Itk Negatively Regulates Induction of T Cell Proliferation by CD28 Costimulation

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Summary

CD28 is a cell surface molecule that mediates a costimulatory signal crucial for T cell proliferation and lymphokine production. The signal transduction mechanisms of CD28 are not well understood. Itk, a nonreceptor protein tyrosine kinase specifically expressed in T cells and mast cells, has been implicated in the CD28 signaling pathway because of reports that it becomes phosphorylated on tyrosines and associates with CD28 upon cross-linking of the cell surface molecule. To determine whether Itk plays a functional role in CD28 signaling, we compared T cells from Itk-deficient mice and control mice for their responses to CD28 costimulation. T cells defective in Itk were found to be fully competent to respond to costimulation. Whereas the CD3-mediated proliferative response was severely compromised in the absence of Itk, the calcineurin-independent CD28-mediated response was significantly elevated when compared with cells from control animals. The augmented proliferation was not due to increased production of interleukin-2. The results suggest that Itk has distinct roles in the CD3 versus the CD28 signaling pathways. By negatively regulating the amplitude of signaling upon CD28 costimulation, Itk may provide a means for modulating the outcome of T cell activation during development and during antigen-driven immune responses.

Induction of T cell proliferation and effector functions requires recognition by the TCR of antigen bound to MHC molecules and subsequent induction of a signaling cascade by way of the TCR-associated CD3 complex. In addition, costimulatory signals are required for full activation to proceed. The major costimulatory signal has been shown to involve the CD28 molecule (1), a transmembrane homodimer expressed on resting and activated T cells. CD28 binds to two glycoproteins, B7-1 and B7-2, expressed on APC (1). Using transfected cell lines expressing B7-1 or B7-2, it has been shown that B7–CD28 interactions provide costimulatory signals to T cells. A similar costimulatory signal can also be delivered with antibody against CD28 in conjunction with anti-TCR antibodies. CD28 ligation in the absence of cognate antigen interaction with the TCR does not alter immune responses and has no obvious effect on resting T cells. However, CD28 stimulation in conjunction with TCR stimulation can dramatically augment T cell proliferation and the production of multiple cytokines (2).

The signaling pathways induced by TCR ligation have been studied extensively (3). Cross-linking of the TCR results in the activation of CD3-associated tyrosine kinases, which further leads to calcium mobilization, activation of protein kinase C (PKC)[1] and the Ras signaling cascade, and subsequent IL-2 production and cell proliferation. However, the signal transduction pathway for CD28 costimulation remains poorly understood. Cross-linking of CD28 with antibodies or with cell surface B7-1 has been reported to result in phosphorylation of CD28 and cellular substrates, such as phospholipase Cγ1 (PLCγ1) (4–7). However, the effect of CD28 cross-linking on Ca^{2+} flux remains controversial (8–12). The cytoplasmic region of CD28 has been shown to associate with phosphatidylinositol 3′ kinase (PI3K) (13–17). Such an association is dependent on the

X.C. Liao and S. Fournier should be considered co–first authors of this study.

[1]*Abbreviations used in this paper:* CsA, cyclosporin A; IL-2Rα, interleukin-2 receptor α chain; JNK, c-Jun NH_2 terminus kinase; PI3K, phosphatidylinositol 3′ kinase; PKC, protein kinase C; PLCγ1, phospholipase Cγ1.

SH2 domain of the p85 subunit of PI3K and on phosphorylation of a tyrosine residue in the CD28 cytoplasmic domain. The identity of the kinase that phosphorylates CD28 after antigen stimulation remains unknown. Furthermore, the functional significance of PI3K association with CD28 remains unresolved (18–22).

Another molecule reported to associate with CD28 is the nonreceptor protein tyrosine kinase Itk, which is expressed specifically in T cells, mast cells, and human NK cell lines (23–27). After cross-linking of CD28 on human T cells, Itk has been shown to associate with the CD28 molecule and to become phosphorylated on tyrosines (28). To determine whether this association reflects a functional role for Itk in CD28 signaling, we compared T cells from Itk-deficient mice (27) and control mice for responses to CD28 costimulation. In T cells lacking Itk, the proliferative response to CD28-mediated costimulation was found not only to be intact, but also to be markedly elevated. Thus, in contrast with its requirement for efficient TCR-mediated signal transduction, Itk appears to regulate negatively the amplitude of the proliferative responses to CD28 costimulation, thereby providing a means to modulate the strength and potentially the outcome of T cell activation.

