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Authors

McArthur, JG
Raulet, DH

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CD28-induced Costimulation of T Helper Type 2 Cells Mediated by Induction of Responsiveness to Interleukin 4

By James G. McArthur and David H. Raulet

From the Department of Molecular and Cell Biology, University of California at Berkeley, Berkeley, California 94720

Summary

Type 1 and type 2 cloned T helper (Th) cells are believed to require different antigen-presenting cell (APC)-derived costimuli for proliferation. In the case of Th1-cloned T cells, CD28 signaling costimulates production of autocrine interleukin 2 (IL-2). Th2 cells produce their autocrine growth factor, IL-4, without costimulation, but require APC-derived costimuli, or IL-1, to respond to IL-4. Here we demonstrate that engagement of CD28 on Th2 cells with anti-CD28 antibody or with APC-associated B7 costimulates Th2 responsiveness to IL-4 but does not affect IL-4 or IL-2 production by Th2 cells. Costimulation of Th2 cells via CD28 appears to involve the induction of IL-1 production by Th2 cells, which acts in an autocrine fashion to induce IL-4 responsiveness. These results suggest that CD28-induced costimulation plays an important role in responses mediated by both types of Th cells.

Two major groups of cloned Th cells can be distinguished on the basis of the cytokines they produce. Most cloned Th1 cells secrete IL-2 and IFN- γ as signature cytokines, whereas cloned Th2 cells secrete IL-4, IL-5, IL-6, and IL-10 (1–5). The proliferative response to antigen of Th1 and Th2 cells requires the action of the autocrine growth factors IL-2 and IL-4, respectively. Activation of the growth pathway of both types of clones requires not only the engagement of the TCR, but additional costimulatory signals provided by APC. However, costimulatory signaling of the proliferation of Th1 and Th2 cells occurs at different steps of T cell activation. Costimulation of Th1 cells is mediated in large part by increasing the production of IL-2 and other cytokines by the cells (5, 6). In contrast, evidence suggests that engagement of the TCR of Th2 cells is a sufficient signal to activate production of the autocrine growth factor IL-4, but costimulation is necessary to render the cells responsive to IL-4 (7–9).

The costimulation of Th1 and Th2 cells has been thought to be mediated by distinct APC-derived signals. In the case of Th1 cells and freshly isolated murine CD4⁺ T cells, costimulation can be mediated by engagement of cell surface CD28, by anti-CD28 mAb, or by its natural ligand B7, which is expressed by most APC. CD28 is a member of the Ig superfamily expressed on most T cells (10, 11). CD28 costimulation of Th1 cells results in a dramatic increase in the levels of IL-2 and other lymphokine transcripts, as a result of elevated transcript stabilization as well as increased cytokine gene transcription (12–15). Engagement of the TCR of cloned Th1 cells in the absence of costimulatory signals is not a neutral

event, but induces long-term T cell unresponsiveness, or anergy, and may represent a mechanism of nondeletional tolerance in vivo (5, 6). Costimulation of cloned Th1 cells with anti-CD28 mAb prevents the induction of anergy (16).

In contrast, previous studies suggest that APC-derived IL-1 plays an important role as a costimulatory signal for Th2 cells. Exogenously supplied IL-1 costimulates responses of Th2 cells stimulated with mitogens, anti-TCR antibodies, or fixed antigen-pulsed APC (2, 7, 8). Furthermore, exogenously supplied IL-1 renders resting Th2 cells sensitive to proliferation induced by IL-4. Some APCs, such as macrophages, produce high levels of IL-1 (17). However, some potent APCs, such as dendritic cells, reportedly do not produce IL-1 (18). Hence, APC-derived IL-1 may not be the sole costimulatory signal for Th2 cells.

In this report, we show that cloned Th2 cells express CD28 on their surface and that engagement of CD28 costimulates Th2 cell proliferation. CD28 engagement induced increased Th2 IL-4 responsiveness through an IL-1-dependent mechanism and was required for the stimulation of Th2 cell proliferation by splenic APC and antigen. The results suggest that the CD28 costimulation pathway is common to both major classes of Th cell clones, although the stage of action of CD28 costimulation differs.

Materials and Methods

Cell Lines and Reagents. The AKR-derived D10.G4 (19) and BALB/c-derived CDC-35 (20) cloned Th2 cell lines have been de-

scribed previously. The T32 Th1 clone was kindly provided by Drs. T. Briner and M. Geffer (Massachusetts Institute of Technology, Cambridge, MA), and has been described previously (16). The HT-2 indicator cell line has also been previously described (21). RPMI media was obtained from Bio Whittaker, Inc. (Walkersville, MD) and fetal bovine serum (FBS)¹ from Sigma Chemical Co. (St. Louis, MO). The anti-CD28 mAb (37.51) was generously provided by Dr. J. P. Allison (University of California, Berkeley, CA) and has been described previously (22). CTLA4-Ig is a fusion molecule containing the cytoplasmic domain of mouse CTLA4 and the hinge, C γ 2, C γ 3, and C γ 4 domains of human IgG1. CTLA4-Ig-expressing transfectants were generously provided by P. Lane (Basel Institute for Immunology, Basel, Switzerland). CTLA4-Ig was isolated from the culture supernatant of these cells using a protein A-Sepharose column. IL-1 receptor agonist (RA) (23) and murine IL-2 were purchased from R&D Systems, Inc. (Minneapolis, MN) and Cetus Corp. (Emeryville, CA), respectively. Murine IL-4 and human IL-1 α were generously provided by the DNAX Research Institute (Palo Alto, CA) and Hoffmann-La Roche (Nutley, NJ), respectively.

