The capacity of the natural ligands for CD28 to drive IL-4 expression in naïve and antigen-primed CD4+ and CD8+ T cells

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Abstract

The B7/CD28 costimulatory pathway plays a critical role in T cell activation including Th1/Th2 differentiation. However, little is known about whether CD28 costimulation favors polarization of either Th1 and Th2 or both. Here, we show a critical role of the natural ligands for CD28 molecules (B7.2–Ig or B7.1–Ig fusion proteins), particularly in the induction of type 2 T cell polarization. Upon TCR-triggering with suboptimal doses of anti-CD3, costimulation of naïve CD4+ T cells with anti-CD28 mAb or B7–Ig fusion proteins led to comparable levels of IFN-γ production. Naïve T cells could produce IL-4 when CD28 costimulation was done with B7–Ig, but not with anti-CD28. IL-4-selective upregulation was also observed when T cells from anti-OVA TCR transgenic mice were stimulated with OVA in the presence of B7–Ig. Correlating with IL-4 expression, GATA-3 expression was induced much more potently by costimulation with B7–Ig than with anti-CD28 mAb, while T-bet induction by these two costimulatory reagents was comparable. This B7 effect was also applied for naïve and antigen-primed CD8+ T cells: IL-4-expressing CD8+ T cells were generated when naïve and alloantigen-primed T cells were stimulated with anti-CD3 and recall antigens, respectively, in the presence of B7–Ig costimulation. Importantly, such CD8+ T cell differentiation required the coexistence of CD4+ T cells during the initial TCR stimulation. These observations indicate that both type 2 CD4 and CD8 T cell polarizations are efficiently induced via costimulation of CD28 with its natural ligands, although the differentiation of CD8+ T cells is dependent on CD4+ cells.

Introduction

Naïve CD4+ T cells differentiate into two distinct subsets, Th1 and Th2, with different cytokine secretion profiles. Th1 cells secrete IL-2 and IFN-γ, whereas Th2 lymphocytes produce IL-4, IL-5 and IL-13 (1,2). CD8+ T lymphocytes are also classified into Tc1 and Tc2 based on the cytokine-secreting patterns similar to those of CD4+ T cells (3,4). While the selective differentiation of either subset is established during priming, this process can be significantly influenced by a variety of factors. One of these factors, the cytokine environment, has been put forward as the major variable influencing Th differentiation (5,6): the presence of APC-derived IL-12 during priming directly augments Th1 development, whereas IL-4 produced in the environment by various types of cells has the greatest influence in driving Th2 differentiation.

In addition, the valency of TCR stimuli and the mode of costimulatory signals have been shown to affect Th subset differentiation [reviewed in (7)]. Several studies (8–12) have demonstrated that priming to different doses of antigen induces the opposite dichotomy in Th responses, with high vs low doses of antigen heading to Th1 vs Th2 responses, respectively. T cell differentiation is also regulated by the involvement of costimulatory molecules such as CD28 and CTLA-4. Initially, the role of CD28 costimulation was demonstrated in the activation of type 1 CD4+ T cells (Th1) for Th1 cytokine (IL-2/IFN-γ) gene expression (13). However, CD28 costimulation also promotes Th2 differentiation and the production of Th2 cytokines (14–17). Therefore, the B7/CD28 costimulation pathway has the potential to costimulate T cells for both Th1 and Th2 differentiation. This was made more complicated by the reports that costimulation of CD28 with its two natural ligands, B7.1 and B7.2, regulates Th1/Th2 differentiation differently (18–21). Because the actions of costimulatory
molecules would integrate with the dose of antigen (the strength of TCR signals) to regulate T cell differentiation, the manipulation of Th1/Th2 polarization should be reconsidered in the context of the integration of antigen dose with costimulatory signals. Including this issue, it remains to be seen which type of manipulation for CD28 costimulation leads to preferential induction of Th1 or Th2 differentiation. It is also unclear whether such a manipulation is applied for the regulation of Tc1 vs Tc2 differentiation.

