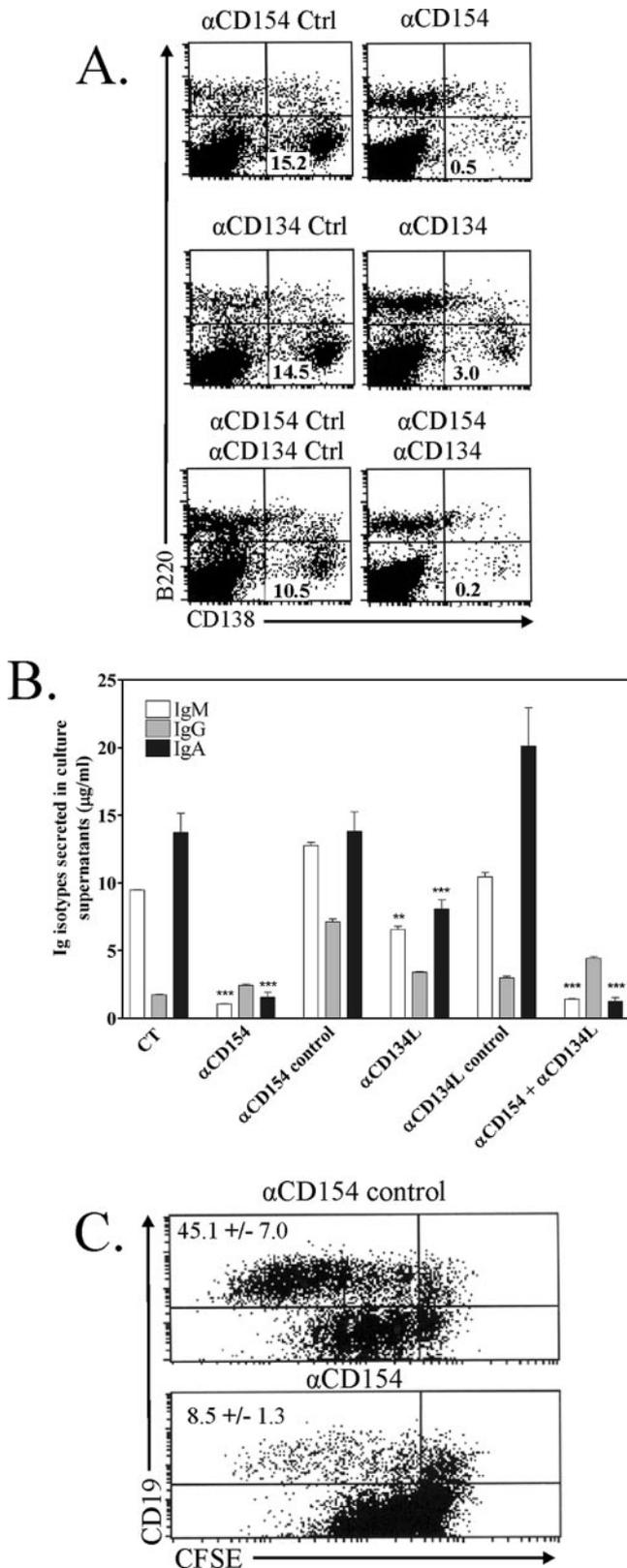


FIG. 6. Blockage of CD134 or CD154 inhibits Ig-SC development and Ig production. (A) Splenic cell suspensions (unlabeled or labeled with CFSE) were depleted of CD138<sup>+</sup> cells and cultured for 7 days with 2  $\mu$ g/ml ConA plus 1  $\mu$ g/ml CT in the presence of  $\alpha$ CD134L,  $\alpha$ CD154,  $\alpha$ CD134 plus  $\alpha$ CD154, or an appropriate isotype control Ab.