Cell Culture Conditions. The Th2 cell lines used in this study were maintained as described previously (19, 20). Briefly, 10⁵ D10.G4 or CDC-35 cloned Th2 cells were restimulated weekly in 24-well plates with 200 μ g/ml conalbumin (ConAlb) or 100 μ g/ml rabbit gamma globulin (RGG), respectively, in complete RPMI media supplemented with 10% FBS, 2 \times 10⁶ T cell-depleted (CD4⁻, CD8⁻, Thy-1⁻) C3H/HeJ or BALB/cByJ (Jackson Laboratories, Bar Harbor, ME) irradiated (3,000 rad) splenocytes, 5% Con A-stimulated rat spleen cell culture supernatant, and 25 mM α -methyl mannoside. At the end of a 7-d stimulation cycle, the T cells were isolated by centrifugation on a Ficoll-Isopaque gradient. Where indicated, Th2 cells were further purified by passing the cells over a nylon wool column to remove adherent cells, and IE⁺ cells were depleted by incubating the cells with an anti-IE mAb (14.4.4) (24) and complement. Viable Th2 cells were then isolated by centrifugation on a Ficoll-Isopaque gradient.

Proliferation Assays. For proliferation assays involving splenocytes, 10⁴ Th2 cells were cultured with the indicated number of T cell-depleted irradiated (3,000 rad) syngeneic spleen cells, antigen, and the indicated additions for 48 h at 37°C in a total volume of 100 μ l in 96A/2 flat-bottom microtiter plates (Costar, Cambridge, MA). T cell proliferation was determined by pulsing cultures with 0.5 μ Ci of [³H]thymidine for an additional 18 h, harvesting with a sample harvester (Skatron, Sterling, VA) and counting incorporated [³H]thymidine.

Antibody-coated plates were prepared with 96 A/2 flat-bottom microtiter plates (Costar) as described previously (25). In brief, wells were incubated with 50 μ l PBS containing 4 μ g/ml of the anti- α/β TCR mAb H57-597 (26) at 22°C for 2 h, followed by a post-coat with 5% FBS in PBS at 37°C for 30 min and multiple washes with PBS containing 5% FBS. 10⁴ Th2 cells were cultured in mAb-coated wells with the indicated additions in a total volume of 100 μ l for 48 h at 37°C, pulsed with 0.5 μ Ci [³H]thymidine for 18 h, and harvested for [³H]thymidine counting.

Lymphokine Assays. HT-2 cells (21) were obtained from A. Abbas (Harvard Medical School, Boston, MA) and maintained in complete media supplemented with 5% FBS, 10% Con A-stimulated rat spleen cell culture supernatant, and 25 mM α -methyl man-

noside. To determine IL-2 and IL-4 levels, dilutions of T cell culture supernatants, harvested 24 h after T cell stimulation, were cultured with 2,500 HT-2 cells in 96-well A/2 plates for 24 h at 37°C. The cultures were pulsed with 0.5 μ Ci [³H]thymidine the last 18 h, and the [³H]thymidine incorporation was determined. IL-2 versus IL-4 content was determined by including 10 μ g/ml of either purified anti-IL-4, 11B11 (27), or purified anti-IL-2, S4B6 (28) mAb in the cultures.

Flow Cytometry. 10⁶ cells/ml were incubated in PBS containing 5% FBS alone or with anti-CD28 mAb (1/20 dilution of ascites fluid) for 30 min on ice, washed extensively, and incubated with a 1/12 dilution of FITC-conjugated anti-hamster IgG mAb (CALTAG Laboratories, South San Francisco, CA) for an additional 20 min on ice. After washing the cells extensively, they were analyzed on a FACS[®] IV analyzer (Becton Dickinson & Co., Mountain View, CA).

IL1 PCR Analysis. RNA was prepared from stimulated D10.G4 or resident peritoneal cells with a guanidinium-isothiocyanate extraction in the presence of carrier bacterial rRNA and ultracentrifugation through a cesium chloride cushion (29). The D10.G4 cells were purified before activation by passage over nylon wool and depleted of IE⁺ cells to minimize contamination with APC (see above). Variable amounts of total cellular RNA corresponding to calculated input cell equivalents were subjected to RT-PCR to measure the levels of IL-1 transcripts. PCR was carried out using conditions suggested by the manufacturer of the IL-1 primers (Stratagene Inc., La Jolla, CA). To amplify IL-1 α transcripts, reverse transcription was primed with an 820–843-bp antisense primer, and the cDNA was amplified with the same primer and a 301–324-bp sense primer. To amplify IL-1 β transcripts, a 756–776-bp antisense primer and a 330–350-bp sense primer were used. The positive control β -tubulin transcripts were amplified with a 24–54-bp sense transcript and 210–239-bp antisense transcript. PCR was carried out for 50 cycles (30). The PCR products were analyzed by electrophoresis on a 4% agarose gel.

Results

Anti-CD28 mAb Can Costimulate Th2 Cell Proliferation. To determine if Th2 cells express surface CD28, the AKR-derived Th2 clone D10.G4 (19) and BALB/c-derived Th1 clone T32 were stained with mAb 37.51 (anti-CD28) (22) and a FITC-conjugated anti-hamster IgG mAb. Analysis of the cells by flow cytometry demonstrated that D10.G4 Th2 cells and T32 Th1 cells express similar levels of cell surface CD28 protein (Fig. 1 a).

Engagement of the TCR by antigen-MHC class II complexes can be mimicked by aggregation of the TCR with the use of immobilized anti-TCR antibodies. To determine if anti-CD28 mAb (mAb 37.51) costimulated Th2 proliferation, D10.G4 and CDC-35 cells were cultured in wells coated with anti-pan TCR- β mAb, H57-597 (26) and variable concentrations of soluble anti-CD28 mAb. Anti-CD28 mAb costimulated vigorous proliferation of both T cell clones (Fig. 1, b and c). Both clones could also be costimulated by IL-1 α (Fig. 1, see legend). As expected, little proliferation was observed when Th2 cells were cultured with anti-TCR mAb alone, or with anti-TCR mAb and an irrelevant control hamster mAb (anti-V γ 3 TCR) (Fig. 1, b and c). In no case did anti-CD28 mAb alone stimulate D10.G4 cell proliferation (data not shown). These experiments indicate that CD28 sig-

¹Abbreviations used in this paper: ConAlb, conalbumin; FBS, fetal bovine serum; RA, receptor agonist; RGG, rabbit gamma globulin.