Materials and Methods

Antibodies. Monoclonal antibodies used for immunofluorescence staining have been described (27). Antibodies used for cell purification include anti-HSA (M1/69), anti-CD8α (53-6.72 and 3.155), anti-I-$A^{b,d}$ (28-16-8S), anti-I-$A^{b,d,j,p,q,u}$ (BP107), anti-rat immunoglobulins, and anti-mouse immunoglobulins (American Type Culture Collection, Rockville, MD). Antibodies used in cell culture include anti-CD3 (500A2 for Figs. 1, 2, 4, and 145-2C11 for Fig. 3), anti-CD28 (37.51), anti-CTLA-4 (9H10), anti-IL-2 (S4B6), anti-IL-2Rα (3C7), and anti-IL-4 (11B11) (PharMingen, San Diego, CA). Antibodies used in the IL-2 ELISA include the capture mAb JES6-1A12 and the detecting mAb JES6-5H4 (PharMingen, San Diego, CA).

Mice. Itk-deficient mice have been described (27). Littermates of $itk^{+/-}$ and $itk^{-/-}$ (50:50% contribution of the C57Bl/6 and 129/Sv backgrounds) were used in experiments shown in Figs. 1, 2, and 4. Experiments in which $itk^{-/-}$ mice were compared with age-matched B6 animals (Fig. 3) were performed with mutant mice that had undergone a further five generations of backcrosses to C57Bl/6.

Cell Purification. Lymph node cells were harvested from the mice. B cells, $CD8^+$ T cells, and adherent cells were depleted by negative selection, using nylon wool columns followed by antibodies coupled to magnetic beads as described before (27), or using complement killing followed by anti-immunoglobulin panning (29). The resulting cells were 85–90% $CD4^+$ from $itk^{+/+}$ or $itk^{+/-}$ mice and 75-90% $CD4^+$ from $itk^{-/-}$ mice. The reason for a consistently lower purity of $itk^{-/-}$ cells is likely due to the fact that $itk^{-/-}$ mice had a significantly reduced proportion of $CD4^+$ T cells (27).

Cell isolation and activation was done in RPMI 1640 with 10% FCS, 2 mM l-glutamine, 100 μM nonessential amino acids, 50 μM β-mercaptoethanol, 110 μg/ml sodium pyruvate, 50 U/ml penicillin, and 50 μg/ml streptomycin.

Stimulation with Plate-bound Anti-CD3. Round-bottomed 96-well microtiter plates were coated overnight at 4°C with purified anti-CD3 mAb (500A.2) at different concentrations (from 0.025–12 μg/ml). Before addition of cells, the plates were washed extensively with PBS and incubated with the medium. For each well of the 96-well plates, 5×10^4 $CD4^+$ T cells were cultured in a final volume of 200 μl. Anti-CD28 (37.51) ascites was used at 1:500, 1:1,000 or 1:3,000, depending on the batch. For some experiments, culture supernatants were used at 1:6.7 for CTLA-4Ig, 1:4 for anti-IL-2 (S4B6), and 1:200 for anti-IL-2R (PC61). Recombinant human IL-2 (PharMingen, San Diego, CA) was used at 100 U/ml.

Cells were stimulated for 24, 48, 72, or 96 h before 50 ml of culture supernatant were removed and 1 μCi of [^{3}H]thymidine (in 50 μl medium) was added for 16–20 h. Cells were then harvested with a LS6000IC harvester (Beckman Instruments, Fullerton, CA).

Stimulation with Antibodies Coated on Beads. Polystyrene beads were coated with anti-CD3 (1.5 μg/ml 500.A2) in the absence or presence of anti-CD28 (1.5 μg/ml 37.51), anti–CTLA-4 (1.5 μg/ml 9H10), or a combination of anti-CD28 and anti–CTLA-4. Isotype-matched hamster mAb (536) was used to maintain a final protein concentration of 5 μg/ml (30). Typically, 10 μl of beads were added to 190 μl of 5×10^4 cells in round-bottomed 96-well plates. Cells were stimulated for 24, 48, or 72 h before addition of 1 μCi [^{3}H]thymidine (in 10 μl medium) and harvested 16 h later with a Matrix 9600 harvester (Packard Instrument, Meriden, CT).

