B7.2-Ig fusion proteins enhance IL-4-dependent differentiation of tumor-sensitized CD8\(^+\) cytotoxic T lymphocyte precursors

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Abstract

The B7/CD28 co-stimulatory pathway plays a critical role in T cell activation and differentiation. Our previous study demonstrated that administration of B7.2-Ig fusion proteins to tumor-bearing mice elicits IL-4-dependent, CD8\(^+\) T cell-mediated tumor regression. Here, we investigated whether B7.2-Ig stimulation of tumor-sensitized CD8\(^+\) CTL precursors during \textit{in vitro} antigen re-sensitization actually results in their differentiation into mature CTLs and if so, whether such a process depends on IL-4 signals. Splenocytes from tumor-sensitized (tumor-bearing or tumor-immunized) mice exhibited low levels of anti-tumor CTL responses upon culturing alone, but induced strikingly enhanced CTL responses when stimulated \textit{in vitro} with B7.2-Ig fusion proteins. Because CTLs were not generated from normal splenocytes even by B7.2-Ig stimulation, the expression of the B7.2-Ig effect required the \textit{in vivo} tumor sensitization of CD8\(^+\) CTL precursors. Administration of anti-CD4 or anti-CD40 ligand (CD40L) to mice before tumor sensitization resulted in almost complete inhibition of CTL responses generated in the subsequent culture containing B7.2-Ig. In contrast, anti-IL-4 did not influence \textit{in vivo} tumor sensitization required for CTL induction. However, B7.2-Ig stimulation of tumor-sensitized splenocytes enhanced IL-4 production and neutralization of this IL-4 with anti-IL-4 potently down-regulated CTL responses. These results indicate that B7.2-Ig enhances IL-4-dependent differentiation of anti-tumor CD8\(^+\) CTL precursors that can be sensitized \textit{in vivo} depending on collaboration with CD4\(^+\) T cells involving CD40L function.

Introduction

CD8\(^+\) CTLs are a major anti-tumor effector cell population. CTL responses against various antigens including tumor antigens have been shown to be induced via cross-presentation by antigen-presenting cells (APCs) (1–3). Recent studies (4, 5) have shown that the cross-presentation is mediated by specialized APCs such as dendritic cells. For this presentation pathway, these APCs take up and process cell-derived antigens and present class I MHC-restricted peptides to CD8\(^+\) CTL precursors (3). While CD8\(^+\) CTL responses require CD4\(^+\) T cell help (6), the molecular mechanism underlying the requirement of CD4\(^+\) T cells was revealed as follows: CD4\(^+\) T cells expressing CD40 ligand (CD40L) activate APCs through CD40-CD40L interactions and then such APCs can mediate the differentiation of CTL precursors into activated CTLs (7–9). More recently, it was also proposed that IL-4 is required for the final stage of the CTL differentiation during CD8 T cell–APC interactions (10, 11).

In an earlier study (12), we showed the previously undescribed effect of the B7/CD28 co-stimulation on the enhanced induction of CD8\(^+\) T cell-mediated tumor regression: administration of B7.2-Ig fusion proteins to tumor-bearing mice induced tumor regression by promoting the differentiation of anti-tumor effector CD8\(^+\) T cells. In this model, B7.2-Ig-stimulated CD8\(^+\) T cells could eradicate tumors even in the absence of CD4\(^+\) T cells (12). However, the induction of this CD8\(^+\) T cell-mediated immunity was inhibited either by eliminating CD4\(^+\) T cells during the tumor-bearing stage prior to B7.2-Ig therapy or by neutralizing IL-4 produced during B7.2-Ig treatment. The obligatory roles for CD4\(^+\) T cells and IL-4 in our \textit{in vivo} model resembled their requirements for...
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either APC activation or CD8+ CTL differentiation in the above-mentioned CTL induction (6–11).