Here, we will show that comparable levels of IFN-γ expression were induced in anti-CD3-triggered naïve CD4+ T cells when CD28 costimulation was provided by anti-CD28 mAb or the fusion proteins of Ig and the natural ligands for CD28 (B7.2-Ig or B7.1-Ig). In contrast, IL-4-producing cells were generated preferentially by B7-Ig costimulation when naïve conventional CD4+ T cells were stimulated with OVA plus APC instead of anti-CD3. B7-Ig-induced IL-4 expression was more manifest upon TCR stimulation with low rather than high doses of anti-CD3. Moreover, the B7 effect on IL-4 induction was observed in anti-CD3-triggered primary cultures of naïve CD8+ T cells as well as in restimulation cultures of CD8+ T cells primed in vivo to alloantigens. Thus, the natural ligands for CD28 have a critical role in inducing the generation of IL-4-producing T cells in both CD4 and CD8 subsets.

**Methods**

**Mice**

Female BALB/c mice were purchased from Shizuoka Laboratory Animal Center (Hamamatsu, Japan). Mice transgenic for an αβ TCR recognizing OVA (323-339) (DO11.10; BALB/c genetic background) were obtained from Jackson Laboratory (Bar Harbor, ME) and bred in our laboratory.

**Reagents**

In order to prepare B7.2–Ig as well as B7.1–Ig fusion proteins, expression plasmids encoding mouse B7.2 or B7.1 signal and extracellular domains were fused to the Fc region of mouse IgG2a as previously described in detail (22). The inducible costimulator (ICOS) ligand (ICOSL)-IgG2a construct was made by linking the DNA element of ICOSL (Genbank accession number AF199027, nucleotides 205–897) to a genomic DNA segment encoding the hinge CH2–CH3 domain for mouse IgG2a. B7–Ig and ICOSL–Ig fusion proteins were collected from culture supernatants or ascitic fluids with the recombinant plasmids carrying the above fused DNA fragment and purified on a protein A–Sepharose Fast Flow column (PharmaciaBiotech). More than 99% of the protein was passed through the column and that were eluted from the column and that were eluted from the column were used as CD62L+ and CD62L− cell populations, respectively.

**Coating of culture plates with anti-CD3, B7 fusion proteins or anti-CD28 mAb**

mAbs and B7–Ig or ICOSL–Ig fusion proteins were diluted to indicated concentrations in PBS and immobilized to individual wells of 48-well culture plates (Corning # 3548, Corning Glass Works, Corning, NY) in a final volume of 0.25 ml at room temperature. After 4 h, solutions were discarded, and the plates were washed with PBS twice.

**In vitro stimulation of T cells with [anti-CD3 + B7-fusion proteins or anti-CD28] or with [OVA antigen + APC]**

Purified BALB/c CD4+ T cells or a mixture of purified CD4+ and CD8+ T cells were stimulated with anti-CD3 (0.13–1.0 μg/ml) alone or together with 5 μg/ml (unless otherwise indicated) of B7–Ig fusion proteins or anti-CD28 mAb. All of these stimulatory reagents were immobilized (unless otherwise indicated) to wells of 48-well culture plates. B cell/CD8+ T cell-depleted splenocytes from DO11.10 mice were stimulated with 100 μg/ml OVA in wells that had been coated with B7–Ig or anti-CD28 mAb.

**Preparation of CD8+ T cells primed to alloantigens**

BALB/c mice (H-2d) were primed to H-2d+ alloantigens by inoculating i.p. with 2.5 × 106 of mitomycin C (MMC)-treated
EL-4 [C57BL/6 (H-2b) origin] cells. The mice were injected i.p. with 250 μg of anti-CD4 (GK1.5) mAb twice every other day before or after alloantigen priming: for the 3rd, after the second anti-CD4 injection, the mice were primed to alloantigens. For the latter 2 weeks after priming, the mice were treated with anti-CD4, and 3 days after the second anti-CD4 injection, splenocytes were used as a source of alloantigen-primed CD8+ T cells. These splenocytes were confirmed to be depleted of CD4+ T cells by flow cytometry before in vitro cultures.