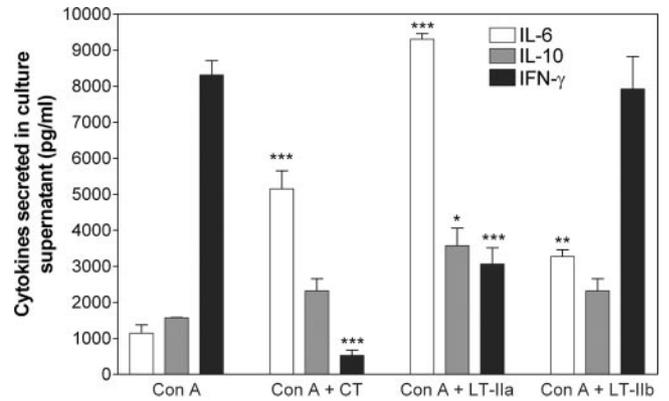


FIG. 7. Modulation of cytokine production by CT, LT-IIa, and LT-IIb. Splenic cell suspensions were cultured with 2  $\mu$ g/ml of ConA for 4 days in the presence or absence of 1  $\mu$ g/ml of CT, LT-IIa, or LT-IIb, at which time the levels of IL-6, IL-10, and IFN- $\gamma$  secreted into the culture supernatants were measured by a multiplex cytokine assay. \*, \*\*, and \*\*\*, significant differences at *P* values of  $\leq 0.05$ ,  $\leq 0.01$ , and  $\leq 0.001$ , respectively, compared to cells stimulated with ConA alone.

this hypothesis, we first investigated the effect of exogenously administered IFN- $\gamma$  on the induction of polyclonal Ig synthesis by CT. Populations of cells isolated from spleens of naïve mice and depleted of CD138<sup>+</sup> cells were activated with ConA plus CT for 7 days in the presence or absence of IFN- $\gamma$  or a control cytokine (IL-4), and the frequencies of CD138<sup>+</sup> cells containing intracellular IgA, IgM, or IgG were determined by FACS (data not shown). Levels of IgA, IgG, and IgM in the supernatants from these cultures were also measured as a direct evaluation of Ig-SC activity (Fig. 8A). Treatment with IFN- $\gamma$  at doses ranging from 1 to 10 ng/ml substantially reduced (10-fold reduction) the frequencies of CD138<sup>+</sup> cells expressing intracellular IgA, IgG, or IgM in comparison to those in untreated cultures or cultures treated with IL-4 (data not shown). This reduction was correlated with a significant decrease in the levels of IgA, IgG, and IgM secreted into the supernatant (Fig. 8A). Next, we investigated the types and frequencies of IFN- $\gamma$ -producing cells in cultures treated with CT, LT-IIa, or LT-IIb. Splenic cells were stimulated for 24 h with ConA in the presence or absence of each of the three enterotoxins. CD4<sup>+</sup> and CD8<sup>+</sup> T cells were subsequently analyzed for intracellular contents of IFN- $\gamma$  by FACS (Fig. 8B). Frequencies of IFN- $\gamma$ -producing CD4<sup>+</sup> and CD8<sup>+</sup> T cells in ConA-stimulated cultures were higher than the frequencies of IFN- $\gamma$ -producing CD4<sup>+</sup> and CD8<sup>+</sup> T cells in unstimulated control cultures (data not shown). Frequencies of IFN- $\gamma$ -producing CD4<sup>+</sup> and CD8<sup>+</sup> T cells in cultures treated with ConA plus LT-IIa or LT-IIb

Frequencies of cells expressing the B220<sup>low</sup>, CD138<sup>+</sup> phenotype were determined and are given as percentages. (B) Levels of IgA, IgM, and IgG secreted into culture supernatants were determined by ELISA. (C) Frequencies of dividing CD19<sup>+</sup> cells in cultures treated with  $\alpha$ CD154 or control Ab were analyzed by FACS at day 4 of culture and are given as percentages (means  $\pm$  standard deviations). Representative data from three independent experiments are shown. \*\* and \*\*\*, significant differences at *P* values of  $\leq 0.01$  and  $\leq 0.001$ , respectively, compared to the  $\alpha$ CD134L and  $\alpha$ CD154 controls.