Stimulation with PMA and/or Ionomycin. When PMA and/or ionomycin were used in conjunction with anti-CD28 antibodies, cells were cultured in 96-well flat-bottomed microtiter plates. Anti-CD28 mAb was used at 1:3,000 for the ascites, and 2.5 μg/ml for the purified antibody, unless otherwise noted. Anti-CD3 mAb (145-2C11) was used at 2.5 μg/ml unless otherwise noted. Antibody against murine IL-2 (S4B6) and antibody against the murine IL-2Rα chain (3C7) were used at 10 μg/ml each. Recombinant human IL-2 (R&D Systems, Minneapolis, MN) was used at 2 U/ml. Cells were stimulated for 48 h before 50 μl supernatant was removed from each well for IL-2 measurement. [^{3}H]-thymidine was added to cells 72 h after stimulation (1 μCi in 50 μl medium) and incubated for 20 h before harvesting using a microtiter plate cell harvester (Tomtec, Gaithersburg, MD).

IL-2 Measurement. IL-2 produced from the cultures was measured by ELISA or with an IL-2–dependent cell line (CTLL-2).

Results

Enhanced CD28 Costimulation of TCR Signaling in Itk-negative T cells. To study CD28 costimulation, $CD4^+$ lymph node T cells were cultured in microtiter plates coated with anti-CD3 mAb at suboptimal concentrations. Addition of soluble anti-CD28 mAb to the culture substantially increased proliferation as well as IL-2 production (Fig. 1), consistent with previous studies (10, 29, 31). Cells from Itk-deficient mice also responded to addition of anti-CD28 Ab with increased proliferation and IL-2 secretion, compared with cells stimulated with anti-CD3 alone, or with anti-CD3 and a control isotype-matched hamster Ab. These results indicate that Itk is not necessary for CD28 costimulation.

Surprisingly, cells lacking Itk showed consistently higher proliferation than the control cells from $itk^{+/-}$ littermates or age-matched $itk^{+/+}$ mice (Fig. 1 *A*; data not shown).