In vitro stimulation of alloantigen-primed CD8+ T cells with the same alloantigens
Splenocytes containing alloantigen-primed CD8+ T cells, but depleted of CD4+ T cells, were cultured together with EL-4 cells in 48-well culture plates that had been coated with B7.2-Ig or anti-CD28 mAb. Anti-CD4 mAb (10 μg/ml) was included in cultures to abrogate the function of residual CD4+ T cells, if any.

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).

Analyses of intracellular cytokine production by flow cytometry
Cultured T cells were stimulated with 50 ng/ml PMA and 500 ng/ml ionomycin in the presence of 2 μM monensin for 4 or 24 h. The cells harvested (10^6) were incubated for 30 min at 4°C with allophycocyanin (APC)-conjugated anti-CD4 or anti-CD8 mAb in 20 μl of staining buffer [HBS, 0.1% BSA and 0.1% sodium azide (NaNO2)]. Then cells were washed with staining buffer and fixed for 20 min at room temperature with 500 μl of PBS containing 4% paraformaldehyde. After washing, the cells were incubated in PBS containing 0.5% BSA, 0.1% NaN3 and 0.5% saponin for cell membrane permeabilization. For intracellular staining, fixed cells were stained for 60 min at room temperature with rat anti-IFN-γ (XMG1.2)-FITC and rat anti-IL-4 (11B11)-PE, as described previously (29). PE-rat IgG1 and FITC-rat IgG1 antibodies were used as an isotype control. All staining reagents were purchased from BD PharMingen. Flow cytometric analysis was performed on a FACSCalibur, and cells were analyzed by CellQuest software (Becton Dickinson, San Jose, CA).

Reverse transcription–polymerase chain reaction (RT-PCR)
Total RNA was prepared from cultured T cells by StrataPrep™ Total RNA Miniprep Kit (Stratagene Cloning Systems, La Jolla, CA). Total RNA (1 μg) was reverse transcribed into cDNA in a total volume of 20 μl using random primers and SuperScript™ III RNase H− Reverse Transcriptase (LifeTechnologies, Rockville, MD). PCR amplification was carried out in a total volume (25 μl) of 1 × PCR buffer (Applied Biosystems, Foster City, CA) containing 0.5 μl of the first strand cDNA, 0.25 mM of each dNTP, 2 μM of each primer and 25 U/ml AmpliTaq Gold™ DNA polymerase (Applied Biosystems). The following oligonucleotides were used: GATA-3 sense primer 5′-CCAGCACAGAGGCAGGGAG-3′, GATA-3 anti-sense primer 5′-CCCCATTAGGGTCTTCCTC-3′, T-bet sense primer 5′-TATGTCACCCGACTCCCACACCC-3′, T-bet anti-sense primer 5′-CTTCTAGGTCAGCCTGACCG-3′, IL-4 sense primer 5′-GAATGTACGAGGCACAGCAT-3′, IL-4 anti-sense primer 5′-CTCGAGCTCAAGCTGTC-3′, IFN-γ sense primer 5′-CTTCTGCTATTGCTGATCCG-3′, IFN-γ anti-sense primer 5′-ACTCTGGTTCCTGCTGATC-3′, β-actin sense primer 5′-AGAAGAGCTAGTGGCTGCTGAGC-3′, and β-actin antisense primer 5′-CTTCTGATCCTGTCAGGAATGCC-3′. Cycle parameters were: annealing 30 s at 55°C, elongation 30 s at 72°C, denaturation 30 s at 94°C. Resulting PCR products were separated in 1.5% agarose gel and visualized by staining with ethidium bromide nucleic acid gelstain (FMC Bioproducts, Rockland, ME). Sequences of the GATA-3, T-bet, IL-4, IFN-γ and β-actin (for standardization) were amplified from each cDNA batch of 236, 559, 386, 393 and 236 baspairs with 35, 30, 35, 30 and 26 amplification cycles, respectively.

