ELSEVIER

Vaccine 21 (2003) 1613–1619

Vaccine

www.elsevier.com/locate/vaccine

Induction of cytotoxic T lymphocyte responses by cholera toxin-treated bone marrow-derived dendritic cells

Myoung Ho Jang [a], Mi-Na Kweon [a], Takachika Hiroi [a], Masafumi Yamamoto [a,b], Ichiro Takahashi [a,c], Hiroshi Kiyono [a,d,*]

[a] *Department of Mucosal Immunology, Research Institute for Microbial Diseases, Osaka University, Osaka 565-0871, Japan*
[b] *Department of Oral Medicine, Nihon University, School of Dentistry at Matsudo, Chiba 271-8587, Japan*
[c] *Department of Host Defense and Preventive Dentistry, Hiroshima University Graduate School of Biomedical Sciences, Hiroshima 734-8553, Japan*
[d] *Devision of Mucosal Immnuology, Institute of Medical Science, The University of Tokyo, Tokyo 108-8639, Japan*

Received 6 August 2002; received in revised form 24 October 2002; accepted 6 November 2002

Abstract

Cholera toxin (CT), a powerful mucosal adjuvant, is a potent inducer of Th2-type responses via activation of co-stimulatory molecules for the induction of IgA antibody responses. Less appreciated is the ability of CT to induce and regulate cytotoxic T lymphocyte (CTL) responses. In order to help for clarifying mechanisms underlying the CTL-inducing ability of CT, we have examined the effects of CT on dendritic cells (DCs) that could lead to the induction of cytotoxic $CD8^+$ T cells. When bone marrow-derived DCs (BM-DCs) were cultured with CT in vitro, B7-1 but not B7-2 molecules were significantly enhanced and allogenic CTL responses were induced. Also, increased numbers of IFN-γ-secreting $CD8^+$ T cells were elicited when CT-treated BM-DCs were co-cultured with allogenic $CD8^+$ CTLs. Antibody blockade of B7-1 on CT-treated BM-DCs suppressed allogenic CTL responses, further indicating the importance of CT-induced B7-1 molecules on DCs for the acquisition of cytolytic function by CTL precursors. CD40 signaling was proven not necessary for the CT-induced CTL response since CT-treated $CD40^{-/-}$ BM-DCs developed CTL responses equivalent to those detected in CT-treated BM-DCs derived from normal mice. Our results suggest that CT-treated DCs are effective inducers of $CD8^+$ CTL, and this induction is mediated through CT's ability to enhance B7-1 expression on DCs.

Keywords: Cholera toxin; Dendritic cells; CTL; B7-1

1. Introduction

The induction of potent immune responses in vaccination requires adjuvant to deliver danger/stimulation signals to dendritic cells (DCs) [1]. The activation of DC is crucial in the initiation of many adaptive immune responses, including lymphocyte-mediated immunity via pattern-recognition innate immunity. DCs are potent antigen-presenting cells (APCs) that initially recognize danger signals via an array of pattern-recognition molecules for the subsequent induction of acquired immunity through internalization and processing of foreign infectious antigens [1]. DCs usually exist in a quiescent state in most tissues, but certain stimuli, such as microbial products (e.g. LPS) and inflammatory cytokines (e.g. TNF-α), trigger the DCs to become mature or activated [2]. During the maturation process, DCs undergo numerous phenotypic and functional changes: a redistribution of the major histocompatibility complex (MHC) from intracellular endocytic compartments to the cell surface, down-regulation of antigen internalization, increase in the surface expression of co-stimulatory molecules, and morphologic changes [2–4].

Cholera toxin (CT), a powerful mucosal adjuvant produced by *Vibrio cholerae*, can support the induction of antigen (Ag)-specific secretory-IgA (S-IgA) and serum IgG antibody (Ab) responses to co-administered protein after oral or nasal immunization [5–8]. Some evidence indicates that CT exerts its adjuvanticity through action on APCs, including DCs and macrophages: two independent groups have reported that CT-treated human monocyte-derived DC enhanced Th2-dominated T cell responses via suppression of IL-12 production and up-regulation of co-stimulatory molecules [9,10]. Also, CT induced the expression of the co-stimulatory molecule B7-2, which is responsible for Th2 priming [11,12] on murine B cells and macrophages in vitro [13,14]. Results of earlier studies suggested that the mucosal adjuvanticity of CT is closely associated with

* Corresponding author. Tel.: +81-6-6879-8292; fax: +81-6-6878-6765.
E-mail address: kiyono@biken.osaka-u.ac.jp (H. Kiyono).

0264-410X/02/$ – see front matter © 2002 Elsevier Science Ltd. All rights reserved.
PII: S0264-410X(02)00734-X

ADP–ribosyltransferase activity [7], but we have reported that a single amino acid substitution-mutant of CT (S61F and E112K), lacking ADP–ribosyltransferase-activity, retained adjuvanticity [15–17].