Aside from cytolytic properties of CD8+ T cells, cytokine-secreting CD8+ T cells can also exhibit growth-inhibitory effects on tumor cells via non-cytolytic mechanisms (13, 14). Therefore, it remains unclear whether administration of B7.2-Ig contributed to the generation of mature CD8+ CTLs in our in vivo model (12). Here, we investigated whether stimulation in vitro of in vivo tumor-sensitized T cells with B7.2-Ig results in enhanced generation of anti-tumor CD8+ CTLs and if so, which factors and conditions including CD4+ T cell help and IL-4 involvement are required for the generation of the B7.2-Ig effect. The results show that in vitro B7.2-Ig stimulation of tumor-sensitized T cells from tumor-bearing or tumor-immunized mice induces strikingly enhanced generation of anti-tumor CD8+ CTLs. Administration of anti-CD4 or anti-CD40L mAb to mice during tumor sensitization resulted in almost complete abrogation of CTL induction in the subsequent B7.2-Ig-stimulated cultures. Stimulation in vitro of tumor-sensitized splenocytes with B7.2-Ig resulted in enhanced production of IL-4 and neutralization of the produced IL-4 with anti-IL-4 potently down-regulated CTL differentiation. These observations are compatible with the views that CD4+ T cells function for the earlier stage of CTL induction by activating APCs via the CD40L-involving mechanism (6–11) and that IL-4 is also required for the later stage of CTL differentiation (10, 11). Thus, the present study suggests that B7.2-Ig up-regulates the induction of anti-tumor CD8+ CTLs by enhancing IL-4 production of tumor-sensitized T cells and consequently promoting the IL-4-dependent CTL differentiation process.

Methods

Mice

Male BALB/c mice were purchased from Shizuoka Laboratory Animal Center (Hamamatsu, Japan). IL-4-deficient (IL-4−/−) mice were obtained from Jackson Laboratory (Bar Harbor, ME, USA) and bred in our laboratory.

Tumor cell lines

The following tumor cell lines were used: LSTRA leukemia and CSA1M fibrosarcoma (both BALB/c origin).

Reagents

In order to prepare B7.2-Ig fusion proteins, expression plasmids encoding mouse B7.2 signal and extracellular domains were fused to the Fc region of mouse IgG2a as previously described in detail (15). B7.2-Ig fusion proteins were collected from culture supernatants of CHO cells transfected with the recombinant plasmids carrying the above fused DNA fragment and purified on a protein A-Sepharose Fast Flow column (Pharmacia Brotec, Uppsala, Sweden). More than 99% of the protein was in the dimeric, non-aggregated form (15). Mouse recombinant IL-12 (rIL-12) was provided by Wyeth Research (Cambridge, MA, USA). Mouse rIL-4 was purchased from Peprotech EC, Ltd (London, UK). The following mAbs were purified from ascitic fluids of the relevant hybridoma cells: anti-CD4 [American Type Culture Collection (ATCC) clone GK1.5], anti-CD8 (ATCC clone 2.43), anti-IL-4 (ATCC clone 11B11), anti-CD40L (HM40L-1) (16) and anti-IL-12 (C17.8) (17).

Preparation of tumor-bearing or tumor-immunized mice

Mice were inoculated subcutaneously (s.c.) with CSA1M cells (2 × 105 per mouse) and used as tumor-bearing ones after 10–14 days. Mice were inoculated intra-peritoneally (i.p.) with mitomycin C (MMC)-treated LSTRA leukemia cells (2 × 107 per mouse) and used as tumor-immunized ones after 7–10 days.

Preparation of various lymphoid cell populations

T cell populations. Spleen cells were depleted of B cells and Ia+ APCs by immunomagnetic negative selection as described (18). Briefly, Ia+ APCs in spleen cells were allowed to react with mouse anti-I-A\(^{\text{eb}}\) mAb. Spleen cells containing the labeled cells and surface Ig+ cells (B cells) were incubated with magnetic particles conjugated to BioMag goat anti-mouse Ig (Qiagen, Tokyo, Japan). Surface Ig− and Ia− cells (B cell- and APC-depleted population) were obtained by removing cell-bound magnetic particles with a rare earth magnet (Polysciences Inc., Warrington, PA, USA). CD4+ and/or CD8+ T cell-depleted populations. Spleen cells were incubated with rat anti-mouse CD4 and/or rat anti-mouse CD8 mAbs followed by incubation with BioMag goat anti-rat IgG-conjugating magnetic particles (Qiagen). T cell-depleted populations were obtained by removing cell-bound magnetic particles. A splenic population depleted of both CD4+ and CD8+ cells was used as an APC population.

Depletion in vivo of CD4+ T cells

In order to deplete the CD4 subset of T cells, 200 μg per mouse anti-CD4 mAb was injected i.p. twice at a 2-day interval. The efficacy of CD4+ T cell depletion was confirmed by flow cytometric analysis of spleen cells from mAb-treated mice as previously described (19).