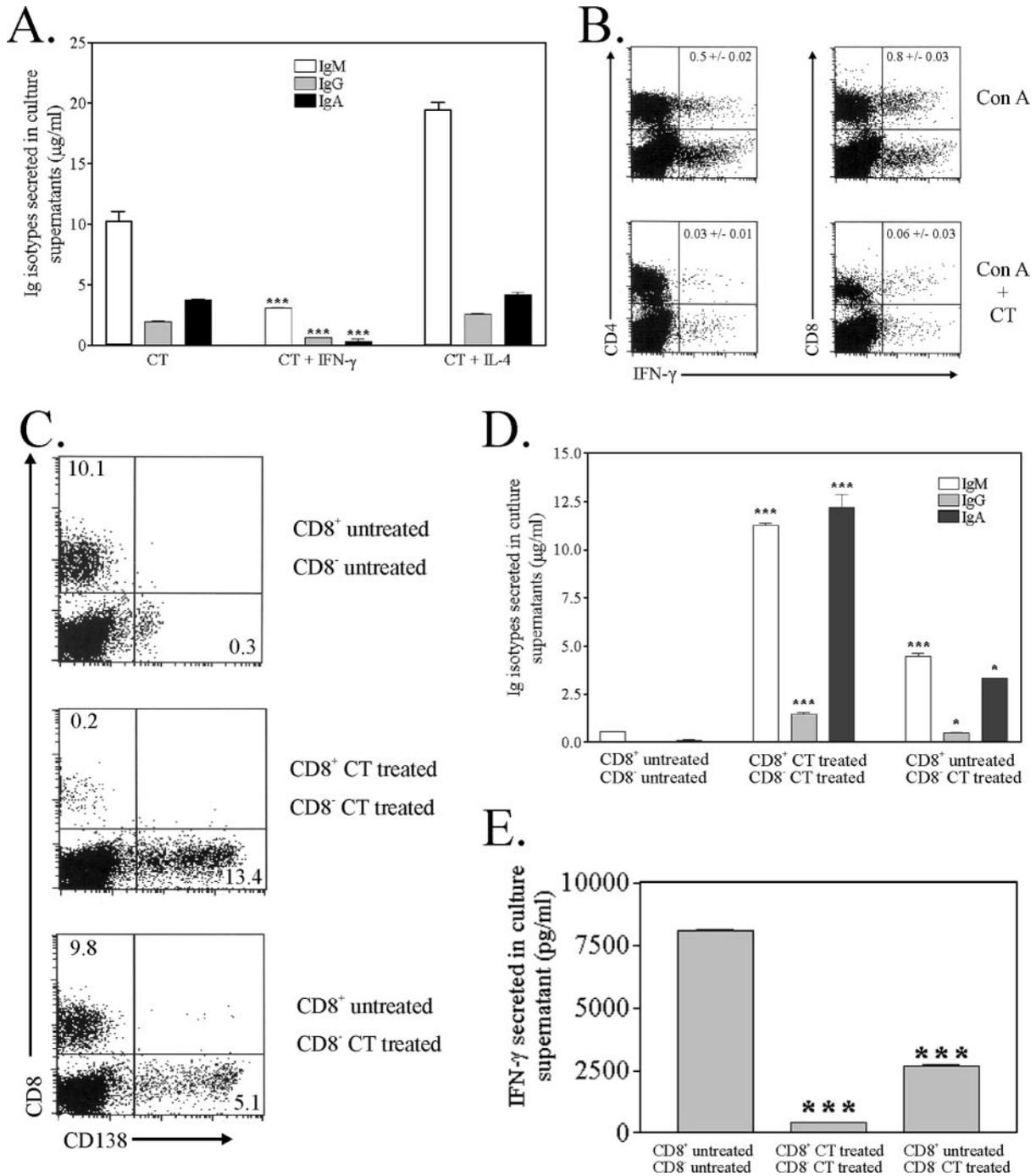


FIG. 8. Inhibition of IFN-γ production by CT favors Ig-SC development and Ig production. Splenic cell suspensions were depleted of CD138<sup>+</sup> cells and cultured for 7 days with 2 µg/ml ConA plus 1 µg/ml CT in the presence or absence of 10 ng/ml of IFN-γ or 10 ng/ml of IL-4. (A) Levels of IgA, IgM, and IgG secreted into culture supernatants were determined by ELISA. \*\*\*, significant differences at a *P* value of 0.001 compared to CT or CT + IL-4. (B) Frequencies of IFN-γ-producing CD4<sup>+</sup> or CD8<sup>+</sup> T cells in cultures treated with ConA plus CT or ConA alone were determined by FACS at day 2 of culture and are given as percentages (means ± standard deviations). (C) Purified preparations of CD8<sup>+</sup> or CD8<sup>-</sup> cells, preincubated in the presence or absence of CT, were mixed together as described in the text and stimulated for 7 days with ConA, and the frequencies of cells expressing CD8 and CD138 were determined by FACS and are given as percentages. Representative data from three independent experiments are shown. (D) Levels of IgA, IgG, and IgM secreted into culture supernatants were determined by ELISA. (E) Levels of IFN-γ secreted into culture supernatants were measured by a Luminex cytokine assay. \* and \*\*\*, significant differences at *P* values of ≤0.01 and ≤0.001, respectively, compared to CD8<sup>+</sup>-untreated and CD8<sup>-</sup>-untreated preparations.

were similar to the frequencies of these cells in cultures treated solely with ConA (data not shown). CT, however, dramatically reduced the frequencies of IFN-γ-producing CD4<sup>+</sup> and CD8<sup>+</sup> T cells in ConA-activated cultures (Fig. 8B). Since CT induces

the apoptosis of ConA-activated CD8<sup>+</sup> cells (3), and consequently inhibits IFN-γ production by these cells, the apoptosis of CD8<sup>+</sup> T cells may indirectly favor the development of Ig-SC and Ig production. To explore this possibility, fractions of