In addition to CT's well-known role in inducing Th2-type immune responses [8,16,18], CT also is capable of supporting cytotoxic T lymphocyte (CTL) responses [19–22]. CTLs are critical in controlling viral infectious diseases and may enhance protective immunity against mucosally transmitted viral pathogens [23]. The differentiation of naïve $CD8^+$ CTL into effector cells is thought to require at least two signals. The first is provided during the engagement of the T cell receptor (TCR) with the peptide/MHC class I on APCs. The second is provided by the interaction between accessory molecules, such as CD28 on the surface of $CD8^+$ T cells, and the ligands B7-1 or B7-2 on the APCs [24,25]. Ligation of CD28 by B7 molecules provokes the release of IL-2 from Ag-specific $CD8^+$ T cells, leading to the cells' acquisition of cytolytic capability [26,27].

The cellular and molecular mechanisms responsible for CT's adjuvant effects, especially on CTL, are still poorly understood. To help define some of these mechanisms, we have herein examined the contribution of CT-treated DCs for the generation of CTL responses.

2. Materials and methods

2.1. Mice

BALB/c and C57BL/6 mice were purchased from Japan Clea Co. (Tokyo, Japan). $CD40^{-/-}$ mice of C57BL/6 background were obtained from Jackson Laboratory (Bar Harbor, ME). All mice were maintained in the experimental animal facility at the Research Institute for Microbial Diseases, Osaka University (Osaka, Japan).

2.2. Antibodies (Abs) and adjuvants

The following Abs, all of which were purchased from BD PharMingen (San Diego, CA), were used in this study: FITC-anti-CD11c (HL3), PE-anti-B7-1 (16-10A1), PE-anti-B7-2 (GL1), FITC-anti-CD40 (HM40-3), FITC-anti-IFN-γ, (XMG1.2), PE-anti-TNF-α (MP6-XT22), PE-anti-IL-12(p40/p70) (C15.6), Cy-Chrome-anti-CD4 (RM4-5), Cy-Chrome-anti-CD8α (53-6.7), PE-rat IgG1 (R3-34), anti-CD16/CD32 (2.4G2), anti-CD3ε (145-2C11) and anti-CD28 (37.51). CT was obtained from List Biologic Laboratories (Campbell, CA), and LPS (*Escherichia coli* 026:B6) was purchased from Sigma (St. Louis, MO).

2.3. DC culture

Bone marrow (BM) cells were removed from the femurs of mice and depleted of erythrocytes by hypotonic lysis. BM cells were cultured for 6 days in RPMI medium plus 10% FCS, 20 mM HEPES, sodium pyruvate, β-mercaptoethanol, penicillin–streptomycin, 10 ng/ml granulocyte–monocyte–colony-stimulating factor (GM–CSF) (BD Pharmingen, San Diego, CA) and 5 ng/ml IL-4 (PeproTech, London, UK) [3]. At days 2 and 4, nonadherent cells were removed, and fresh medium with the cytokines was added. At day 6, nonadherent cells were harvested and used as immature BM-derived DCs (BM-DCs). Because of variability in the level of constitutive activation as well as in stimulation, each experiment had its own positive (LPS) and negative (untreated) controls; results were compared only within and not between experiments. Thus, BM-DCs were then cultured with CT (10 ng/ml), LPS (1 μg/ml) or medium only. Concentration of CT (10 ng/ml) and LPS (1 μg/ml) was selected based on the previous studies [3,9]. Also, we found this concentration of CT had no effect on the viability of BM-DCs. Further, biological activity of the dose of CT used in this study was confirmed by the suppression of TNF-α production from LPS-treated BM-DCs (data not shown; 9). Splenic lymphocytes were co-cultured with the CT- or LPS-stimulated allogenic BM-DCs for 3–5 days in 12-well culture plates and harvested for phenotypic analysis, intracellular cytokine expression and cytotoxicity (see below).

2.4. Cytotoxicity assay

The cytotoxicity mediated by CTL generated in mixed lymphocyte culture (MLC) was measured by use of a ^{51}Cr-release assay, as described [28]. Briefly, H-2^d-specific cytotoxicity was determined, using BALB/c-derived P815 mastocytoma cells (H-2^d) as target cells. As control, C57BL/6-derived EL4 cells (H-2^b) were used. Different numbers of effector cells were cultured with ^{51}Cr-labeled target cells (1×10^4 cells per well) for 5 h at the indicated E:T ratio in 200 μl of complete RPMI medium in 96-well round-bottom plates. After 5 h incubation, 100 μl of supernatant from triplicate cultures were collected and the level of ^{51}Cr-release was counted. The percent cytotoxicity was calculated according to the formula: (experimental release − spontaneous release) / (maximum release − spontaneous release) × 100, where spontaneous release represents the counts obtained when the target cells were cultured in medium in the absence of effectors and maximum release represents the counts obtained when the target cells were lysed with 1% Triton X-100.