Cytotoxicity assays (DNA fragmentation assays)

Anti-tumor cytotoxic activity was measured by employing DNA fragmentation assay as previously described (20). Briefly, target tumor cells (5 × 105) were labeled with 200-kBq [3H]thymidine by incubating overnight in a culture dish containing 5 ml culture medium. Labeled cells (1 × 105) were cultured with different numbers of effector cells for 6 h (LSTRA target) or 8 h (CSA1M target) in 96-well flat-bottomed microculture plates. The plates were harvested on a microtiter plate cell harvester, and radioactivity trapped on the filters was counted on a liquid scintillation counter. Cytotoxic activity was measured with triplicate wells per group, calculated as follows and expressed as the mean of percent cytotoxicity: percent cytotoxicity = [counts per minute (c.p.m.) in effector-negative wells – c.p.m. in effector-positive wells]/[c.p.m. in effector-negative wells] × 100. SEMs were excluded from the figures because they were consistently <5%.

Measurement of cytokine concentrations

The concentrations of IL-4 and IFN-γ in culture supernatants were determined using ELISA kits purchased from Genzyme Corp. (Cambridge, MA, USA).
Analyses of intracellular cytokine production by flow cytometry

The procedure was essentially the same as that previously described (21). Cultured spleen cells were stimulated with 50 ng ml⁻¹ phorbol myristate acetate and 500 ng ml⁻¹ ionomycin in the presence of 2 μM monensin for 4–6 h. The cells harvested were stained with allophycocyanin-conjugated anti-CD4 (RM4-5) or anti-CD8 (53-6.7) mAb. After fixing with PBS containing 4% PFA, the cells suspended in PBS containing 0.5% BSA, 0.1% NaNO₃ and 0.5% saponin were intracellularly stained with FITC-conjugated rat anti-IFN-γ (XMG1.2) and PE-conjugated rat anti-IL-4 (11B11). FITC-rat IgG1 and PE-rat IgG1 antibodies were used as an isotype control. All staining reagents were purchased from BD PharMingen, San Diego, CA, USA. Flow cytometric analysis was performed on a FACSCalibur, and cells were analyzed by CellQuest software (Becton Dickinson, San Jose, CA, USA).

Results

The generation in vitro of anti-tumor CTLs from splenocytes of tumor-sensitized mice is enhanced by addition of B7.2-Ig fusion proteins to cultures

Our previous study (12) showed that stimulation of tumor-bearing mice induced CD8⁺ T cell-mediated tumor regression. Our previous studies (22, 23) showed that unlike normal splenocytes, splenocytes from tumor-bearing and tumor-immunized mice generated weak albeit detectable CTL responses upon in vitro cultures. Stimulation of these splenocytes with immobilized B7.2-Ig resulted in strikingly enhanced CTL responses. In addition, such enhancement of CTL generation was also induced by the co-stimulation of APCs. Figure 2 shows that a similar mechanism works for the induction of CTL responses. As long as splenocytes containing both T cells and APCs were from tumor-immunized mice, B7.2-Ig-enhanced CTL responses were induced irrespective of whether tumor cells were included as a source of tumor antigens (Fig. 2A). However, when purified T cells from tumor-sensitized mice were cultured with APCs from normal mice,

![Fig. 1](https://example.com/fig1.png)

**Fig. 1.** B7.2-Ig enhances the generation in vitro of CTLs from splenocytes of tumor-sensitized mice. BALB/c mice were (A) inoculated s.c. with 2 × 10⁶ viable CSA1M fibrosarcoma cells or (B) immunized i.p. with 2 × 10⁷ MMC-treated LSTRA leukemia cells. After 7–14 days, splenocytes (5 × 10⁶ per well) from CSA1M tumor-bearing or LSTRA-immunized mice or normal control mice were cultured in 24-well culture plates coated or uncoated with B7.2-Ig for 5 days. Cytotoxicity was determined by DNA fragmentation assays as described in detail in Methods. SEs of cytotoxicity in each group were excluded for simplicity because they were usually <5%. The results are representative of eight independent experiments.

![Fig. 2](https://example.com/fig2.png)

**Fig. 2.** APCs from tumor-sensitized mice can stimulate tumor-sensitized T cells without requiring exogenous tumor antigens. (A) Unfractionated splenocytes (5 × 10⁶ per well) from LSTRA tumor-sensitized mice were cultured in the presence or absence of MMC-treated LSTRA tumor cells (10⁷ per well) in B7.2-Ig-coated or -uncoated culture plates. (B) Purified T cells (5 × 10⁶ per well) from LSTRA-immunized mice were cultured with 10⁵ per well of LSTRA cells plus 10⁵ per well of APC-enriched fractions from normal mice in B7.2-Ig-coated or -uncoated plates. The results are representative of three similar experiments.
the induction of CTL responses enhanced by B7.2-Ig required tumor antigens (Fig. 2B). Co-culture of normal T cells and APCs from tumor-sensitized mice in the presence of tumor cells also failed to generate CTL responses. These observations confirm that splenocytes from tumor-immunized mice contain tumor-sensitized T cells and APCs capable of stimulating these T cells and indicate that B7.2-Ig can enhance CTL responses induced through T-cell–APC interactions.