Results
The capacity of B7.2-Ig to induce IL-4 production in naïve CD4+ T cells
Previously, it was reported that freshly isolated BALB/c CD4+ T cells produce large amounts of IL-4 in response to anti-CD3 mAB immobilized to culture plates at a high concentration (5 μg/ml), while C57BL/6 CD4+ T cells produce small amounts of IL-4 (30). The results also demonstrated that the CD4+ T cells responsible for IL-4 production in BALB/c mice were of a CD62L− memory-type, whereas CD62L+ naïve T cells produce only small amounts of IL-4 (30). We examined whether a similar functional difference is observed between CD62L+ (naïve) and CD62L− (memory) BALB/c CD4+ T cells are stimulated with a low concentration (0.25 μg/ml) of immobilized anti-CD3 together with anti-CD28 mAb (5 μg/ml) for 5 days (Fig. 1). IFN-γ production was produced by either naïve or memory T cells, although the levels of IFN-γ production were appreciably higher in the latter than in the former. However, IL-4 production was only marginally induced by naïve CD4+ T cells (Fig. 1A).

A recent study (31) showed that Th2 cytokines, including IL-4, can be produced much more effectively by anti-CD3-triggered T cells upon costimulation with natural ligands for CD28 (B7.2-Ig or B7.1-Ig fusion proteins) than by anti-CD28 although the level was approximately one order lower than that in memory T cells stimulated with anti-CD3 plus anti-CD28 (Fig. 1B). It should be noted that B7.2-Ig-mediated enhancement of IL-4 and IFN-γ production is not affected by the addition of anti-CTLA-4 mAb (Fig. 1C). Thus, it appears that the B7.2-Ig effect occurs without necessarily engaging CTLA-4 molecules with which this reagent is potentially reactive.
Further, Fig. 1(D) shows that the effect of B7.2–Ig on the upregulation of IL-4 production is observed when TCR stimulation is performed with native antigen plus APC instead of anti-CD3. Namely, CD4+ T cells from anti-OVA TCR transgenic mice produced markedly enhanced amounts of IL-4 in response to OVA, depending on B7.2–Ig costimulation. We also examined the effect of anti-CTLA-4 mAb in this culture system. Because APC were present in these cultures, we included the Fab monomer of anti-CTLA-4 mAb instead of the whole mAb. Figure 1(E) shows that the upregulation of IL-4 production by B7.2–Ig costimulation is also unaffected by anti-CTLA-4 Fab in this system, consistent with the result of Fig. 1(C).

**Time course of IL-4 expression in naïve T cells costimulated with B7.2–Ig**

We examined the time course of IL-4 expression in naïve CD4+ T cells stimulated with anti-CD3 plus B7.2–Ig. Figure 2 shows that IL-4 production enhanced by B7.2–Ig costimulation is detected on day 3 and reaches high levels on days 5 and 6. IFN-γ production was again induced by either B7.2–Ig or anti-CD28 costimulation and was observed at a time course similar to that of IL-4 production.

GATA-3 and T-bet are master transcription factors for the expression of IL-4 and IFN-γ mRNAs, respectively (32,33). Figure 3 shows the time course of mRNA expression for these transcription factors and cytokines. B7.2–Ig costimulation of naïve CD4+ T cells induced both GATA-3 and T-bet mRNA expression on day 3. Consistently, these T cells expressed high levels of IL-4 and IFN-γ mRNAs. In contrast, GATA-3 and IL-4 mRNA expression were only weakly induced by anti-CD28 costimulated T cells under conditions in which the same T cells expressed high levels of T-bet and IFN-γ mRNAs. The patterns for IL-4 and IFN-γ mRNA expression are consistent with the production of these two cytokines in Fig. 2.

**The conditions for the induction of IL-4 production by B7.2–Ig costimulation**

We defined various conditions for B7.2–Ig-induced IL-4 production in naïve CD4+ T cells. When CD28 costimulation was performed using soluble B7.2–Ig instead of immobilized B7.2–Ig, neither IL-4 nor IFN-γ production was enhanced (Fig. 4A). While a soluble form of anti-CD28 was capable of enhancing IFN-γ production more potently than an immobilized form of anti-CD28, soluble anti-CD28 failed to induce as large an enhancement of IL-4 production as immobilized B7.2–Ig.