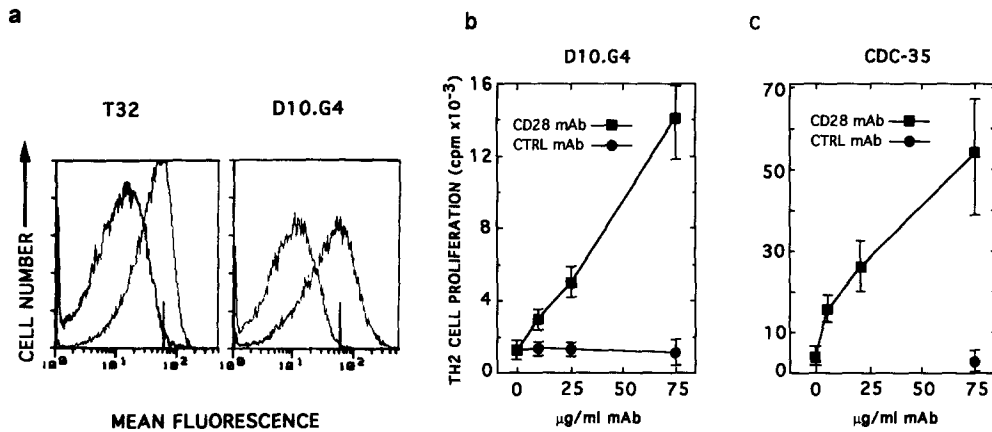


Figure 1. Anti-CD28 mAb can costimulate cloned Th2 cell proliferation. (a) Cloned Th2 cells express cell surface CD28 as shown in this FACS[®] analysis of cloned T32 Th1 cells and D10.G4 Th2 cells stained with the anti-CD28 mAb 37.51 and a goat anti-hamster IgG-FITC. Staining with the second-step antibody alone is also shown. (b and c) Anti-CD28 mAb can costimulate cloned Th2 cells. D10.G4 Th2 cells (b) or CDC-35 Th2 cells (c) were cultured in 96 A/2 well plates coated with anti-TCR- β mAb (H57-597) and the indicated concentrations of soluble purified anti-CD28 antibody (■) or an irrelevant anti-V γ 3 TCR mAb (●). The proliferative responses were determined 48 h after stimulation. The proliferative response after stimulation with anti-TCR- β mAb and 1 U/ml IL-1- α was 39,729 cpm in the case of D10.G4 cells (b) and 55,416 cpm in the case of CDC-35 cells (c).

naling can substitute for exogenously added IL-1 in costimulating Th2 cell proliferation.

Costimulation of Th2 Cell Proliferation by Anti-CD28 mAb Results from Stimulation of Responsiveness to IL-4. The response of D10.G4 cells stimulated with immobilized anti-TCR mAb and anti-CD28 mAb was completely blocked by the addition of neutralizing anti-IL-4 mAb, 11B11 (27) but largely unaffected by anti-IL-2 mAb, S4B6 (28) (Fig. 2 a). These

data indicate that costimulation of Th2 cells mediated by anti-CD28 mAb acts through an IL-4 growth factor-dependent pathway, and not by induction of IL-2 production.

To test whether anti-CD28 mAb increases the responsiveness of Th2 cells to IL-4, D10.G4 cells were incubated with 5 U/ml of rIL-4 and the indicated concentration of purified anti-CD28 mAb (Fig. 2 b). As has been shown previously, rested Th2 cells proliferate minimally when cultured with IL-4 alone ([7-9], Fig. 2 b). However, coculture with soluble anti-CD28 mAb increased D10.G4 cell proliferation in response to IL-4 approximately sixfold. Similar results were obtained with the CDC-35 Th2 clone (see below, Fig. 4 d). A control hamster mAb, against the TCR V γ 3, did not augment D10.G4 or CDC-35 cell proliferation mediated by IL-4 (Fig. 2 b and data not shown). These results demonstrate that CD28 costimulation induces responsiveness of Th2 cells to IL-4 in the absence of TCR engagement. The increase in Th2 cell responsiveness to IL-4 was not IL-2 dependent, as shown by the inability of the anti-IL-2 mAb S4B6 to inhibit D10.G4 proliferation (Fig. 2 b).

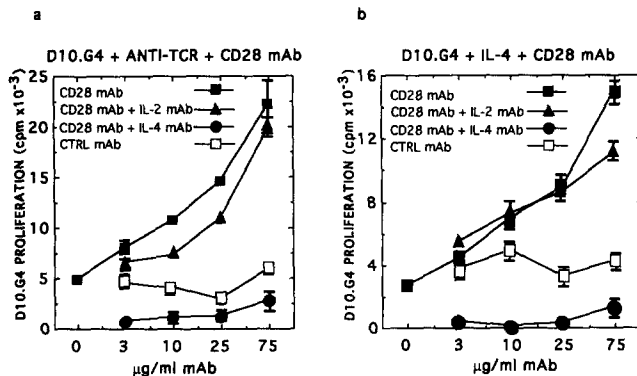


Figure 2. CD28 costimulation acts at the level of Th2 cell responsiveness to IL-4. (a) Costimulation of D10.G4 cell proliferation with immobilized anti-TCR antibody and anti-CD28 mAb is IL-4 dependent. D10.G4 cells were cultured with plate-bound anti-TCR- β mAb and the indicated concentrations of soluble purified anti-CD28 mAb (■). Parallel sets of cultures also contained 10 μ g/ml anti-IL-4 mAb (●) or 10 μ g/ml anti-IL-2 mAb (▲). The proliferative responses of cultures stimulated with plate-bound anti-TCR and an irrelevant hamster anti-V γ 3 TCR mAb are also shown (□). (b) Anti-CD28 mAb potentiates D10.G4 cell proliferation to exogenous IL-4. D10.G4 cells were cultured with 5 U/ml of IL-4 and the indicated concentrations of purified anti-CD28 mAb (■) or an irrelevant anti-V γ 3 TCR mAb (□). The T cell proliferation observed in cultures containing anti-CD28 is IL-4 dependent, as shown by its inhibition with 10 μ g/ml anti-IL-4 mAb (●), but not 10 μ g/ml anti-IL-2 mAb (▲).