CD8<sup>+</sup> or CD8<sup>-</sup> splenocytes depleted of CD138<sup>+</sup> cells, each preincubated in the presence or absence of CT, were combined (i.e., untreated CD8<sup>+</sup> cells plus untreated CD8<sup>-</sup> cells, CT-treated CD8<sup>+</sup> cells plus CT-treated CD8<sup>-</sup> cells, and untreated CD8<sup>+</sup> cells plus CT-treated CD8<sup>-</sup> cells), stimulated for 7 days with ConA, and analyzed by FACS for the expression of CD8 and CD138 (Fig. 8C). Levels of IgA, IgG, and IgM and of IFN- $\gamma$  in the supernatants from these cultures were also measured by ELISA (Fig. 8D) and a Luminex cytokine assay (Fig. 8E), respectively. As expected, few CD138<sup>+</sup> cells in ConA-stimulated mixtures of CD8<sup>+</sup> plus CD8<sup>-</sup> cells that had been cultured in the absence of CT were observed (Fig. 8C). Small amounts of IgA, IgG, and IgM and high levels of IFN- $\gamma$  were also detected in the supernatants of these cultures (Fig. 8D and 8E). A substantial induction of CD138<sup>+</sup> cells concomitant with a loss of CD8<sup>+</sup> cells was observed when both CD8<sup>+</sup> and CD8<sup>-</sup> cells were pretreated with CT (Fig. 8C). High levels of IgA, IgG, and IgM and reduced levels of IFN- $\gamma$  were secreted into the supernatant (Fig. 8D and E). When mixtures of untreated CD8<sup>+</sup> cells plus CT-treated CD8<sup>-</sup> cells were analyzed, however, a reduction in the numbers of CD138<sup>+</sup> cells was observed relative to those in both cell populations treated with CT (Fig. 8C). This reduction was reversed by the addition of neutralizing  $\alpha$ IFN- $\gamma$  monoclonal Ab to the cell culture (data not shown). Decreased amounts of IgA, IgG, and IgM and high levels of IFN- $\gamma$  were also found in the supernatants of these cultures (Fig. 8D and E). Taken together, these results indicated that both CD4<sup>+</sup> and CD8<sup>+</sup> T cells exert inhibitory effects on polyclonal Ab production that are mediated by IFN- $\gamma$  and that the induction of apoptosis in CD8<sup>+</sup> T cells and the suppression of IFN- $\gamma$  production in CD4<sup>+</sup> and CD8<sup>+</sup> T cells are important mechanisms by which CT enhances Ab responses.