To determine the amounts of anti-CD3 mAb and B7.2–Ig fusion protein for the induction of the peak IL-4 response, either one of these reagents was titrated (Fig. 4B and C). IL-4
production by CD4+ T cells stimulated with a given concentration (0.25 μg/ml) of anti-CD3 was strengthened in parallel to an increase in the amount of immobilized B7.2–Ig, showing a plateau at the concentration of 5 μg/ml. On the contrary, low concentrations of anti-CD3 were found to be suitable for the induction of the peak IL-4 responses. Notably, the reduction of IL-4 production at high doses of anti-CD3 did not appear to result from preferential differentiation into IFN-γ-producing Th1 cells, because IFN-γ levels did not change significantly (25, 17, 27 and 19 ng/ml at 0.13, 0.25, 0.5 and 1.0 μg/ml of anti-CD3 stimulation, respectively).

Comparison of the capacities of various B7 family molecules to costimulate naïve T cells for IL-4 production

We compared the capacity to preferentially enhance IL-4 production between B7.2 and other members of the B7 family such as B7.1 and ICOSL. Figure 5 shows that IL-4 production is enhanced by either B7.2–Ig or B7.1–Ig costimulation, whereas ICOSL–Ig failed to costimulate anti-CD3-stimulated naïve T cells for IL-4 production. While the ICOS pathway has been reported to enhance IL-4 expression (34,35), it appears that the ICOS signal is insufficient to induce IL-4 production in purified naïve CD4+ T cells when they are stimulated with low doses of anti-CD3 without CD28 involvement. Because ICOSL could enhance IFN-γ production, its failure to costimulate naïve CD4+ T cells for IL-4 expression is not ascribed to the overall inability of this costimulatory molecule to stimulate naïve T cells. Thus, IL-4 production of naïve CD4+ T cells is induced preferentially by two B7 molecules (B7.2 and B7.1).

Fig. 2. Time course of IL-4 and IFN-γ production enhanced by B7.2–Ig and anti-CD28 costimulation. Purified naïve CD4+ T cells (3 × 10^5/well) were stimulated with plate-coated anti-CD3 (0.25 μg/ml) and coimmobilized B7.2–Ig or anti-CD3 (5 μg/ml) for the indicated days.

The effect of B7.2–Ig costimulation on the induction of IL-4 production in anti-CD3-stimulated naïve CD8+ T cells

We examined whether IL-4 production is induced in anti-CD3-triggered naïve CD8+ T cells by B7.2–Ig costimulation. Purified naïve CD8+ T cells failed to survive in cultures containing coimmobilized anti-CD3 plus B7.2–Ig or anti-CD28, irrespective of the doses of these stimulating reagents or the concentrations of responding CD8+ T cells (data not shown). However, concurrent anti-CD3 stimulation of naïve CD8+ and CD4+ T cells in a single well coated with B7.2–Ig or anti-CD28 resulted in the expansion of CD8+ T cells along with CD4+ T cell proliferation. Because the capacity of CD8+ T cells to produce IL-4 was not determined by measuring IL-4 in cultures containing CD4+ T cells as well, we took advantage of detecting intracellular IL-4 expression in CD8+ T cells. Figure 6 shows intracellular staining of IL-4 and IFN-γ in proliferating CD8+ (upper) and CD4+ (lower) T cells. Because cultures without costimulation generated only small numbers of viable cells, they were excluded from the analysis. B7.2–Ig costimulation induced high levels of IL-4 expression in the CD4+ T cell fraction. Although at an apparently reduced level, IL-4 expression was also induced in naïve CD8+ T cells when they were costimulated with B7.2–Ig. IFN-γ induction was observed in both CD8+ and CD4+ T cells irrespective of the type of costimulation, but was more potent in the former T cells than in the latter. It should be noted that contrary to IL-4 expression, the level of IFN-γ induction in CD8+ T cells was lower in the B7.2–Ig costimulated culture than in the anti-CD28-costimulated culture. Thus, B7.2–Ig has the capacity to induce IL-4 expression in anti-CD3-triggered naïve CD8+ T cells so far they are stimulated by anti-CD3 together with CD4+ T cells.
The capacity of B7.2–Ig to promote IL-4 production in antigen-primed CD8+ T cells