CD28-B7-mediated Costimulation Does Not Increase Growth Factor Production by Th2 Cells. CD28 costimulation with anti-CD28 mAb did not increase production of the growth factors IL-4 or IL-2 by Th2 cells. This was shown by testing the capacity of activated D10.G4 or CDC-35 cell culture supernatants to induce proliferation of HT-2 indicator cells (21), which respond to both IL-4 and IL-2 (4). The titration curves revealed that the IL-4 content of culture supernatants of CD28-costimulated cells was similar to that of cells stimulated by anti-TCR mAb alone (Fig. 3, a and b). The active growth factor in the culture supernatants was identified as IL-4 by inhibition of the proliferation of the HT-2 indicator cells with anti-IL-4 mAb but not with anti-IL-2 mAb (Fig. 3, a and b). Titrations of rIL-4 are shown for comparison. Therefore,

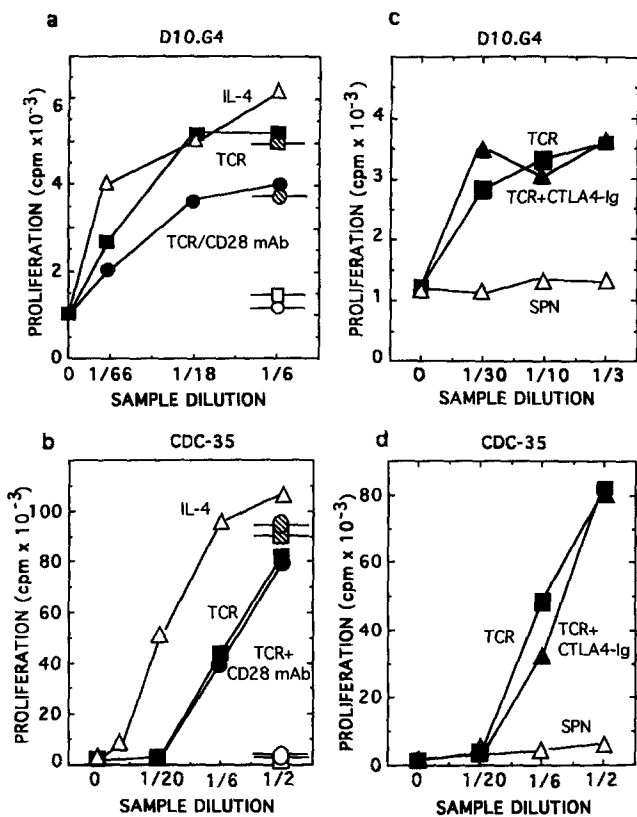


Figure 3. CD28-B7 costimulation of Th2 cell proliferation does not affect IL-4 production. (a and b) Costimulation with anti-CD28 mAb does not increase IL-4 or IL-2 release by D10.G4 or CDC-35 cells. Cell culture supernatants of D10.G4 cells (a) or CDC-35 cells (b) were titrated for growth-promoting activity on the IL-2/IL-4-responsive HT-2 indicator cells. Supernatants from cells activated with plate-bound anti-TCR- β mAb (4 μ g/ml) (■) or with anti-TCR- β mAb and 75 μ g/ml soluble CD28 mAb (●) were tested. In both cases the active growth factor was IL-4, as shown by the inhibition of HT-2 proliferation with anti-IL-4 mAb (open symbols), but not anti-IL-2 mAb (hatched symbols). Titrations of rIL-4 (initial sample concentrations were 180 U/ml (Fig. 3 a) and 60 U/ml (Fig. 3 b) are shown for comparison (Δ). The assays shown in a, b, and c were performed on separate days. (c and d) CTLA4-Ig does not inhibit IL-4 release by Th2 cells. Similar levels of HT-2 proliferation were induced by cell culture supernatants of D10.G4 cells (c) or CDC-35 cells (d) that were activated with plate-bound anti-TCR- β mAb (4 μ g/ml) alone (■) or with anti-TCR- β mAb and 18 μ g/ml CTLA4-Ig (\blacktriangle). Supernatants from D10.G4 or CDC-35 cells cultured with 10⁵ T cell-depleted syngeneic splenocytes without antigen served as a control to show that IL-4 release required TCR engagement (Δ). The [³H]thymidine incorporation was determined 48 h later. The data in b and d are from the same experiment.

although CD28-mAb-mediated costimulation induced increased growth factor (IL-2) production by Th1 cells, it failed to induce production of IL-2 by Th2 cells and did not increase the level of IL-4 production by these cells.

Some evidence suggests that Th2 cells costimulate proliferative responses of Th1 cells (31, 32), raising the possibility that Th2 cells express costimulatory molecules such as B7. These observations raise the possibility that Th2 cells can receive costimulatory signals from other Th2 cells in culture. However, the level of B7 expressed by Th2 cells is apparently insufficient to costimulate IL-4 responsiveness or proliferation of Th2 cells (Figs. 1 and 2). Nevertheless, the possibility

remained that the interaction of CD28 on Th2 cells with B7 on other Th2 cells is necessary for IL-4 production by Th2 cells in our experiments. To test this possibility, we used the B7 antagonist CTLA4-Ig, a genetically engineered soluble fusion protein derived from CTLA4, a second high-affinity receptor for B7. A saturating dose of CTLA4-Ig, sufficient to prevent B7-mediated costimulation of resting T cells and Th1 cells ([33]; Fig. 6 and data not shown), was included in Th2 cultures stimulated with anti-TCR mAb, and the IL-4 content of the culture supernatants was subsequently assayed with the use of HT-2 indicator cells. A comparison of the titration curves revealed that the inclusion of CTLA4-Ig in