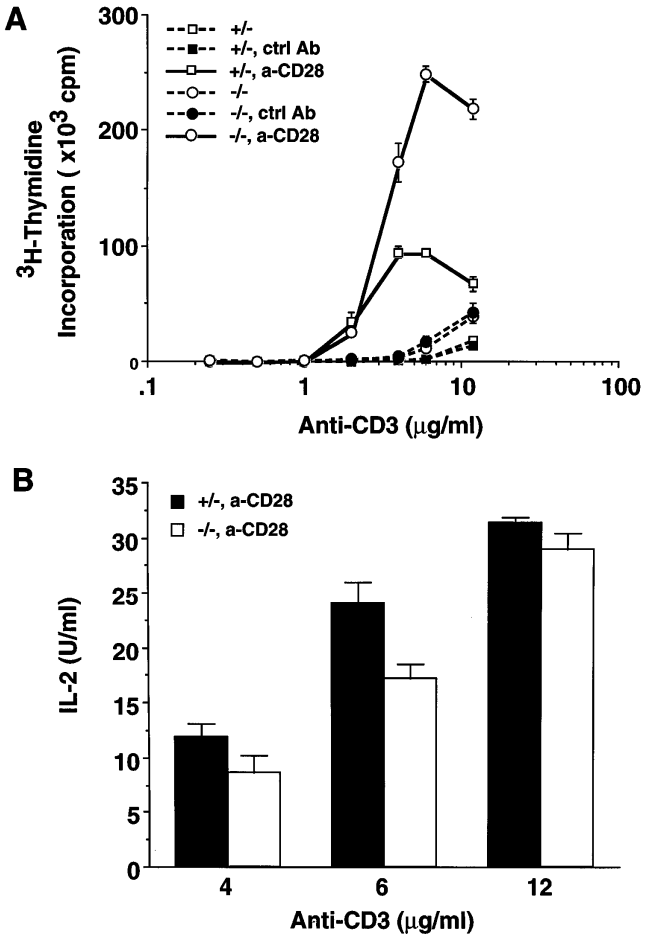

Figure 1. CD28 costimulation of TCR-induced proliferation and IL-2 production. (*A*) CD4+ T cells from *itk*$^{-/-}$ mice (*circles*) and *itk*$^{+/-}$ littermates (*squares*) were stimulated with plate-bound anti-CD3 at different concentrations as indicated, in the presence (*solid lines*) or absence (*broken lines*) of anti-CD28 ascites (1:500). Cell proliferation was measured 48 h after stimulation. (*B*) IL-2 production in the culture supernatant after 48 h of stimulation was measured by ELISA. No detectable IL-2 was produced from cells stimulated with anti-CD3 alone.

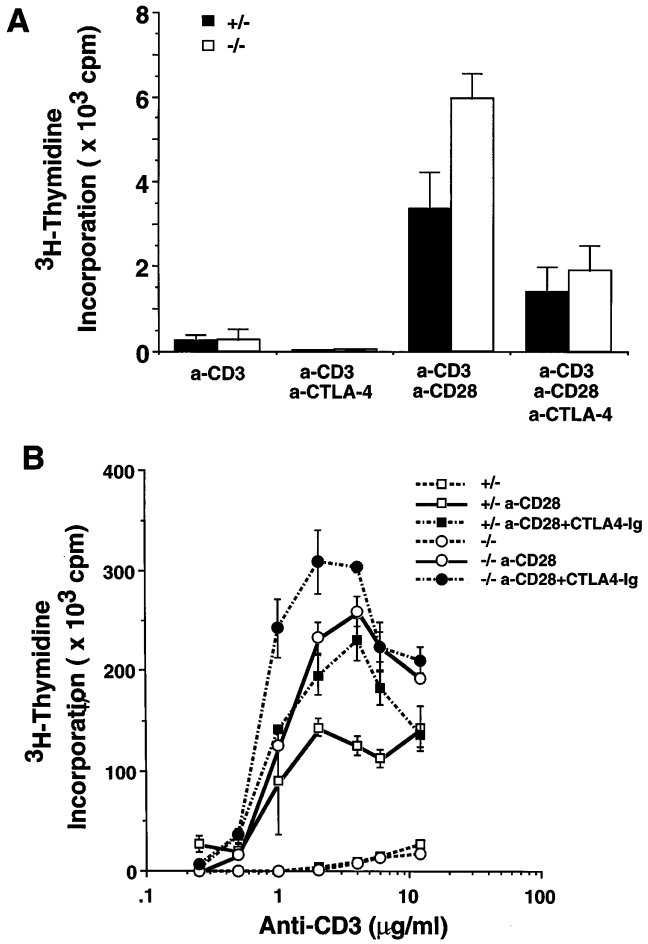

Figure 2. Itk is not required for the inhibitory signal mediated by CTLA-4. (*A*) CD4+ T cells were stimulated with polystyrene beads coated with various antibodies as indicated. Cell proliferation was measured 72 h after stimulation. (*B*) CD4+ T cells from *itk*$^{-/-}$ mice (*circles*) and *itk*$^{+/-}$ littermates (*squares*) were stimulated with plate-bound anti-CD3 at different concentrations as indicated, in the presence or absence of anti-CD28 ascites (1:500). CTLA-4Ig was included at the beginning of culture (*closed symbols*) together with anti-CD28. Cell proliferation was measured 72 h after stimulation.

This increased proliferative response did not correlate with IL-2 levels produced by the stimulated cells. In the absence of anti-CD28, no IL-2 was detectable in the culture supernatant. When anti-CD28 was added to wells coated with anti-CD3, IL-2 was detected in supernatants of both *itk*$^{+/-}$ and *itk*$^{-/-}$ cells, but the level was consistently lower in the mutant cells, despite their higher proliferative response (Fig. 1 *B*).