2.5. Intracellular cytokine assay

For intracellular cytokine analysis, cells were restimulated with soluble anti-CD28 mAb (2 μg/ml) in 24-well flat-bottomed plates coated with anti-CD3 mAb (10 μg/ml) for 4 h in the presence of GolgiStop (BD PharMingen). Cytoplasmic staining was performed, using Cytofix/Cytoperm Kits (BD PharMingen). Specific mAb-labeled cells were analyzed in a flow cytometry analysis, using FACScalibur (Becton Dickinson, San Jose, CA).

2.5.1. In vitro blocking studies with anti-B7-1 mAb

After stimulation with CT or LPS, BM-DCs were washed in cold PBS and incubated on ice for 30 min in the presence of 25 μg/ml of purified anti-B7-1 mAb (1G10; BD PharMingen), anti-B7-2 mAb (GL1; BD PharMingen) or control monoclonal Ab rat IgG2a (R35-95; BD PharMingen) [29]. DCs were then washed in cold PBS and the conventional MLC assay for generation of CTL, as described above, was conducted.

3. Results

3.1. CT enhanced B7-1 expression on BM-DCs

Since several studies have demonstrated that CT influences the expression of B7 molecules on professional APCs [10,13,14], we initially determined the effects of CT on BM-DCs. BM-DCs were co-cultured with 10 ng/ml of CT for 24 h and analyzed for expression of the co-stimulatory molecules B7-1, B7-2 and CD40. As illustrated in Fig. 1, CT enhanced B7-1 expression on the BM-DCs as early as 24 h but did not induce expression of B7-2 or CD40. In contrast to stimulation with CT, stimulation with LPS up-regulated B7-2 and CD40 as well as B7-1. Thus, in these in vitro conditions, CT was a potent, rapid and specific inducer of B7-1 on BM-DCs.

3.2. Induction of allogenic CTL responses by CT-treated BM-DCs

A recent study has shown that co-stimulatory signals provided by B7-1 molecules are important for the acquisition of cytolytic capacity by $CD8^+$ T cells [29]. Therefore, we next evaluated whether CT-treated BM-DCs could enhance CTL responses. To minimize the possibility of experimental error introduced by contamination with Ag-derived endotoxin, we employed an allogenic CTL assay system. Splenic lymphocytes from C57BL/6 ($H\text{-}2^b$) mice were co-cultured with allogenic BM-DCs ($H\text{-}2^d$) which had been preactivated with CT or LPS for 24 h. After 5 days cultivation, live cells were harvested and a standard CTL assay was performed, with P815 mastocytoma cells ($H\text{-}2^d$) as target cells. As shown in Fig. 2A, CT significantly enhanced the ability of DC to induce a CTL response against the P815 cells, and the level of the CTL response was comparable to responses induced by LPS. The results of these in vitro experiments suggested that CT can provide stimulation signals for the induction of CTL responses.

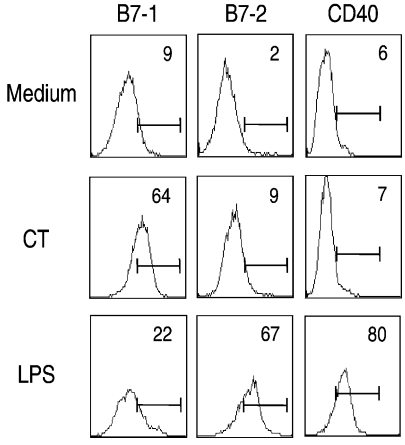

Fig. 1. CT selectively up-regulates membrane expression of B7-1 on immature DCs. BALB/c bone marrow-derived DCs (BM-DCs) were grown for 6 days; then CT (10 ng/ml) or LPS (1 μg/ml) was added to the wells for an additional 24 h incubation. Cells were then collected and first stained for the DC-specific marker CD11c, then stained for B7-1, B7-2 and CD40 by use of respective mAbs conjugated with appropriate fluorescence dyes. The data were electronically gated for $CD11c^+$ cells and are representative of four separate experiments. Numbers in histogram represent the percentage of positive cells.

3.3. High levels of IFN-γ production by CT-treated BM-DCs stimulated splenic $CD8^+$ T cells

A direct correlation between IFN-γ synthesis and CTL activity by $CD8^+$ T cells has been established [30]. Thus, we next measured IFN-γ synthesis by $CD8^+$ T cells co-cultivated with CT-treated BM-DCs. Intracellular cytokine analysis showed that the frequency of IFN-γ-producing $CD8^+$ T cells was greater in the co-cultures containing CT-treated BM-DCs than in those containing untreated BM-DCs (Fig. 2B). The IFN-γ secreting $CD8^+$ T cells simultaneously produced TNF-α. The frequency of $CD8^+$ T cells producing IFN-γ and TNF-α (double positive) induced by CT-treated BM-DCs was similar to the frequency induced by LPS-treated DCs. As expected, the frequency of IFN-γ-producing $CD4^+$ T cells also was increased in the presence of CT or LPS-treated BM-DCs. The net results of these experiments indicate that the CTL response resulting from CT's activation of BM-DCs is ultimately mediated by IFN-γ producing $CD8^+$ T cells.