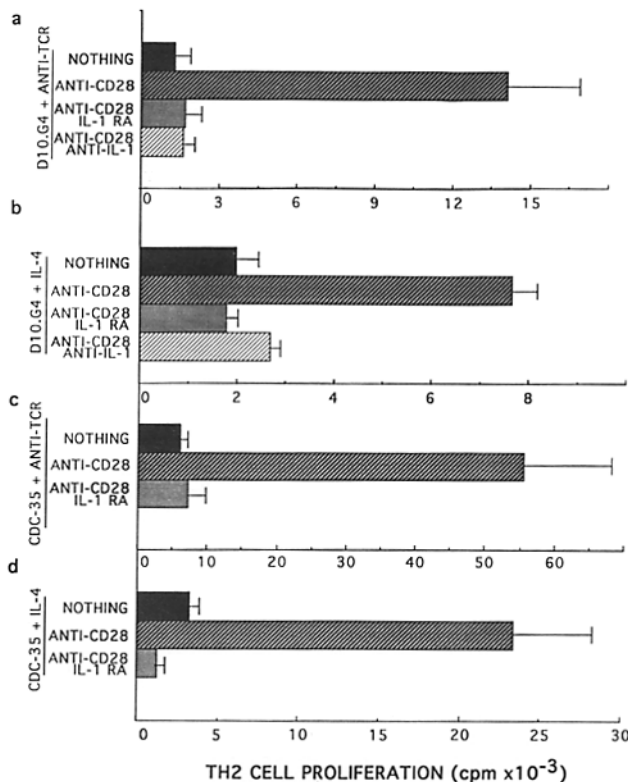


Figure 4. Anti-CD28 mAb costimulation of Th2 cell proliferation is IL-1 dependent. (a and c) CD28 costimulation of anti-TCR stimulated Th2 cells is IL-1 dependent. D10.G4 (a) or CDC-35 (c) cells were stimulated with plate-bound anti-TCR- β mAb with or without the addition of 75 μ g/ml purified anti-CD28 mAb. Parallel sets of cultures stimulated with plate-bound anti-TCR mAb and anti-CD28 mAb were also incubated with 250 ng/ml IL-1 receptor antagonist (IL1 RA) or a mixture of 10 μ g/ml anti-IL-1- α mAb and 10 μ g/ml anti-IL-1- β mAb (ANTI-IL1; a). The inhibition by IL-1 antagonists was not due to nonspecific toxicity because D10.G4 cell proliferation was restored with the addition of 5 U/ml IL-2 (IL-1 RA/IL-2, 42,099 cpm; ANTI-IL-1/IL-2, 24,885 cpm). Both D10.G4 and CDC-35 cells were depleted of IE⁺ cells, and D10.G4 cells were further depleted of adherent cells as described in Materials and Methods. (b and d) Responsiveness of Th2 cells to IL-4 is IL-1 dependent. D10.G4 (b) or CDC-35 (d) cells were stimulated with 5 U/ml IL-4 alone or with the further addition of 75 μ g/ml purified anti-CD28 mAb, and the proliferative responses were measured. Parallel cultures contained in addition 250 ng/ml IL-1 receptor antagonist (IL1 RA) or a cocktail of 10 μ g/ml anti-IL-1- α mAb and 10 μ g/ml anti-IL-1- β mAb (ANTI-IL1; b). The inhibition by IL-1 antagonists was not due to nonspecific toxicity because D10.G4 cell proliferation was restored with the addition of 5 U/ml IL-2 (IL-1 RA/IL-2, 42,738 cpm; ANTI-IL-1/IL-2, 26,180 cpm).

cultures of D10.G4 or CDC-35 cells had no effect on the levels of IL-4 release induced by anti-TCR mAb (Fig. 3, *c* and *d*). HT-2 proliferation induced by each of these culture supernatants was abrogated with the addition of an anti-IL-4 mAb, but not with an anti-IL-2 mAb (data not shown). Together these data suggest that IL-4 production by Th2 cells is independent of the B7-CD28 signaling pathway.

CD28 Costimulation of Th2 Cells Is IL-1 Dependent. Because responsiveness of Th2 cells to IL-4 was stimulated by either anti-CD28 mAb or by IL-1, it was possible that the effects of CD28 signaling were mediated indirectly by IL-1. IL-1 inhibitors were used to test this possibility. Vigorous D10.G4 cell proliferation was observed in cultures stimulated with anti-TCR mAb and anti-CD28 mAb, but the response was inhibited to background levels by purified IL-1 RA or by a mixture of anti-IL-1 α and anti-IL-1 β mAbs (Fig. 4 *a*). Similarly, the costimulation of CDC-35 cell proliferation with anti-CD28 mAb was inhibited by the addition of IL-1 RA (Fig. 4 *c*). The IL-1 inhibitors also prevented the induction by anti-CD28 mAb of enhanced responsiveness of D10.G4 and CDC-35 cells to exogenously added IL-4, in the absence of TCR engagement (Fig. 4, *b* and *d*). These results demonstrate that costimulation through CD28 of Th2 cell IL-4 responsiveness and proliferation involves an IL-1-dependent mechanism.

In the preceding experiments, no APC or other cells were added to the Th2 cell cultures, and possible contaminating APC were depleted by passage of the Th2 cells over nylon wool columns and/or cytotoxic elimination of MHC class II⁺ cells with anti-IE mAb plus complement (see legend to Fig. 4). These considerations suggest that the source of the IL-1 is the Th2 cells themselves.