Inhibition of CD28-mediated Costimulation by CTLA-4. Our data indicated that stimulation of CD4+ T cells with anti-TCR plus anti-CD28 Ab induced stronger proliferation in the absence than in the presence of Itk. Because CTLA-4, a cell surface molecule sharing extensive homology with CD28, was shown to mediate a negative signal in T cell activation (32–34), we sought to determine whether the increased proliferation in the absence of Itk was due to a lack of negative signaling through CTLA-4. CD4+ T cells were stimulated by incubation with polystyrene beads coated with a constant amount of various hamster antibodies. Using beads coated with anti-CD3 and anti-CD28, we observed that CD28 costimulation occurs in the absence of Itk (Fig. 2 *A*), consistent with what was observed with plate-bound anti-CD3 and soluble anti-CD28 (see Fig. 1 *A*). CTLA-4 did not have a positive costimulatory function because beads coated with anti-CD3 and anti–CTLA-4 did not stimulate proliferation, and even slightly inhibited anti-CD3–induced proliferation (Fig. 2 *A*). As has been reported (32–34), CTLA-4 has a negative effect on the response of T cells to stimulation because there was reduced proliferation upon stimulation with beads coated with anti-CD3, anti-CD28, and anti–CTLA-4 compared with beads coated with only anti-CD3 and anti-CD28 (Fig. 2 *A*). Addition of anti–CTLA-4 Ab to anti-CD3 and anti-CD28 also reduced the costimulatory effect of anti-CD28 on *itk*$^{-/-}$