3.4. Blocking of B7-1 on CT-treated DCs suppressed allogenic CTL responses

Our results thus far suggested that CT initiated allogenic CTL responses by its ability to enhance the expression of B7-1 on BM-DCs. To confirm this possibility, we performed blocking experiments with anti-B7-1 mAb, anti-B7-2 mAb or isotype control mAb (rat IgG2a). BM-DCs treated with either CT or LPS were incubated with optimal concentration of anti-B7-1 or anti-B7-2 mAb prior to the MLC assays. The anti-B7-1 antibody pretreatment of CT-treated BM-DCs resulted in the significant reduction of allogenic CTL responses (Fig. 3A). In case of anti-B7-2 antibody treatment, the level of CTL activity was slightly decreased. In contrast to CT-treated BM-DCs, neither anti-B7-1 nor anti-B7-2 influenced on the LPS-treated BM-DCs (Fig. 3B). These findings further implicated B7-1 expression induced on DCs by

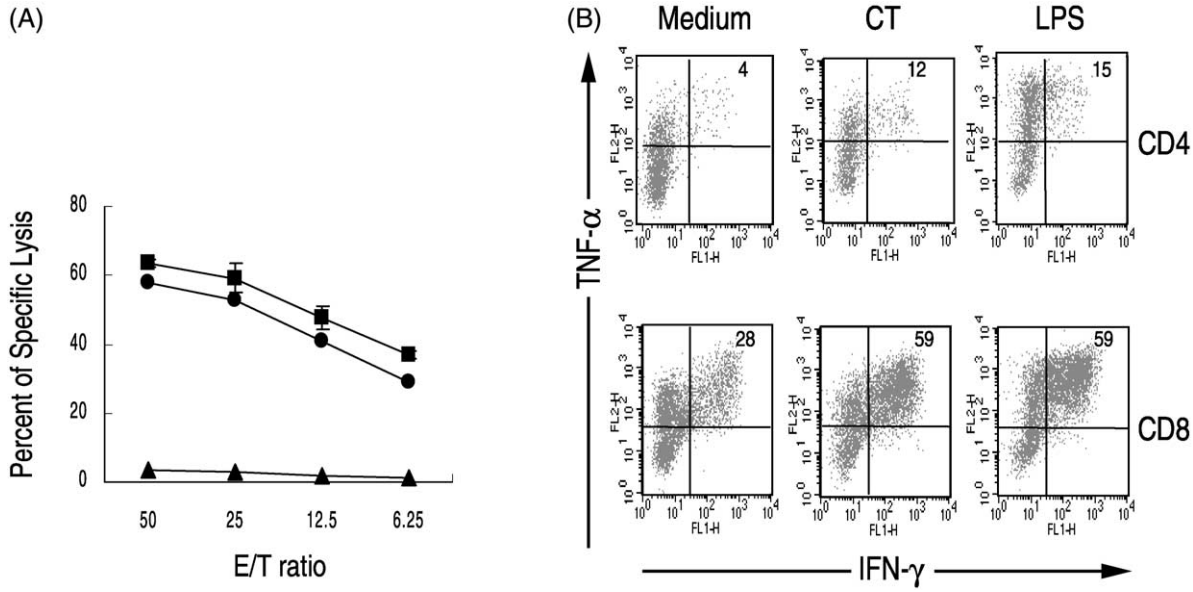

Fig. 2. Induction of IFN-γ producing $CD8^+$ CTLs by CT-treated BM-DCs. (A) CT enhances the ability of allogenic DCs to induce CTL responses. C57BL/6 mouse spleen cells (H-2^b) were stimulated with BM-DCs (▲), CT-treated BM-DCs (●) or LPS-treated BM-DCs (■) derived from BALB/c BM (H-2^d) in MLC for 5 days. Cells were harvested from the MLC and examined for their cytotoxicity against P815 (H-2^d) cells in a 5 h ^{51}Cr-release assay. (B) CT-treated allogenic BM-derived DC enhanced the frequency of IFN-γ producing $CD8^+$ T cells. Intracellular staining of TNF-α and IFN-γ was carried out as described in Section 2. Numbers represent the percentage of cells in each quadrant. The results shown are representative of three separate experiments.

CT in the acquisition of cytolytic function by $CD8^+$ CTL precursors.

3.5. CD40 signaling is not necessary for the induction of CTL responses by CT

Although signaling through CD40–CD40L interaction is one of the important co-stimulatory pathways for enhancement of cytotoxic responses and IFN-γ production by CTLs [31,32], CT failed to induce CD40 expression on BM-DCs in vitro (Fig. 1). To verify that CT treatment did not lead to enhanced CTL activity via a CD40-dependent pathway, we performed CTL assays and measured IFN-γ production in experiments using CD40-deficient BM-DCs pre-treated or not pre-treated with CT. CTL and intracellular IFN-γ analyses showed that the CTLs killing activity and IFN-γ production were maintained after co-culture with CT-treated allogenic CD40-deficient BM-DCs, but not with LPS-treated allogenic CD40-deficient DC (Fig. 4A and B). These data are evidence that CD40–CD40L co-stimulation signals were not involved in the CT-induced CTL responses.