To determine if IL-1 transcripts were induced in Th2 cells after culture with anti-CD28 mAb, RT-PCR analysis was performed. IL-1 α transcripts were detected as a 265-bp PCR product in RNA from D10.G4 cells costimulated with anti-CD28 mAb (Th2/TCR/CD28), but not in RNA from cultures stimulated with anti-TCR- β mAb alone (Th2/TCR) (Fig. 5 *a*). This was confirmed by Southern blot analysis with an internal IL-1 α -radiolabeled probe (Fig. 5 *b*). Based on comparisons of the titration of the RNA samples in this and another experiment, IL-1 α transcript levels were approximately 5–10-fold lower in costimulated Th2 cells than in LPS-activated peritoneal cells, a source of macrophages. Equivalent amounts of intact RNA were used, as shown by RT-PCR amplification of β -tubulin transcripts. It is unlikely that the IL-1 α transcripts in the Th2 cultures arise from contaminating macrophages, because the LPS-activated macrophages, a potent source of IL-1, produce only 5–10 times more IL-1 α transcripts than the Th2 cells. Furthermore, the Th2 cells had been depleted

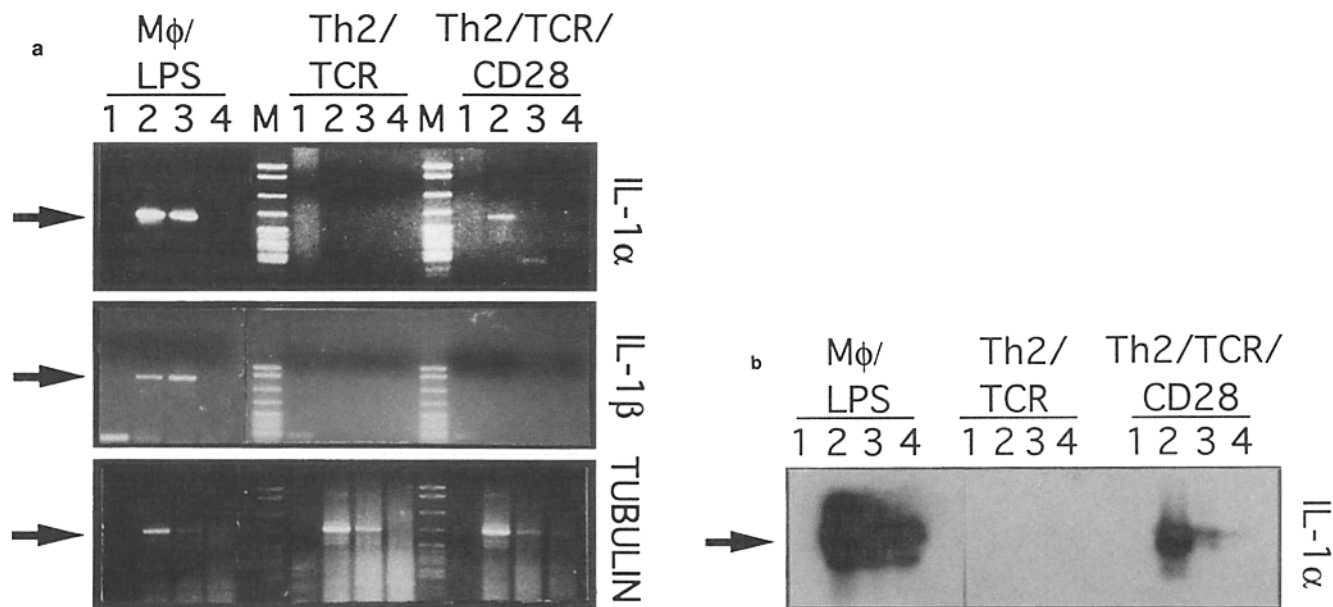


Figure 5. CD28 signaling increases IL-1 α transcript levels in Th2 cells. (a) Total RNA was prepared from D10.G4 cells stimulated for 12 h with anti-TCR- β mAb (Th2/TCR), alone or with the addition of anti-CD28 mAb (Th2/TCR/CD28). RNA from LPS-activated resident peritoneal cells (M ϕ) was also analyzed as a positive control. Each RNA was analyzed by reverse transcription followed by PCR amplification of the cDNA from 25,000, 5,000, and 1,000 cell equivalents, with primer pairs for mouse IL-1 α (lanes 2–4) or IL-1 β (lanes 2–4). PCR with each primer pair was performed on all cell types in parallel. To control for the amount of RNA in each sample, 1,000, 200, and 40 cell equivalents of RNA were also analyzed by reverse transcription and PCR DNA amplification with a primer pair for β -tubulin (TUBULIN; lanes 2–4) as described previously (43). PCR DNA amplification was also performed without reverse transcriptase to ensure that the observed bands did not result from amplification of genomic DNA. For the IL-1 α and IL-1 β primers, 25,000 cell equivalents were used and for tubulin, 1,000 cell equivalents were used (lane 1). The PCR products were fractionated on a 2% agarose gel and visualized by ethidium bromide staining. MspI-digested pBR322 DNA fragments served as markers (M). (b) Southern blot analysis of IL-1 α PCR products. The gel shown in the top of *a* was blotted to a nitrocellulose membrane and the blot was hybridized with a ³²P-end-labeled IL-1 α homologous oligonucleotide probe (516–546 bp) (44) by standard protocols. The resulting autoradiogram from the Southern blot is shown.

of APC before stimulation (see Materials and Methods). Also arguing against the possibility that contaminating macrophages were the source of the IL-1 transcripts was the observation that no IL-1 β transcripts (expected product, 447 bp) were detected in the costimulated Th2 cell RNA. IL-1 β transcripts were easily detected in RNA from control-activated macrophages (M ϕ /LPS). These results indicate that CD28 costimulation induces IL-1 α (but not IL-1 β) transcripts in Th2 cells, and suggests that induction of IL-1 α production is the mechanism of CD28-mediated costimulation of Th2 cells.

Costimulation of Th2 Cells by Splenic APC Is Mediated by the CD28-B7 Pathway. The previous experiments demonstrate that engagement of CD28 with an mAb is costimulatory to Th2 cells, but do not indicate whether APC can costimulate Th2 cells via a B7-CD28-mediated interaction. To address this question, we investigated the effects of the B7 antagonist, CTLA4-Ig, on proliferation of Th2 cells induced by antigen and splenic APC (Fig. 6). CTLA4-Ig inhibited antigen-dependent proliferation of both D10.G4 and CDC-35 Th2 cells in a dose-dependent fashion (Fig. 6, *a* and *b*). The proliferative responses of the CTLA4-Ig-containing cultures were restored by addition of IL-2, demonstrating that the CTLA4-Ig was not toxic for the cells. The effects of CTLA4-Ig on these responses suggest that B7 signaling plays an important role in costimulation of Th2 cell proliferation by splenic APC.