## DISCUSSION

The analysis of enterotoxin-treated splenic cells activated with ConA demonstrated that CT exerted distinct stimulatory effects on Ig production within splenic cell populations that were less apparent or absent in cultures treated with either LT-IIa or LT-IIb. Specifically, treatment with CT significantly enhanced the levels of secretion of IgA and IgM, but not that of IgG, by splenic cells. The enhanced levels of IgA and IgM in CT-treated cells correlated with the emergence of a population of CD138<sup>+</sup>, B220<sup>low</sup> Ig-SC that was the source of Ig secreted into the culture supernatant. That the CT-induced Ig-SC population expressed CD138 is in accord with results from other studies that demonstrated that the expression of this surface marker correlates strongly with the generation of Ig-SC both in vitro and in vivo (18, 39). Relevant phenotypic and functional differences within the Ig-SC compartment of mice have revealed that at least two discrete populations of Ig-SC can be identified: (i) plasma blasts and (ii) plasma cells. Plasma blasts are formed in the extrafollicular region of the secondary lymphoid tissues during the early response to Ag, exhibit a short life span, and proliferate. In contrast, plasma cells reside predominantly in the bone marrow as nondividing cells that have reached a stable, prolonged survival state (18). While MHC-II is expressed on plasma blasts, the expression of this molecule is down-modulated in mature plasma cells (29). The expression of MHC-II, therefore, correlates well with the expansion phase

of the Ig-SC response and, thus, can be employed as a surrogate marker to distinguish proliferating plasma blasts from noncycling plasma cells (18, 29). We found that the population of Ig-SC induced by CT expressed high levels of MHC-II that corresponded to the levels expressed by undifferentiated B cells. These data strongly indicated that the Ig-SC induced by CT were plasma blasts. Additionally, the CD138<sup>+</sup> cells induced by ConA plus CT proliferated and expressed CD19 at levels comparable to those of B cells. Recent studies demonstrated that normal Ig-SC in inductive sites express CD19 at high levels and that this molecule is progressively down-regulated during the plasma blast growth phase (28). The proliferative phenotype and the CD19 expression profile of the CD138<sup>+</sup> population reinforced our conclusion that the cells induced by treatment with ConA plus CT were plasma blasts.

Collectively, these results indicate that the Ig-SC in the splenic population are probably the in vitro equivalents of extrafollicular Ig-SC and distinctive from the long-lived, bone marrow-localized plasma cells.

Despite their ability to serve as adjuvants in vivo, LT-IIa and LT-IIb exhibited much weaker capacities to enhance Ig production in vitro by splenic cells. Nor did these two enterotoxins promote plasma blast differentiation of B cells. Studies to elucidate the mechanisms responsible for this differential effect revealed that CT up-regulated the expression of CD25, CD69, CD80, CD86, and MHC-II in a substantial proportion of isolated B cells. Neither LT-IIa nor LT-IIb, however, substantially up-regulated the expression of these same activation markers. Also, the B pentamer of CT is known to enhance MHC-II expression in B cells and the B pentamer of LT-I up-regulates CD25, B7, and MHC-II in B lymphocytes (12, 33). Thus, the up-regulation of these activation markers in B lymphocytes by CT is likely due to signals generated by the high-affinity binding of the enterotoxin to GM<sub>1</sub>, a ganglioside located on the B-cell surface (3, 33). GM<sub>1</sub> associates with lipid rafts that harbor numerous proteins associated with cell signaling (10). The binding of CT to GM<sub>1</sub> within lipid rafts could mediate interactions of the enterotoxin with signaling molecules that modulate B-lymphocyte activation. Although we do not rule out a possible contribution of the A subunit to the stimulatory effect, the different ganglioside binding specificities of CT, LT-IIa, and LT-IIb may be strong determinants of their distinct stimulatory effects on B cells (1, 3).