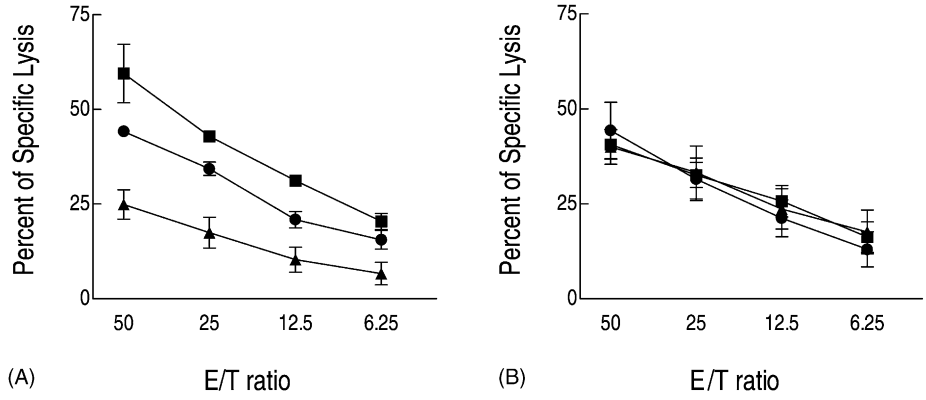

Fig. 3. Blocking of B7-1 on CT-treated DCs suppressed the induction of allogenic CTL responses. BM-DCs (H-2^d), treated with CT (A) or LPS (B), were incubated with anti-B7-1 Ab (▲), anti-B7-2 Ab (●) or rat IgG Ab (■) as isotype control, then co-cultured with C57BL/6 mouse spleen cells (H-2^b) for 5 days. Cells were harvested from the MLC and examined for their cytotoxicity against P815 (H-2^d) cells by use of 5 h ^{51}Cr-release assay. The results shown are representative of three separate experiments.

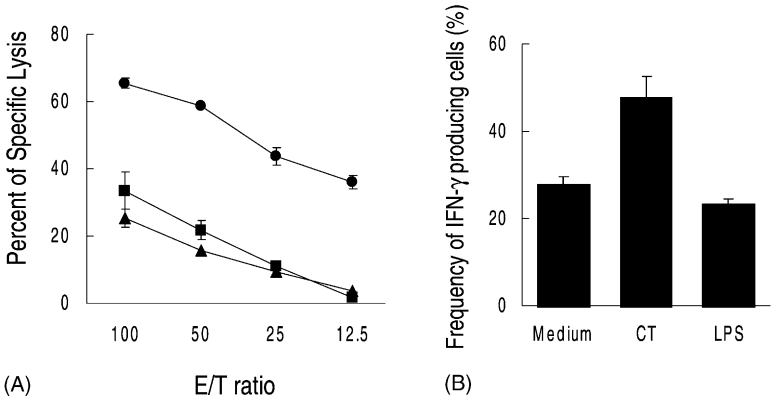

Fig. 4. Induction of IFN-γ producing $CD8^+$ CTLs by CT-treated CD40-deficient DCs. (A) BALB/c mice spleen cells ($H\text{-}2^d$) were stimulated with BM-DCs (▲), CT-treated BM-DCs (●) or LPS-treated BM-DCs (■) derived from $CD40^{-/-}$ mice (C57BL/6 background) BM ($H\text{-}2^b$) for 5 days. Cells were harvested from the MLC and examined for their cytotoxicity against EL4 ($H\text{-}2^b$) cells by use of ^{51}Cr-release assay. (B) Intracellular staining of IFN-γ was carried out as described in Fig. 2 legend. The bars represent mean ± S.E. of triplicate samples.

4. Discussion

CT is an effective mucosal adjuvant which elicits classical IL-4-mediated Th2-type immune responses for the induction of systemic IgG and mucosal IgA Ab responses [8,16,18]. CT also has been a useful adjuvant for the induction of CTL responses [20,21], which are not normally associated with strong Th2-type immune activity. In order to help for defining mechanisms involved in the induction of CTL responses by CT, we have explored the effects of CT on B7-1 and B7-2 expression on DCs since the previous studies had shown that these co-stimulatory molecules are involved in CT's adjuvant effects [13,14,33]. We focused on DCs because these cells are effective professional APCs for the stimulation of naïve T cells to initiate primary Th1, Th2 and CTL immune responses [34].