Requirement of CD4+ T cells for enhanced in vitro induction of CD8+ CTLs

As shown in Fig. 3(A), addition of anti-CD8, but not anti-CD4, to CTL cultures markedly down-regulated the generation of CTL activity. Anti-CSA1M and anti-LSTRA effectors exhibited cytotoxicity only against the corresponding CSA1M and LSTRA target cells (data not shown). Thus, the major effector population induced by B7.2-Ig stimulation is tumor-specific CD8+ CTLs. We next investigated whether this CD8+ CTL induction requires CD4+ T cells at either in vivo CTL priming or in vitro CTL differentiation phase. To determine the CD4+ T cell requirement at the former stage, the CD4+ T cell subset was eliminated by two injections of anti-CD4 mAb prior to tumor sensitization in vivo. The efficacy of depletion was confirmed by flow cytometric analysis of spleen cells from anti-CD4-treated mice (data not shown). Figure 3(B) shows that depletion of CD4+ T cells at the in vivo tumor-sensitization stage abrogates the capacity of CD8+ T cells to differentiate into mature CTLs in the subsequent in vitro culture even when B7.2-Ig stimulation was provided.

Recent studies (6–9) revealed the mechanism underlying the requirement for CD4+ T cells in CD8+ CTL induction: CD4+ T cells are required to activate APCs that in turn cross-present processed tumor antigens to CD8+ CTL precursors. This APC activation has also been reported to depend on the molecular interaction between CD40L on CD4+ T cells and CD40 on APCs. Based on this view, we examined whether the blocking of CD40L by anti-CD40 mAb at the tumor sensitization phase influences B7.2-Ig-mediated CTL induction in the subsequent culture. As expected, administration in vivo of anti-CD40L mAb prior to tumor sensitization induced almost complete inhibition of the subsequently induced CTL generation (Fig. 4A).

We also examined the requirement of CD4+ T cells and CD40L molecules at the in vitro CTL differentiation phase. Addition of anti-CD4 mAb to cultures resulted in complete inhibition of CTL induction even by B7.2-Ig stimulation (Fig. 3C). However, B7.2-Ig-mediated CTL generation was not affected by anti-CD40L mAb (Fig. 4B). These observations suggest that CD4+ T cells are required at the in vivo priming as well as in vitro differentiation phases of CD8+ CTL induction but they contribute to the former and latter stages of CTL induction via the CD40L-dependent and -independent mechanisms, respectively.

Role for IL-4 in CTL differentiation enhanced by B7.2-Ig

Recently, it was proposed that IL-4 is required for the later stage of CD8+ CTL differentiation (10, 11). We examined whether this is also the case in the in vitro CTL differentiation enhanced by B7.2-Ig stimulation. Splenocytes from tumor-sensitized (tumor-bearing or tumor-immunized) mice were stimulated with B7.2-Ig in the presence of anti-IL-4 or anti-IL-12 as control (Fig. 5). Addition of anti-IL-12 produced only a slight influence on the in vitro induction of CTLs, but did not largely reduce CTL induction. CTL induction was not influenced by anti-IFN-γ mAb, another antibody with the capacity to inhibit the type 1 T cell response (data not shown). In contrast, anti-IL-4 exhibited potent down-regulatory effects on B7.2-Ig-enhanced CTL induction (Fig. 5).

Fig. 3. CD8+ CTLs are not induced in the absence of CD4+ T cells even by stimulation in vitro with B7.2-Ig. (A) Effector cells generated from splenocytes of LSTRA-sensitized mice were assayed for cytotoxicity in the presence of 10 µg ml−1 indicated mAbs. (B) Splenocytes from mice sensitized to LSTRA after in vivo CD4+ T cell depletion were used as responding cells. (C) Anti-CD4 mAb (10 µg ml−1) was included in 5-day CTL cultures to block the function of CD4+ T cells. The results are representative of three (Fig. 3A), five (Fig. 3B) and eight (Fig. 3C) independent experiments.
We further investigated whether the role for IL-4 is limited to the later (in vitro CTL induction) phase or also observed at the priming (tumor sensitization) phase. Administration of anti-IL-4 mAb did not affect CTL induction in the subsequent in vitro cultures (Fig. 6A). Figure 6(B) shows that B7.2-Ig-mediated CTL induction from splenocytes of IL-4−/− mice is considerably weak compared with that from wild-type (WT) splenocytes. Addition of rIL-4 to CTL differentiation cultures of IL-4−/− splenocytes resulted in strikingly enhanced CTL generation. These results indicate that IL-4 is required for B7.2-Ig-enhanced CTL induction particularly at its later stage (in vitro CTL differentiation rather than in vivo CTL-priming phase).