In T cell-dependent Ab responses, the differentiation of resting B cells into Ig-SC requires both direct physical interactions between B cells and T cells and soluble cytokines derived from T helper cells (34). The major entity on the surfaces of activated CD4<sup>+</sup> T cells responsible for contact-dependent B-cell activation is CD154. CD154<sup>-/-</sup> mice show a profound defect in T-cell-dependent Ab responses, particularly those involving Abs of switched isotypes (45). Treatment with CT induced the up-regulation of CD154 and sustained the levels of expression of this molecule for extended periods of time in ConA-activated CD4<sup>+</sup> T cells, an effect that was not elicited by either LT-IIa or LT-IIb. The binding of CD154 to CD40 initiates the signals required for the activation, proliferation, and differentiation of B cells (34, 35, 45). The blockage of CD154-CD40 interactions completely inhibited the CT-dependent induction of B-cell responses in these experiments, thus demonstrating that CD154-CD40 interactions are directly responsible

for the stimulatory effect of CT on B cells. The enhancement of CD154-CD40 interactions by CT may facilitate the development of Ig responses by inducing antiapoptotic genes and the reexpression of telomerase activity in B cells, thereby promoting the survival of these lymphocytes (44). In addition, cross-linking of CD154 delivers costimulatory signals that induce IL-4 synthesis by CD4<sup>+</sup> T cells (6). Thus, triggering of CD154 on CD4<sup>+</sup> T cells may lead to the development of Th2 cells and eventually to increased Ig production.

The expression of CD154 could also have been differentially regulated in the CD4<sup>+</sup> T cells by the modulation of cytokines induced by the various enterotoxins. The treatment of splenic cells with CT significantly reduced IFN- $\gamma$  production in cultures stimulated with ConA; effects on IFN- $\gamma$  production were less evident in cultures treated with LT-IIa or LT-IIb. Since IFN- $\gamma$  inhibits CD154 expression in activated CD4<sup>+</sup> T cells, decreased levels of this cytokine could indirectly enhance the expression of CD154 in ConA-activated CD4<sup>+</sup> T cells (37). Additionally, triggering of CD28 in conjunction with the T-cell receptor-CD3 complex stabilizes CD154 expression in CD4<sup>+</sup> T cells (24). Since CT, but not LT-IIa or LT-IIb, induced the up-regulation of CD80 and CD86 in B cells, interactions of these B-cell surface receptors with constitutively expressed CD28 could induce and maintain the high-level expression of CD154 in activated CD4<sup>+</sup> T lymphocytes.

In the absence of Ag, the proliferation and differentiation of memory B cells are stimulated by bystander T cells via CD154. This mechanism has been hypothesized to be involved in the maintenance of immunological memory in humans (5). It is possible, therefore, that the up-regulation of CD154 by CT facilitates the development of humoral memory by promoting bystander activation of memory B lymphocytes. Our studies on the kinetics of the expression of CD154 in ConA-plus-CT-activated CD4<sup>+</sup> T cells revealed that CD154 was down-modulated to undetectable levels after 48 h of culture. Continuous interactions between CD154 and CD40 provide inhibitory signals for B-cell differentiation into Ig-SC (35). Thus, the down-modulation of CD154 expression in CD4<sup>+</sup> T cells has the potential to interrupt those inhibitory signals, thereby permitting B-cell differentiation into Ig-SC.