Our results showed that CT rapidly and specifically enhanced the expression of B7-1 on murine BM-DCs in vitro (Fig. 1). Since B7-1 molecules can enhance CTL activity [29,35–37] and Th1 responses [11,12], we then addressed the influence of CT-treated BM-DCs on the induction of CTL responses and IFN-γ production by $CD8^+$ T cells (Fig. 2). CT significantly enhanced the ability of BM-DCs to induce CTL responses together with high levels of IFN-γ synthesis, and did so via the preferential induction of B7-1 (Fig. 3).

In other work, the two co-stimulatory ligands B7-1 and B7-2 have exhibited different functions: B7-2 preferentially augmented responses associated with MHC class II epitopes, whereas B7-1 generated MHC class I-mediated CTL responses [38]. Reportedly, CT stimulation of bone marrow macrophages and $Mac\text{-}1^+$ cells isolated from Peyer's patches was associated with an increased expression of B7-2, but not B7-1 [13,14]; it is not clear why CT significantly up-regulated B7-1 on BM-DCs but up-regulated B7-2 on macrophages, but perhaps CT-induced intracellular signaling pathway differs between DCs and macrophages.

$CD4^+$ T lymphocytes play a central role in the regulation of both cell-mediated and humoral immune responses. In general, helper signals provided by $CD4^+$ T cells are essential for the induction and maintenance of $CD8^+$ CTL responses [39], as illustrated by the finding that cell-associated ovalbumin [40] and DNA vaccine [41] have required $CD4^+$ T cells for the induction of $CD8^+$ CTL. The $CD4^+$ T cell help is mediated through CD40L signaling of CD40 on APCs [31,32]. In contrast to the $CD4^+$ T cell requirement for the generation of the CTL responses, $CD4^+$ T cell help was not critical in CTL responses to herpesvirus [42], or the induction of $CD8^+$ CTL responses by the injection of class I binding peptides in complete Freund's adjuvant [40]. Taken together, these findings indicate that $CD4^+$ T cell help provided via CD40L and CD40 is involved in the induction and maintenance of CTL responses. In our present work, however, CT elicited CTL responses independent of CD40L and CD40 molecules since CT-treated BM-DCs from $CD40^{-/-}$ mice supported the induction of CTL responses (Fig. 4). Thus, activation signals provided by CT may bypass the requirement of CD40L- and CD40-mediated help from $CD4^+$ T cells. This observation may imply that the induction of CTL via CT-treated DCs could be useful in tumor immunotherapy because tumor cells lack available class II-restricted antigenic determinants.

The cytokine IL-12 is essential for the generation of CTL responses to mucosally delivered peptide immunogens [23], and it has contributed to ovalbumin-specific CTL responses induced by immune stimulating complexes [43]. A role for IL-12 in the induction of CTL also is implied by the observation that the administration of exogenous IL-12 at the time of immunization with either a recombinant protein [44] or encoded in a DNA vaccine [45] enhanced the induction of Ag-specific CTL responses. Although the co-administration of CT has supported the generation of CTL responses [23], CT also has inhibited Th1 immune responses by suppressing IL-12 production from human DCs [9,10]. In our work,

perhaps CT-treated BM-DCs maintained a low-level of IL-12 synthesis, sufficient for the generation of $CD8^+$ CTL. To this end, our recent preliminary result has demonstrated that low levels of IL-12 are detected in BM-DCs treated with CT associated molecules including CT-B and mutant CT (E112K) (data not shown). In support of this view, less IL-12 is required for the induction of $CD8^+$ CTL than for the generation of $CD4^+$ Th1 responses [46,47].

In summary, in this work we have demonstrated that CT provides adjuvant activity for the induction of CTL responses by specifically stimulating the expression of B7-1 on DCs, resulting in the generation of IFN-γ producing $CD8^+$ CTLs. The possibility that these observations will be useful in the application of CT adjuvanticity to the development of viral vaccines and cancer therapy deserves investigation.

Acknowledgements

This work was supported by grants from the Ministry of Health, Labor and Welfare of Japan, the Ministry of Education, Culture, Sports, Science and Technology of Japan and the Japan Health Science Foundation, and CREST, Japan.