The function of CD4+ T cells and IL-4 in the initial 24 h of 5-day CTL cultures are required to induce B7.2-Ig-enhanced CTL differentiation

To investigate how CD4+ T cells and IL-4 are required to induce B7.2-enhanced CTL generation, we first determined which phase of 5-day CTL differentiation cultures requires their function. Figure 7 shows that addition of anti-CD4 (panel A) or anti-IL-4 (panel B) at the staring point of the cultures again induced complete (A) or potent (B) inhibition of CTL induction. However, when these antibodies were included after 24 h, no inhibition was observed. These results indicate that both CD4+ T cells and IL-4 exert its function for B7.2-Ig-mediated enhanced CTL generation in the initial 24 h of 5-day CTL cultures.

B7.2-Ig enhances CD4+ T cell-mediated IL-4, but not IFN-γ, production particularly in the initial 24 h of CTL cultures

We examined whether IL-4 is produced in CTL differentiation cultures and if so, whether IL-4 production is enhanced by B7.2-Ig stimulation. As shown in Fig. 8(A), IL-4 production was up-regulated by B7.2-Ig stimulation at the early phase of CTL cultures, particularly in the first 24 h. In contrast, B7.2-Ig stimulation did not up-regulate IFN-γ production, but rather this cytokine production was reciprocally down-regulated. It should also be noted that IL-4 production induced in the B7.2-Ig stimulation culture exhibited a unique time course. Cultures free of B7.2-Ig displayed gradually but continuously increasing

Fig. 4. Administration of anti-CD40L during in vivo tumor sensitization inhibits CTL generation enhanced by B7.2-Ig in the subsequent in vitro cultures. (A) Anti-CD40L mAb (1 mg per mouse) or control IgG was injected intravenously to BALB/c mice 1 day before and 1 day after LSTRA sensitization. (B) Anti-CD40L mAb (10 μg ml−1) or control Ig was included in 5-day CTL differentiation cultures. The results are representative of four independent experiments.

Fig. 5. Addition of anti-IL-4 to cultures inhibits the enhancement of CTL responses induced by B7.2-Ig stimulation. Splenocytes from CSA1M-bearing (A) or LSTRA-immunized mice were stimulated with B7.2-Ig in the presence of 10 μg ml−1 anti-IL-4 or anti-IL-12 (control) mAb. The results are representative of five (Fig. 5A) and four (Fig. 5B) independent experiments.

Fig. 6. IL-4 is required for the in vitro CTL differentiation stage that is enhanced by B7.2-Ig stimulation. (A) Anti-IL-4 mAb (1 mg per mouse) or control rat Ig was injected i.p. to WT BALB/c mice 1 day before and after LSTRA sensitization. Splenocytes from these mice were unstimulated or stimulated with B7.2-Ig. (B) Splenocytes from WT or IL-4−/− LSTRA-sensitized mice were unstimulated or stimulated with B7.2-Ig in the presence or absence of rIL-4 (10 ng ml−1). The results are representative of four (Fig. 6A) and three (Fig. 6B) independent experiments.
IL-4 production on one hand. On the other hand, enhanced amounts of IL-4 were produced rapidly in the first day of B7.2-Ig cultures, but the level of IL-4 was decreased in the second day, which was reproducibly observed. This suggests accelerated production of IL-4 by B7.2-Ig stimulation and its consumption by splenocytes during culture. B7.2-Ig-enhanced IL-4 production during the initial 24 h of culture was also found to be mediated by CD4+ T cells (Fig. 8B). Moreover, analyses of the intracellular cytokine staining showed that B7.2-Ig co-stimulation of tumor-sensitized spleen cells during in vitro cultures promoted the generation of IL-4-producing cells in the CD4+ but not CD8+ T cell subset (Fig. 8C). Together with the results of Figs 6 and 7, these observations suggest that in addition to the requirement for the in vivo CTL priming, CD4+ T cells play a role in B7.2-Ig-enhanced CTL differentiation by producing IL-4, a critical cytokine.