Other surface molecules expressed by activated CD4<sup>+</sup> T cells deliver contact-dependent help to B cells (34). Herein, we demonstrated that CD134 was up-regulated in CD4<sup>+</sup> T cells by CT but not by either LT-IIa or LT-IIb. The blockage of CD134-CD134L interactions, however, did not exert an additional effect on the inhibition of Ig-SC development produced by the blockage of CD154, indicating that the effect of CD154 was predominant over that of CD134. This pattern of response is in accordance with the observation that the expression of CD134L in activated B cells is dependent upon either CD40 or B-cell receptor signaling (42). CD134-CD134L interactions are critical for the development of T-cell-dependent Ab responses *in vivo*; the blockage of CD134-CD134L interactions is associated with a profound decrease in Ag-specific Ab responses (43). In addition, costimulatory signals delivered through CD134 have been implicated in selectively promoting the differentiation of Th2 cells (11). The potent adjuvant activity of CT towards humoral immune responses may be a direct result of the enhancement of CD134 expression by CT in CD4<sup>+</sup> T cells.

CT induces apoptosis in ConA-activated CD8<sup>+</sup> T cells (3). Although when differentiated, CD8<sup>+</sup> T cells become cytotoxic T cells, these cells are also a major source of IFN- $\gamma$ , a cytokine that can directly inhibit B-cell activation, proliferation, and differentiation (31, 36). Here we show that the reduction of CD8<sup>+</sup> cells by CT decreases the levels of IFN- $\gamma$  and facilitates the development of polyclonal Ig responses. Conversely, the lack of inhibition of IFN- $\gamma$  production by LT-IIa or LT-IIb may partially account for the diminished ability of these HTL to stimulate polyclonal Ab responses *in vitro*.

Early IFN- $\gamma$  production by CD8<sup>+</sup> cells contributes to the priming of CD4<sup>+</sup> T cells for subsequent development into polarized Th1 cells (8). Although Th1 cells can cooperate with B cells to induce Ig-SC differentiation, Th1 cells do not usually exhibit the full complement of cytokines characteristic of Th2 cells. Therefore, Th1 cells are less effective than Th2 cells in providing B-cell help (40). It is possible that the induction of apoptosis of CD8<sup>+</sup> cells by CT and the subsequent reduction in IFN- $\gamma$  levels inhibit the differentiation of CD4<sup>+</sup> T cells into Th1 effectors, indirectly favoring the development of Ab responses *in vivo*.

CT, LT-IIa, and LT-IIb enhanced IL-6 and IL-10 production in cultures of splenic cells stimulated with ConA. Since IL-6 and IL-10 are potent inducers of Ab secretion in various systems of B-cell activation (34), these results suggest that IL-6 and IL-10 are likely to be involved in the regulation of B-cell responses by the three enterotoxins when appropriate contact-dependent signals from T cells are available.

While it is clear from these experiments that the stimulation of Ab responses by CT relies upon the activation of B cells, the up-regulation of CD134 and CD154 in CD4<sup>+</sup> T cells, and the inhibition of IFN- $\gamma$  production by CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes, it is less clear what interactions underlie the ability of LT-IIa and LT-IIb to augment Ab responses *in vitro* or *in vivo*. It is possible that other cell types or different contact-dependent signals are involved in immune stimulation by LT-IIa and LT-IIb. LT-IIa and LT-IIb bind to dendritic cells. The binding of LT-IIa and LT-IIb to dendritic cells is correlated with the up-regulation of MHC-II and B7 molecules in these cells (S. Arce, T. D. Connell, and M. W. Russell, unpublished results). In addition, the B pentamers of LT-IIa and LT-IIb induce the secretion of IL-1 $\beta$ , IL-6, IL-8, and tumor necrosis factor in various antigen-presenting cell types by a mechanism which depends on interactions with TLR-2 (17). Dendritic cells have been shown to respond to TLR-2 signals and to modulate events leading to B-cell activation and differentiation (4, 9). Thus, it is possible that dendritic cells mediate the Ab-enhancing effects of the type II enterotoxins.

CT, LT-IIa, and LT-IIb interact with lymphocytes in markedly different manners. These interactions induce a variety of different events which have the potential to exert dramatic effects on immune responsiveness. Comparing and contrasting the immunomodulatory activities of CT, LT-IIa, and LT-IIb will likely reveal novel regulatory pathways for eliciting Ab responses which will elucidate new cellular and molecular mechanisms that underlie adjuvanticity.

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