References

[1] Gallucci S, Matzinger P. Danger signals: SOS to the immune system. Curr Opin Immunol 2001;13(1):114–9.
[2] Banchereau J, Steinman R. Dendritic cells and the control of immunity. Nature 1998;392(6673):245–52.
[3] Gallucci S, Lolkema M, Matzinger P. Natural adjuvants: endogenous activators of dendritic cells. Nat Med 1999;5(11):1249–55.
[4] Reis e Sousa C, Sher A, Kaye P. The role of dendritic cells in the induction and regulation of immunity to microbial infection. Curr Opin Immunol 1999;11(4):392–9.
[5] Jackson RJ, Fujihashi K, Xu-Amano J, Kiyono H, McGhee JR. Optimizing oral vaccines: induction of systemic and mucosal B-cell and antibody responses to tetanus toxoid by use of cholera toxin as an adjuvant. Infect Immun 1993;61:4272–9.
[6] Lycke N, Holmgren J. Intestinal mucosal memory and presence of memory cells in lamina propria and Peyer's patches in mice two years after oral immunization with cholera toxin. Scand J Immunol 1986;23:611.
[7] Lycke N, Tsuji T, Holmgren J. The adjuvant effect of *Vibrio cholerae* and *Escherichia coli* heat-labile enterotoxins is linked to their ADP–ribosyltransferase activity. Eur J Immunol 1992;22:2277–81.
[8] Marinaro M, Staats HF, Hiroi T, et al. Mucosal adjuvant effect of cholera toxin in mice results from induction of T helper 2 (Th2) cells and IL-4. J Immunol 1995;155:4621–9.
[9] Braun MC, He J, Wu CY, Kelsall BL. Cholera toxin suppresses interleukin (IL)-12 production and IL-12 receptor β1 and β2 chain expression. J Exp Med 1999;189(3):541–52.
[10] Gagliardi M, Sallusto F, Marinaro M, Langenkamp A, Lanzavecchia A, Magistris MD. Cholera toxin induces maturation of human dendritic cells and licences them for Th2 priming. Eur J Immunol 2000;30(8):2394–403.
[11] Kuchroo V, Das M, Brown J, et al. B7-1 and B7-2 co-stimulatory molecules activate differentially the Th1/Th2 developmental pathway: application to autoimmune disease therapy. Cell 1995;80(5):707–18.
[12] Freeman GJ, Boussiotis VA, Anumanthan A, et al. B7-1 and B7-2 do not deliver identical co-stimulatory signals, since B7-2 but not B7-1 preferentially costimulates the initial production of IL-4. Immunity 1995;2(5):523–32.
[13] Yamamoto M, Kiyono H, Yamamoto S, et al. Direct effects on antigen-presenting cells and T lymphocytes explain the adjuvanticity of a nontoxic cholera toxin mutant. J Immunol 1999;162(12):7015–21.
[14] Cong Y, Weaver C, Elson C. The mucosal adjuvanticity of cholera toxin involves enhancement of co-stimulatory activity by selective up-regulation of B7-2 expression. J Immunol 1997;159(11):5301–8.
[15] Yamamoto M, Briles DE, Yamamoto S, Ohmura M, Kiyono H, McGhee JR. A nontoxic adjuvant for mucosal immunity to pneumococcal surface protein A. J Immunol 1998;161(8):4115–21.
[16] Yamamoto S, Kiyono H, Yamamoto M, et al. A nontoxic mutant of cholera toxin elicits Th2-type responses for enhanced mucosal immunity. Proc Natl Acad Sci USA 1997;94:5267–72.
[17] Yamamoto S, Takeda Y, Yamamoto M, et al. Mutants in the ADP–ribosyltransferase cleft of cholera toxin lack diarrheagenicity but retain adjuvanticity. J Exp Med 1997;185:1203–10.
[18] Xu-Amano J, Kiyono H, Jackson RJ, et al. Helper T cell subsets for immunoglobulin A responses: oral immunization with tetanus toxoid and cholera toxin as adjuvant selectively induces Th2 cells in mucosa associated tissues. J Exp Med 1993;178(4):1309–20.
[19] Porgador A, Staats HF, Itoh Y, Kelsall BL. Intranasal immunization with cytotoxic T lymphocyte epitope peptide and mucosal adjuvant cholera toxin: selective augmentation of peptide-presenting dendritic cells in nasal mucosa-associated lymphoid tissue. Infect Immun 1998;66(12):5876–81.
[20] Simmons C, Mastroeni P, Fowler R, et al. MHC class I-restricted cytotoxic lymphocyte responses induced by enterotoxin-based mucosal adjuvants. J Immunol 1999;163(12):6502–10.
[21] Simmons C, Hussell T, Sparer T, Walzl G, Openshaw P, Dougan G. Mucosal delivery of a respiratory syncytial virus CTL peptide with enterotoxin-based adjuvants elicits protective, immunopathogenic, and immunoregulatory antiviral $CD8^+$ T cell responses. J Immunol 2001;166(2):1106–13.
[22] Staats HF, Bradney CP, Gwinn WM, et al. Cytokine requirements for induction of systemic and mucosal CTL after nasal immunization. J Immunol 2001;167(9):5386–94.
[23] Belyakov IM, Derby MA, Ahlers JD, et al. Mucosal immunization with HIV-1 peptide vaccine induces mucosal and systemic cytotoxic T lymphocytes and protective immunity in mice against intrarectal recombinant HIV-vaccinia challenge. Proc Natl Acad Sci USA 1998;95(4):1709–14.
[24] Chambers CA, Allison JP. Co-stimulation in T cell responses. Curr Opin Immunol 1997;9(3):396–404.
[25] Lenschow DJ, Walunas TL, Bluestone JA. CD28-B7 system of T cell costimulation. Annu Rev Immunol 1996;14:233–58.
[26] Cronin DC, Lancki DW, Fitch FW. Requirements for activation of $CD8^+$ murine T cells. I. Development of cytolytic activity. Immunol Res 1994;13(4):215–33.
[27] Harding FA, Allison JP. CD28-B7 interactions allow the induction of $CD8^+$ cytotoxic T lymphocytes in the absence of exogenous help. J Exp Med 1993;177(6):1791–6.
[28] Sato M, Iwakabe K, Ohta A, et al. Functional heterogeneity among bone marrow-derived dendritic cells conditioned by T(h)1- and T(h)2-biasing cytokines for the generation of allogeneic cytotoxic T lymphocytes. Int Immunol 2000;12(3):335–42.
[29] King C, Hoenger R, Cleary M, et al. Interleukin-4 acts at the locus of the antigen-presenting dendritic cell to counter-regulate cytotoxic $CD8^+$ T cell responses. Nat Med 2001;7(2):206–14.
[30] Selin LK, Lin MY, Kraemer KA, et al. Attrition of T cell memory: selective loss of LCMV epitope-specific memory CD8 T cells following infections with heterologous viruses. Immunity 1999;11(6):733–42.