Among conventional antigens, allo-class I MHC antigens could be representative ones that efficiently activate the CD8+ T cell subset. While stimulation in vitro of primary BALB/c splenocytes with allo-class I-expressing EL-4 tumor cells activated both CD4+ and CD8+ T cells, this primary activation did not lead to enhanced generation of IL-4-producing CD8+ T cells even in the presence of B7.2–Ig costimulation (data not shown). We then examined whether B7.2–Ig costimulation affects type 2 polarization of antigen-primed CD8+ T cells during in vitro restimulation. BALB/c mice were immunized i.p. with MMC-treated EL-4 tumor cells, and 2 weeks later, unfracionated splenocytes from these antigen-primed mice (including CD4+ and CD8+ T cells) were restimulated with EL-4 cells in culture plates uncoated or coated with B7.2–Ig or anti-CD28 mAb. Cultured cells were exposed to PMA/ionomycin plus monensin and then examined for intracellular expression of IL-4 and IFN-γ. Figure 7 shows that costimulation with B7.2–Ig, but not with anti-CD28, during in vitro restimulation promotes the generation of IL-4-producing cells in both CD8+ and CD4+ T cell subsets.

The generation of IL-4-expressing cells induced by B7.2–Ig costimulation was apparently low in the CD8+ as well as CD4+ T cell subsets (Fig. 7), although such low frequencies of type 2 T cell differentiation were reproducible in at least three consecutive experiments. To verify that this weak generation of IL-4-expressing CD8+ T cells denotes type 2 CD8 polarization, CD4+ T cells were eliminated from splenocytes by injecting anti-CD4 mAb, and CD4+ T cell-depleted splenocytes were cultured and examined for their intracellular IL-4 expression (Fig. 8) and the secretion of IL-4 in culture supernatants (Fig. 9). In vivo CD4+ T cell depletion was performed either before or after the priming with EL-4 cells (prior to in vitro cultures). Comparison was also made between costimulation with B7.2–Ig and anti-CD28 mAb during restimulation cultures. Figure 8 shows that when costimulated with B7.2–Ig, splenocytes depleted of CD4+ T cells after EL-4 priming generated IL-4-producing CD8+ T cells. In contrast, anti-CD28 costimulation induced a much smaller number of IL-4-producing CD8+ T cells, although the levels of generation of IFN-γ-producing cells were comparable between B7.2–Ig and anti-CD28 costimulation. Importantly, when CD4+ T cells were depleted in mice before allo-priming, splenocytes from these mice generated only few type 2 CD8+ T cells even in restimulation cultures containing B7.2–Ig.

Figure 9 shows IL-4 and IFN-γ production in cultures of the above various groups. High levels of IL-4 production were observed when splenocytes were costimulated with B7.2–Ig, but not with anti-CD28, during in vitro priming. In contrast, anti-CD28 costimulation induced a much smaller number of IL-4-producing CD8+ T cells, although the levels of generation of IFN-γ-producing cells were comparable between B7.2–Ig and anti-CD28 costimulation. Importantly, when CD4+ T cells were depleted in mice before allo-priming, splenocytes from these mice generated only few type 2 CD8+ T cells even in restimulation cultures containing B7.2–Ig.

Discussion

The B7/CD28 costimulatory pathway has been well established to play an indispensable role in T cell activation [reviewed in (36,37)]. However, it remains to be completely understood how this pathway contributes to the polarization of TCR-triggered T cells to a functional Th1/Tc1 or Th2/Tc2
subset. Because signaling pathways downstream to TCR and CD28 form a set of TCR signals in a combinatorial way, the role of the CD28 pathway in type 1 and type 2 T cell differentiation should also be considered in conjunction with the strength and/or mode of TCR/CD28 stimulation.