Discussion

Because CD8+ T cells are a major anti-tumor effector population, an attempt to enhance the induction of this effector population would be an essential aspect in the manipulation of tumor immunity. Full T cell activation requires CD28 co-stimulation in addition to TCR stimulation with antigens (24, 25). Aside tumor vaccination, augmenting the B7/CD28 co-stimulatory pathway could, therefore, lead to enhanced generation of anti-tumor CD8+ T cells. Consistent with this view, our previous study (12) demonstrated that stimulation in vivo of the CD28 pathway by B7.2-Ig fusion proteins in tumor-bearing mice results in the regression of growing tumors by promoting the activation of anti-tumor CD8+ T cells. The results also showed that this CD8+ T cell activation requires CD4+ T cells at the initial tumor sensitization phase prior to B7.2-Ig treatment and IL-4 at the later B7.2-Ig therapeutic phase (12). However, it remains unclear whether CD8+ T cells actually represent anti-tumor CTLs and if so, how stimulation of the CD28 pathway using B7.2-Ig fusion proteins enhances the differentiation of anti-tumor CD8+ CTLs.

To address ourselves to the above issues, we analyzed the role of B7.2-Ig in the in vitro CTL induction system. The results illustrated that B7.2-Ig stimulation of splenocytes from in vivo tumor-sensitized mice results in strikingly enhanced generation of anti-tumor CD8+ CTLs. Depletion of CD4+ T cells or the blockade of CD40L during the in vivo tumor sensitization eliminated the capacity of splenocytes to develop CD8+ CTLs in the subsequent CTL differentiation cultures. B7.2-Ig-enhanced CTL generation required the involvement of IL-4, but not of CD40L, in CTL cultures. Importantly, it was critical.
that IL-4 is produced at the initial phase of CTL cultures. Such IL-4 was provided by B7.2-Ig-stimulated CD4+ T cells. Namely, B7.2-Ig acted on CD4+ T cells to up-regulate IL-4 production in parallel to reciprocal down-regulation of IFN-γ production. Thus, in addition to supporting the previously observed role of CD4+ T cells (6–9) and IL-4 (10, 11) in CTL induction, the present study demonstrates that B7.2-Ig stimulates IL-4 production by tumor-sensitized T cells and enhances IL-4-dependent differentiation of tumor-sensitized CD8+ CTL precursors into mature CTLs.

Tumor cells, even though expressing tumor antigen peptides associated with class I MHC molecules, would be unable to activate anti-tumor CD8+ T cells because, unlike APCs, they fail to deliver co-stimulatory signals required for full T cell activation. In this context, it becomes increasingly evident that the activation of anti-tumor CD8+ T cell precursors occurs through interactions not with tumor cells but with APCs that have picked up and processed tumor antigens. The pathway of CD8+ T cell activation involving the participation of APCs with co-stimulatory function has been described for 'cross-priming' or 'cross-presentation' (1–3). However, such APCs have to be pre-activated by CD4+ T cells via CD40–CD40L interactions before presenting tumor antigens to CD8+ T cell precursors (6–9). This mechanism provided an explanation for the requirement of CD4+ T cells help in the activation of anti-tumor CD8+ T cells. In the present study, elimination of CD4+ T cells at the stage of tumor sensitization completely inhibited B7.2-Ig-enhanced CTL differentiation induced in the subsequent in vitro culture. This is compatible with our previous results that administration of B7.2-Ig to tumor-bearing mice induced CD8+ T cell-mediated tumor regression, whereas the same did not occur in tumor-bearing mice that had been depleted of CD4+ T cells prior to tumor initiation (12). The blockade of CD40L, instead of CD4 T cell depletion, at the tumor sensitization stage also abolished the capacity to generate the subsequent CTL responses. Together, these observations are consistent with the view that CD4+ T cells expressing CD40L are required to activate APCs for the acquisition of the capacity to cross-present tumor antigens to CD8+ T cells (6–9).

In the present study, CTL responses were generated irrespective of whether exogenous tumor antigens were included in cultures. Regarding this phenomenon, our previous studies (22, 23) showed that splenocytes from tumor-bearing (22, 23) or tumor-immunized mice (22) contain T cells primed to tumor antigens and APCs presenting processed tumor antigens and that upon cultures, these splenocytes induce an anti-tumor response through T cell–APC interactions. Consistent with this, co-cultures of purified tumor-sensitized T cells and APCs prepared from normal mice failed to induce B7.2-Ig-mediated CTL responses in the absence of exogenous tumor antigens. While these cultures generated CTL responses depending on exogenous tumor antigens, the levels of the responses were apparently lower than those obtained by unfractionated splenocytes from tumor-sensitized splenocytes. These observations may be explained by the following possibility: APCs harvested from tumor-sensitized mice not only have picked up tumor antigens to present its processed forms but also have been activated by CD4+ T cells to acquire the cross-presenting capacity. Instead, normal APCs begin to process and present tumor antigens after being included in CTL cultures together with tumor cells. These considerations may account for the difference in the levels of CTL responses between cultures containing APCs from tumor-sensitized and normal mice.