[31] Bennett S, Carbone F, Karamalis F, Flavell R, Miller J, Heath W. Help for cytotoxic-T cell responses is mediated by CD40 signalling. Nature 1998;393(6684):478–80.
[32] Schoenberger S, Toes R, Voort Evd, Offringa R, Melief C. T cell help for cytotoxic T lymphocytes is mediated by CD40–CD40L interactions. Nature 1998;393(6684):480–3.
[33] Agren LC, Ekman L, Lowenadler B, Lycke NY. Genetically engineered nontoxic vaccine adjuvant that combines B cell targeting with immunomodulation by cholera toxin A1 subunit. J Immunol 1997;158(8):3936–46.
[34] Lassila O, Vainio O, Matzinger P. Can B cells turn on virgin T cells? Nature 1988;334(6179):253–5.
[35] Gajewski TF. B7-1 but not B7-2 efficiently costimulates $CD8^+$ T lymphocytes in the P815 tumor system in vitro. J Immunol 1996;156(2):465–72.
[36] Matulonis U, Dosiou C, Freeman G, et al. B7-1 is superior to B7-2 costimulation in the induction and maintenance of T cell-mediated antileukemia immunity. Further evidence that B7-1 and B7-2 are functionally distinct. J Immunol 1996;156(3):1126–31.
[37] Fields PE, Finch RJ, Gray GS, et al. B7-1 is a quantitatively stronger costimulus than B7-2 in the activation of naive $CD8^+$ TCR-transgenic T cells. J Immunol 1998;161(10):5268–75.
[38] Corr M, Tighe H, Lee D, et al. Costimulation provided by DNA immunization enhances antitumor immunity. J Immunol 1997; 159(10):4999–5004.
[39] Kalams SA, Walker BD. The critical need for $CD4^+$ help in maintaining effective cytotoxic T lymphocyte responses. J Exp Med 1998;188(12):2199–204.
[40] Bennett SR, Carbone FR, Karamalis F, Miller JF, Heath WR. Induction of a $CD8^+$ cytotoxic T lymphocyte response by cross-priming requires cognate $CD4^+$ T cell help. J Exp Med 1997; 186(1):65–70.
[41] Maecker HT, Umetsu DT, DeKruyff RH, Levy S. Cytotoxic T cell responses to DNA vaccination: dependence on antigen presentation via class II MHC. J Immunol 1998;161(12):6532–6.
[42] Cardin RD, Brooks JW, Sarawar SR, Doherty PC. Progressive loss of $CD8^+$ T cell-mediated control of a γ-herpesvirus in the absence of $CD4^+$ T cells. J Exp Med 1996;184(3):863–71.
[43] Smith RE, Donachie AM, Grdic D, Lycke N, Mowat AM. Immune-stimulating complexes induce an IL-12-dependent cascade of innate immune responses. J Immunol 1999;162(9):5536–46.
[44] Ahlers JD, Dunlop N, Alling DW, Nara PL, Berzofsky JA. Cytokine-in-adjuvant steering of the immune response phenotype to HIV-1 vaccine constructs: granulocyte-macrophage colony-stimulating factor and TNF-α synergize with IL-12 to enhance induction of cytotoxic T lymphocytes. J Immunol 1997;158(8):3947–58.
[45] Iwasaki A, Stiernholm BJ, Chan AK, Berinstein NL, Barber BH. Enhanced CTL responses mediated by plasmid DNA immunogens encoding co-stimulatory molecules and cytokines. J Immunol 1997;158(10):4591–601.
[46] Magram J, Connaughton SE, Warrier RR, et al. IL-12-deficient mice are defective in IFN-γ production and type 1 cytokine responses. Immunity 1996;4(5):471–81.
[47] Piccotti JR, Li K, Chan SY, et al. Alloantigen-reactive Th1 development in IL-12-deficient mice. J Immunol 1998;160(3):1132–8.