CD28 costimulation has been generally performed using anti-CD28 mAb in most previous studies. However, the present study showed that the outcome of CD28 costimulation, particularly with respect to type 1 vs type 2 T cell polarization, differs whether CD28 molecules are stimulated with anti-CD28 mAb or its natural ligands (B7.2–Ig or B7.1–Ig). Anti-CD28 mAb and the natural ligands of CD28 induced comparable levels of IFN-γ expression in TCR-triggered CD4+ T cells, whereas IL-4-producing cells were induced preferentially when CD28 costimulation was performed using CD28 natural ligands. Moreover, B7-mediated generation of IL-4-producing cells from naïve CD4+ T cells was induced more easily by low rather than high levels of TCR stimulation. The B7 effect on the generation of IL-4-producing cells was also observed for the CD8+ subset of T cells. This was the case irrespective of whether they were naïve or antigen-primed. Importantly, the induction of IL-4-expressing CD8+ T cells required the coexistence of CD4+ T cells when CD8+ T cells received initial TCR stimulation. Thus, the present study raises important implications for the role of natural ligand-induced CD28 costimulation in the polarization of type 1 vs type 2 T cell differentiation.

It was first reported by Broeren et al. that CD4+ T cells are activated toward Th2 cytokine expression following stimulation with B7–Ig fusion proteins rather than anti-CD28 mAb (31). We made similar observations by employing an anti-CD28 mAb different from that used in their study (31). We also confirmed that essentially the same results are obtained with their type of anti-CD28 mAb (our unpublished observations). Therefore, it appears that the failure of our anti-CD28 mAb to efficiently costimulate IL-4 production is not ascribed to its own unique property. Further, our present results obtained for CD4+ T cells provided the following new information: (i) B7-mediated polarization to IL-4 expression is associated with preferential induction of GATA-3; (ii) low rather than high doses of anti-CD3 as a TCR stimulating reagent favor the polarizing effect of B7; and (iii) this B7 effect also occurs when TCR stimulation is provided by natural antigen plus APC instead of artificial anti-CD3 mAb. Furthermore, such a capacity of B7–Ig is applied for the induction of IL-4-producing CD8+ T cells. The last point is particularly important because the B7-induced polarization of CD8+ T cells not only has been poorly investigated in previous studies, including that of Broeren et al. (31), but also has a great biological significance, as discussed below.

The strength of TCR signaling governs the early events of T cell activation. However, the optimal strength of TCR stimulation required for either Th1 or Th2 cytokine response appears to be different [reviewed in (7,8–12)]. In general, Th2 cytokine
responses peak upon lower levels of TCR stimulation than Th1 cytokine responses (11,38). Because CD28 costimulatory signals integrate with TCR signals, the valency of functional TCR stimuli should be considered as the combination of TCR and CD28 costimulation. The present study showed that the generation of IL-4-producing cells from anti-CD3-triggered CD4+ T cells is more efficiently induced by CD28 costimulation with its natural ligands rather than with anti-CD28 mAb. In such a case, lower doses of anti-CD3 could induce higher levels of IL-4 expression. Previously, individual molecules as the CD28 natural ligands selectively regulate differentiation into the Th1 or Th2 phenotype. For example, interaction of CD28 with B7.2 has been suggested to promote Th2 differentiation but not Th1 differentiation (17,18,20). In contrast, CD28 interacting with B7.1 has been proposed to be inhibitory for Th2 differentiation and integral to Th1 differentiation (19,21). These contradictory observations remain to be reconciled. One caveat to these studies may be that they were mostly performed in a situation where the TCR stimuli were not varied. In contrast to this contradiction, our present results showed that either B7.2 or B7.1 is capable of effectively promoting Th2 differentiation as evaluated by the generation of IL-4-producing cells, particularly when the B7-mediated CD28 costimulation is combined with the low valency of TCR stimulation.