A more essential aspect of the present study is that B7.2-Ig enhances the differentiation of tumor-sensitized CTLs and that IL-4 is required for this enhanced CTL induction. Among functionally and phenotypically heterogeneous T cell subsets, it has been believed that type 1 T cells, both Tc1 and Th1, play a predominant role in inducing the rejection of established tumors. This notion is consistent with recent observations that administration of IL-12 induces tumor regression that is dependent on the production of another Th1 cytokine IFN-γ (26–28). The development of type 1 and type 2 T cells is cross-regulated by the other subset of T cells or by cytokines required for the differentiation of such T cells [reviewed in detail in (29)]. According to this theory, IL-4 is considered to down-regulate the above-mentioned type 1 T cell-mediated tumor rejection responses. Nevertheless, the positive anti-tumor effect of IL-4 was first reported in 1989 before the paradigm of Tc1/Teff2 was established (30). Recently, the role of IL-4-producing Th2 (31–33) and Tc2 cells (10, 34, 35) themselves in tumor eradication has also been demonstrated in several independent studies. Critically, IL-4−/− mice failed to exhibit the Tc2-mediated anti-tumor effect that was otherwise observed in WT mice (10). Our previous results (12) also showed that administration of B7.2-Ig to tumor-bearing mice induces tumor regression by promoting IL-4-dependent activation of anti-tumor CD8+ T cells. These results indicate that IL-4 does not necessarily counteract Th1-mediated anti-tumor responses but may be capable of exhibiting its own anti-tumor pathway.

More closely relating to the present study, IL-4 was directly shown to play an important role in CTL differentiation (10, 11). These studies showed that IL-4 acts on APCs pre-activated by CD4+ T cells to induce further maturation of APCs and that such IL-4-treated APCs can efficiently activate CD8+ CTLs via the cross-presentation pathway (10, 11). In this study, administration of anti-IL-4 mAb to the mice before tumor initiation and neutralization of produced IL-4, if any, during tumor sensitization did not affect B7.2-Ig-enhanced CTL generation in the subsequent CTL differentiation culture. In contrast, addition of anti-IL-4 to the culture resulted in potent down-regulation of CTL responses. Moreover, splenocytes from IL-4−/− mice were capable of mounting high levels of CTL responses when rIL-4 was included in their CTL differentiation cultures. This confirms the above observations that B7.2-Ig-enhanced CTL responses are affected by IL-4 neutralization not at the tumor sensitization stage but at the CTL differentiation phase, and is consistent with the previous reports (10, 11).

Further, our present study clarified the time course and the cellular basis of IL-4 production required for B7.2-Ig-enhanced CTL differentiation. B7.2-Ig stimulation of splenocytes from tumor-sensitized mice resulted in marked enhancement of IL-4 production in the first day of 5-day CTL differentiation cultures. Addition of anti-IL-4 to cultures on day 0 inhibited CTL differentiation, whereas the effect of IL-4 neutralization was largely reduced when anti-IL-4 was added on day 1. Thus, IL-4 produced in the early phase of cultures depending on
B7.2-Ig stimulation is critical for enhanced CTL induction. Further, our results demonstrated that CD4+ T cells are responsible for IL-4 production during this limited period. Based on the recently proposed model of APC functional maturation (10, 11), B7.2-Ig stimulation allows CD4+ T cells to produce IL-4 and to induce IL-4-mediated maturation of APCs. Thus, CD4+ T cells could exert its two sequential effects on APCs for the acquisition of the capacity to pursue cross-presentation: CD40L-mediated activation and IL-4-mediated functional maturation of APCs.