The effects of CTLA4-Ig and IL-1 RA were compared for their capacity to inhibit proliferative responses of D10.G4 cells induced by antigen and splenic APC (Fig. 6 *c*). As in the previous experiment, CTLA4-Ig strongly blocked D10.G4 proliferation induced by antigen and APC. IL-1 RA also blocked D10.G4 proliferation, consistent with earlier reports (7) and with our finding that costimulation mediated by anti-CD28 mAb is an IL-1-dependent process. The combination of the two inhibitors was only slightly more inhibitory than either alone. The inhibition of D10.G4 proliferation by CTLA4-Ig and IL-1 RA was not due to toxicity of the reagents, because the addition of IL-2 restored the proliferative response. Taken together, these results indicate that B7-CD28-mediated costimulation plays an important role in the responses of Th2 cells to antigen presented by splenic APC. The dependence of the responses on IL-1 production, presumably by the responding Th2 cells, suggests that IL-1 production occurs as an intermediate step.

Costimulation of Th2 Cell Proliferation by IL-1 Is Independent of the B7-CD28 Pathway. The preceding data do not address the possibility that IL-1- and CD28-mediated signals are both essential to synergistically activate Th2 cells. Although Th2 cells are costimulated by exogenous IL-1 in the absence of APC, Th2 cells may themselves express costimulatory molecules, as noted earlier. To determine whether costimulation of Th2 cells mediated by exogenous IL-1 requires B7-mediated interactions, we used the B7 antagonist CTLA4-Ig. The addition of increasing concentrations of CTLA4-Ig, up to 10 μ g/ml, failed to inhibit the proliferative response costimulated by exogenous IL-1 α (Fig. 7). This concentration of CTLA4-Ig is sufficient to strongly inhibit

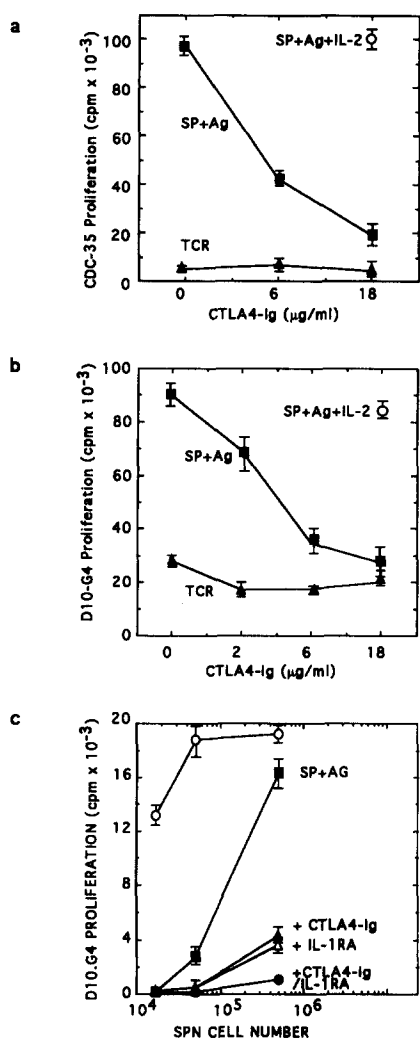


Figure 6. B7-CD28 signaling is involved in stimulation of Th2 cells by splenic APC and antigen. CDC-35 (*a*) and D10.G4 (*b*) cells were stimulated with 2×10^5 T-depleted irradiated (3,000 rad) syngeneic splenocytes and antigen (RGG or Con Alb, respectively) and increasing concentrations of CTLA4-Ig (\blacksquare). As a control for toxicity of the CTLA4-Ig, the inhibition caused by the highest dose of CTLA4-Ig was reversed with the addition of 5 U/ml rIL-2 (\circ). The Th2 cells were also stimulated with 4 μ g/ml plate-bound anti-TCR mAb and the indicated concentration of CTLA4-Ig (\blacktriangle). (*c*) D10.G4 cells were stimulated with the indicated number of T cell-depleted irradiated syngeneic splenocytes and 100 μ g/ml antigen (Con Alb) (\blacksquare). Parallel sets of cultures contained 10 μ g/ml CTLA4-Ig (\blacktriangle), 250 ng/ml IL-1 RA (\triangle), or both (\bullet), and the proliferative responses were determined by [3 H]thymidine incorporation 48 h after stimulation. As a control for toxicity of the reagents, proliferation of antigen-stimulated D10.G4 cell cultures inhibited with a mixture of CTLA4-Ig and IL-1 RA was restored by the further addition of 10 U/ml of rIL-2 (\circ).

various B7-mediated costimulation events, including costimulation of Th1 cells ([34]; data not shown) and costimulation of Th2 cells by splenic APC (Fig. 6). Furthermore, titrations of IL-1 α together with CD28 mAb revealed no synergy in induction of proliferation of Th2 cells stimulated with anti-TCR mAb (data not shown). Together these results suggest that costimulation in vitro by exogenous IL-1 is independent of B7-CD28 interactions.

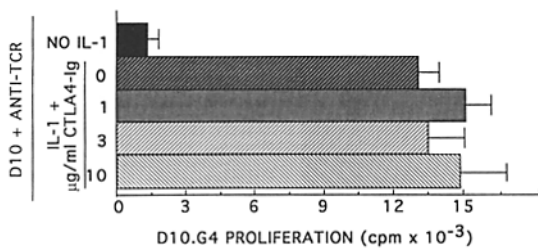


Figure 7. IL-1 costimulation of Th2 cells does not require B7-CD28 signaling. D10.G4 cells were cultured in half-area 96-plate wells coated with 4 µg/ml immobilized anti-TCR-β mAb alone or with the further addition of 1 U/ml IL-1α. The indicated concentrations of CTLA4-Ig were added to parallel cultures, and the proliferative responses were determined by [³H]thymidine incorporation 48 h after stimulation.