B7.2–Ig-mediated upregulation of IL-4 production was observed when TCR stimulation was provided not only with anti-CD3/B7.2–Ig (for conventional naïve CD4+ T cells) but also with native antigen (OVA) plus APC (for CD4+ T cells from anti-OVA TCR transgenic mice). This cytokine, once released from APC into cultures, can strikingly stimulate IFN-γ production (40) irrespective of the presence of B7–Ig.
The biological significance for B7-Ig-mediated facilitation of type 2 CD8+ T cell differentiation comes from a series of our recent observations. Our previous studies (42,43) have shown that tumor-reactive T cells are primed to tumor antigens in tumor-bearing mice through collaboration with APC-presenting processed tumor antigens. While this was initially observed with tumor-reactive CD4+ T cells, the same was recently shown to hold true for CD8+ T cells (44). Administration of B7.2-Ig or B7.1-Ig to tumor-bearing mice resulted in regression of growing tumors (44). This regression was found to associate with enhanced in vivo activation of tumor antigen-sensitized CD8+ T cells by B7-Ig fusion proteins and to depend on IL-4 production (44). The B7-Ig as the natural ligands of CD28 was not replaced by anti-CD28 mAb in inducing this anti-tumor effect. However, it was unclear in such an in vivo study (44) whether B7.2-Ig actually promotes differentiation of antigen-primed CD8+ T cells into the IL-4-producing phenotype. The present study provides a direct demonstration that B7-Ig fusion proteins have the capacity to induce enhanced differentiation of naïve as well as antigen-primed CD8+ T cells into IL-4-producing cells.

While CD8+ T cells, like CD4+ T cells, differentiate into the type 2 phenotype depending on CD28 costimulation with B7-Ig, these two subsets of T cells exhibited an essential difference in the capacity to pursue type 2 differentiation. CD4+ T cells alone were capable of differentiating into Th2, whereas the type 2 differentiation of CD8+ T cells required the co-existence of CD4+ T cells during the initial TCR stimulation. However, antigen-primed CD8+ T cells could pursue type 2 differentiation independent of CD4+ T cells during restimulation in vitro. TCR-triggered Th2 cells need IL-2 for survival and proliferation at initial phases of their activation. In general, CD4+ T cells can produce large amounts of IL-2, whereas IL-2 expression by CD8+ T cells is negligible and insufficient for maintaining TCR-triggered CD8+ T cell proliferation (45,46). Therefore, the upregulating effect of B7-Ig on IFN-γ expression may be rendered obscure in such a condition.

A more important aspect of the present study concerns the effect of B7-Ig-mediated costimulation on the functional differentiation of CD8+ T cells. As in the case of CD4+ T cells, CD8+ T lymphocytes can be classified into two distinct effector cell types (type 1 and type 2 CD8+ T cells; Tc1 and Tc2) depending on their cytokine-secreting profiles following antigen encounter (3,4). Because activation of naïve CD8+ T cells requires CD28 costimulation (41), probably more CD28 costimulatory activity than for the activation of CD4+ T cells, the B7/CD28 costimulation could also regulate type1 and type 2 CD8+ T cell differentiation. However, little is known regarding which type of CD8 T cell differentiation is promoted more efficiently by CD28 costimulation. In this context, the present study demonstrates that the capacity of B7-Ig to induce type 2 T cell differentiation is applied for the CD8 as well as the CD4 T cell subset. This was observed for naïve CD8+ T cells upon stimulation with anti-CD3 in the presence of B7-Ig costimulation, depending on the coculture with naïve CD4+ T cells on the one hand. On the other, type 2 CD8 differentiation was hardly observed during primary stimulation of naïve CD8+ T cells with natural antigens such as alloantigens even in the presence of naïve CD4+ T cells (our unpublished observations). However, antigen-primed CD8+ T cells were facilitated to differentiate into the type 2 phenotype when costimulated with B7-Ig during in vitro restimulation.
be possible that there exists a qualitative difference in these stimuli: for example, antibody aggregates two molecules of CD28 whereas the B7 fusion protein, even in the dimeric form, may react only with a single CD28 molecule. Studies will be required to investigate the differential capacities of the B7–Ig fusion proteins and anti-CD28 mAb in terms of signaling to IL-4 expression. Nevertheless, by directly demonstrating the capacity of the B7-fusion proteins to induce type 2 CD8 T cell differentiation that has a great significance, the present results add to a growing list of research on the CD28/B7 costimulatory pathway.

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Abbreviations
ICOS inducible costimulator
OVA ovalbumin

References