Our present results regarding the cellular basis of IL-4 production differs with the observations previously made by Schuler et al., in which IL-4 was produced by CD8+ T cells interacting with APCs (11). In their model, mice were repeatedly immunized with attenuated tumor cells. In the present study using splenocytes from mice sensitized with a single injection of attenuated tumor cells, IL-4 was provided by CD4+ T cells. Although it appears that the cell source of IL-4 is different between their model and ours, this could be explained as follows: it is obvious that CD8+ CTL responses are induced without exogenous B7 stimulation, if the conditions of CTL induction are appropriately defined. In our model, the conditions in which CTL responses are weakly induced without B7.2-Ig stimulation were defined to examine the effect of exogenous B7.2-Ig stimulation on CTL induction. It is possible that CD8+ T cells do not acquire the ability to produce IL-4 in response to B7.2-Ig co-stimulation unless they have undergone prolonged antigen exposures. Thus, the present particular model may have revealed the capacity of CD4+ T cells to produce IL-4 promptly and intensively in response to B7.2-Ig. However, the possibility that IL-4 is also produced by CD8+ T cells themselves may not be totally excluded. In fact, our previous study (12) demonstrated that when tumor-bearing mice prepared by the inoculation of viable tumor cells ~10 days previously were depleted of CD4+ T cells in vivo and then given a single B7.2-Ig injection, they produced considerable amounts of IL-4 that was prerequisite to inducing tumor regression. In the situation under which mice were given repeated antigen stimulation by growing tumors, CD8+ T cells may develop the capacity to produce IL-4.

Analyses of cytokines produced in CTL cultures showed that B7.2-Ig stimulation enhances IL-4 production but reciprocally down-regulates IFN-γ production. A number of studies (36, 37) using anti-CD28 mAb have indicated that the B7/CD28 co-stimulation pathway is potentially effective for enhancing both Th1 and Th2 cytokine production. Here, a critical question may be raised regarding why Th2 (IL-4) cytokine production was preferentially enhanced by B7.2-Ig stimulation. In this context, a recent study (21, 38) revealed that there was a great difference in the patterns of Th1 versus Th2 cytokine expression between stimulation of CD28 with anti-CD28 mAb and with the ligands of CD28 (B7.1-Ig or B7.2-Ig). Namely, stimulation in vitro of CD4+ T cells with B7.1-Ig or B7.2-Ig leads to much higher levels of Th2 cytokine production than with anti-CD28. Thus, it appears that stimulation of TCR-triggered T cells with B7.2-Ig efficiently leads to the induction of type 2 cytokine production.

B7-Ig also reacts with CTLA-4 expressed on activated T cells (39), whereas anti-CD28 mAb does not. Therefore, it is speculated that the up-regulating effect of B7-Ig on IL-4 production and CTL generation is induced not only via its direct action on CD28 but also via CTLA-4 involvement. In relation to this, Boasso et al. (40) have recently shown that unlike CD28-Ig (Fc) fusion proteins, CTLA-4-Ig (Fc) induces the expression of IFN-γ and IFN-γ-dependent indoleamine 2,3-dioxygenase (IDO) by binding to B7 molecules on APCs. Because IDO functions to down-regulate T cell activation, their model suggested feedback control of T cell responses by CTLA-4 on activated T cells. In the present model, the effect of B7-Ig might be suggested as occurring by competitively inhibiting the interaction of CTLA-4 on activated T cells with B7 on APCs. However, the present results showing the failure of B7-Ig to up-regulate IFN-γ expression could not be interpreted solely by considering their model. Consistently, B7-Ig-mediated enhancement of CTL responses was not affected by the presence of anti-IFN-γ mAb in CTL cultures (our unpublished results).

Our present results illustrate that splenocytes from tumor-sensitized mice are allowed to induce enhanced anti-tumor CD8+ CTL responses when stimulated by a natural ligand of CD28, B7.2-Ig. Consistent with the recent observation (10, 11), the results demonstrate that IL-4 is prerequisite to enhancing CTL induction and that B7.2-Ig stimulation can up-regulate preferentially Th2 cytokine production along with reciprocal down-regulation of IFN-γ (Th1 cytokine) responses. Thus, by showing that B7.2-Ig stimulation induces IL-4-dependent anti-tumor CD8+ CTL differentiation, the present results support our previous observation that CD8+ T cell-mediated, IL-4-dependent tumor regression is induced by a manipulation using B7.2-Ig (12). Further studies will be required to investigate why B7.2-Ig stimulation favors Th2 (IL-4) cytokine production and how produced IL-4 works for CD8+ CTL differentiation.

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**Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>APC</td>
<td>antigen-presenting cell</td>
</tr>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>CD40L</td>
<td>CD40 ligand</td>
</tr>
<tr>
<td>c.p.m.</td>
<td>counts per minute</td>
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<tr>
<td>IDO</td>
<td>indoleamine 2,3-dioxygenase</td>
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<tr>
<td>IL-4</td>
<td>IL-4 deficient</td>
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<tr>
<td>i.p.</td>
<td>intra-peritoneally</td>
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<td>MMC</td>
<td>mitomycin C</td>
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<td>rIL</td>
<td>recombinant IL</td>
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<td>s.c.</td>
<td>subcutaneously</td>
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<td>WT</td>
<td>wild type</td>
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**References**


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