Discussion

Based on the finding that IL-1 costimulates Th2 cell proliferation, it has often been assumed that Th2 cell activation requires costimulation by APC-derived IL-1. We have now shown that CD28-B7 interactions can costimulate proliferation of two independently derived Th2 clones. The costimulation of Th2 cell responses mediated by CD28 provides an explanation for the ability of cells that produce little or no IL-1, such as dendritic cells and activated B cells (18, 34), to costimulate Th2 cell proliferation. Both of these APC types express B7 (35, 36). Further studies will be necessary to determine whether a similar mechanism holds in the case of IL-4-dependent Th cells *in vivo*.

B7-mediated signaling is apparently the predominant source of Th2 costimulation in splenocyte populations, based on the demonstration that the B7 antagonist CTLA4-Ig strongly blocked Th2 cell proliferation. Although cells capable of IL-1 production (e.g., macrophages) are present among splenic APC, and could in principle directly costimulate Th2 cells by releasing IL-1, our results suggest that they fail to secrete sufficient IL-1 to trigger Th2 cells. Perhaps these cells lack a sufficient activating signal to induce maximal IL-1 release, at least in the *in vitro* culture system used. Instead, our results suggest that signaling via direct T cell-APC contact may be required for effective Th2 costimulation, consequent auto-crine IL-1 production, and IL-4 responsiveness. If this holds true for IL-4-dependent T cells *in vivo*, these costimulatory requirements may help to tightly regulate antigen-specific responses of Th2 cells under steady state conditions. On the other hand, in cases of serious infections, where circulating IL-1 can achieve sustained high levels, the requirement for costimulation mediated by cell contact may be relaxed in the interests of promoting a vigorous and rapid response of Th2 cells. In this case, antigen presented even by B7-negative APC, such as resting B cells, could lead to a Th2 cell response.

Costimulation of Th1 cell proliferation has been previously shown to involve increased IL-2 production (5, 37). In contrast, the costimulation of Th2 cell proliferation by anti-CD28 mAb is not mediated through increased IL-2 or IL-4 secretion. Rather, CD28 costimulation induces IL-1 production,

apparently by the Th2 cells themselves, which then acts in an autocrine fashion to induce increased responsiveness of the cells to IL-4. It is unlikely that APC contamination was responsible for the IL-1 production in our experiments, because APCs were depleted from the Th2 population and because IL-1β transcripts, which are present in macrophages ([38]; Fig. 5), were absent in the D10.G4 RNA preparations.

We do not yet know whether costimulation of IL-1 production by Th2 cells involves induction of IL-1 gene transcription and/or stabilization of IL-1 transcripts. Both mechanisms are involved in costimulation of IL-2 gene expression in Th1 cells by CD28 mAb (14, 39). Regulated stabilization of cytokine transcripts has been associated with the presence of specific sequence repeats (AUUUA) in the 3' untranslated segment of the mRNA. These sequences have been found in all of the transcripts previously known to be stabilized by CD28 mAb costimulation (40, 41). Interestingly, such sequences are also found in the 3' untranslated region of the IL-1α transcript (40), suggesting that message stabilization may be involved in costimulation of IL-1 production via CD28 signaling.

Other groups have reported that Th2 cells synthesize IL-1 (42, 43). However, Zubiaga et al. (43) reported that exogenous costimuli are not required for proliferation of Th2 cells cultured at high cell densities. Under those conditions, Th2 cells reportedly produce IL-1α and proliferate after stimulation with a variety of mitogens. This effect may be related to the findings that Th2 cells, in sufficiently high numbers, can costimulate proliferative responses of Th1 cells (31, 32), suggesting that Th2 cells can express costimulatory ligands. Presumably, the dependence of our responses on exogenous costimulation is due to the use of lower Th2 cell concentrations. *In vivo*, it is likely that the concentrations of activated costimulatory Th2 cells are too low in most circumstances to circumvent the necessity for APC-derived costimuli.

Th1 cells can also proliferate in response to exogenously provided IL-4. Interestingly, responsiveness of Th1 cells to IL-4 was shown to depend on a costimulatory signal that is sensitive to chemical fixation (44). Th1 cell responsiveness to IL-4 was not mediated by IL-2. Although the identity of the costimulatory molecule mediating IL-4 responsiveness by Th1 cells has not yet been reported, it appears likely to be B7. Interestingly, in contrast to Th2 cells, there is no evidence to indicate that Th1 cells produce IL-1 or that they can respond to it (7, 8). In fact, Th1 cells reportedly lack cell surface receptors for IL-1 (7, 45). These considerations raise the possibility that IL-4 responsiveness of Th1 cells is mediated by a distinct mechanism from that of Th2 cells.

With the addition of our results, it appears that CD28-B7-mediated costimulation plays a role in the activation of most if not all types of T cells, suggesting that this signaling mechanism arose early in the evolution of T cells. In a practical vein, these findings enhance optimism that therapeutic approaches based on blocking the B7 costimulation pathway of Th cells will be effective. A therapeutic approach with particular promise is based on the premise that blocking costimulatory signals during immune responses to transplanted tissue, autoantigens, or allergens will render the individual

tissue, autoantigens, or allergens will render the individual unresponsive to subsequent challenges. However, anergy induction has not been demonstrated in the case of Th2 cells, raising some doubts as to whether the approach would be

effective in the case of Th2-dependent responses. With the knowledge that Th2 cells are responsive to CD28-B7-mediated costimulatory signals, the possibility that blockade of B7 will lead to anergy of Th2 cells can be directly examined.

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Address correspondence to David H. Raulet, Department of Molecular and Cell Biology, University of California at Berkeley, Berkeley, CA 94720.

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