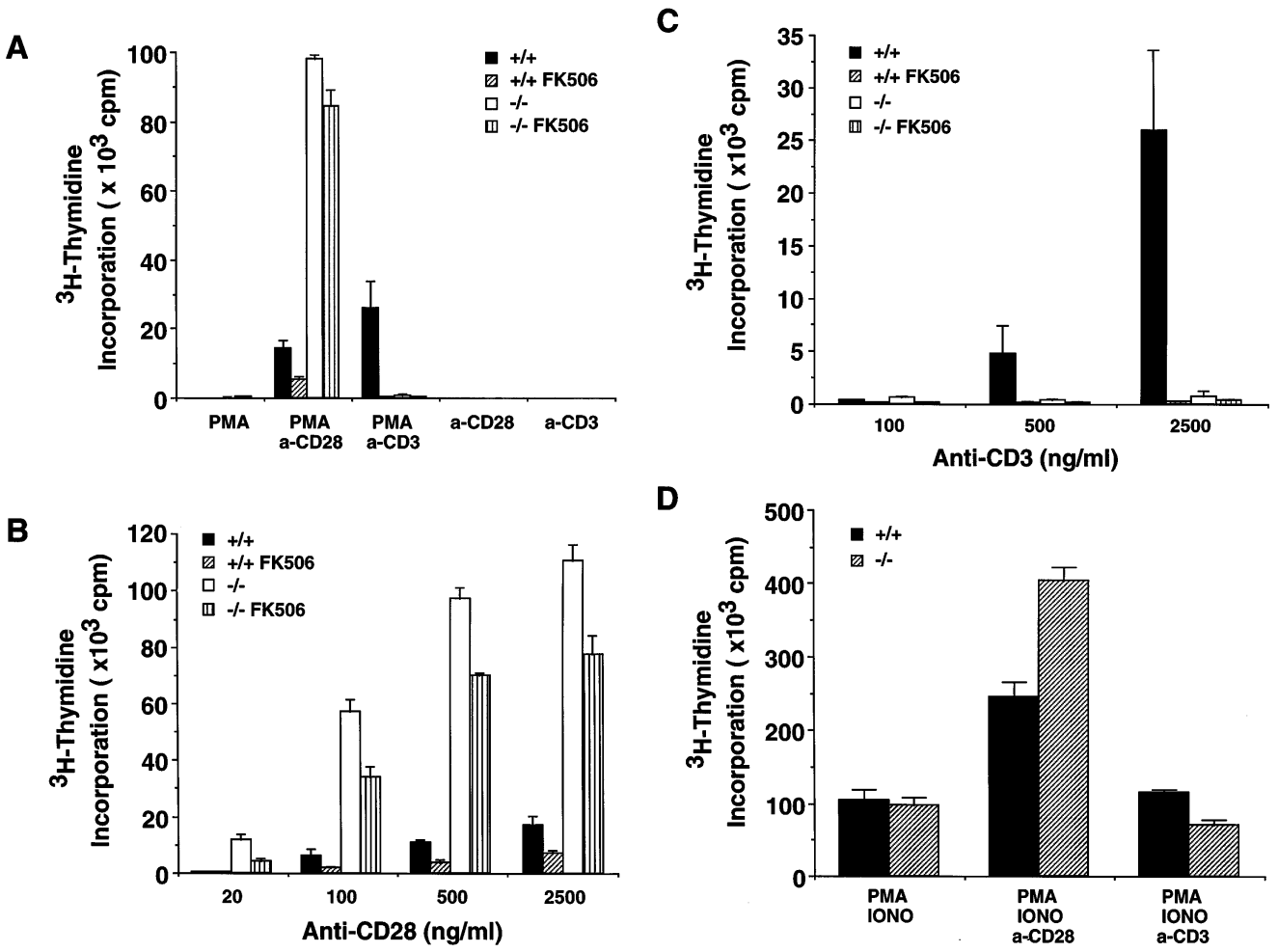

Figure 3. Opposite functions of Itk in CD28 costimulation– and TCR stimulation–induced proliferation. (*A*) $CD4^+$ T cells were stimulated with PMA (10 ng/ml) in the presence or absence of anti-CD28 or anti-CD3 as indicated. The immunosuppressive agent drug FK506 was used at 100 ng/ml. Cell proliferation was measured 72 h after stimulation. a-CD28, purified anti-CD28 mAb (2.5 μg/ml); a-CD3, purified anti-CD3 mAb (2.5 μg/ml). (*B*) $CD4^+$ T cells were stimulated with PMA (10 ng/ml) in the presence of different concentrations of purified anti-CD28 mAb as indicated. FK506 was used at 100 ng/ml. Cell proliferation was measured 72 h after stimulation. (*C*) $CD4^+$ T cells were stimulated with PMA (10 ng/ml) in the presence of different concentrations of purified anti-CD3 mAb as indicated. FK506 was used at 100 ng/ml. Cell proliferation was measured 72 h after stimulation. (*D*) $CD4^+$ T cells were stimulated with PMA (10 ng/ml) and ionomycin (125 ng/ml) in the presence or absence of anti-CD28 or anti-CD3 as indicated. Cell proliferation was measured 72 h after stimulation. IONO, ionomycin (125 ng/ml); a-CD28, purified anti-CD28 mAb (2.5 μg/ml); and a-CD3, purified anti-CD3 (2.5 μg/ml).

cells (Fig. 2 *A*), suggesting that the inhibitory signal delivered via CTLA-4 was not affected by the absence of Itk.

It has been shown that activated T cells express B7-1 and B7-2 (30), which are the ligands for both CD28 and CTLA-4 (1, 35). Because CTLA-4 has considerably higher affinity than CD28 for B7-1 and B7-2 (36), it is conceivable that B7 expressed on activated T cells could stimulate CTLA-4 on these cells. A fusion protein comprised of the extracellular region of murine CTLA-4 and the Fc region of human immunoglobulin (CTLA-4Ig) will compete for B7 binding, thus effectively blocking signaling via CTLA-4 on T cells (30, 34, 37). Addition of CTLA-4Ig to T cells in anti-CD3–coated wells further increased their proliferation in response to anti-CD28 (Fig. 2 *B*), suggesting that CTLA-4 delivered an inhibitory signal for T cell proliferation under these conditions. Increased proliferation in response to CTLA-4Ig was also observed for stimulated $itk^{-/-}$ cells (Fig. 2 *B*). Taken together with the results using anti-CTLA-4 Ab, we conclude that Itk does not appear to have a major role in mediating the inhibitory signal from CTLA-4. The enhanced proliferation observed in the $itk^{-/-}$ cells in response to anti-CD28–mediated costimulation therefore is not due to a defect in inhibitory signaling by CTLA-4.

CD28 Costimulation in the Presence of Phorbol Esters. To determine whether the observed enhancement in costimulation is due to changes in relevant cell surface receptors, FACS® analysis was performed with antibodies specific for CD28, CTLA-4, CD25, and CD69 before and after costimulation. There was no significant difference in levels of expression of these molecules in $itk^{+/-}$ versus $itk^{-/-}$ cells (data not shown). However, the level of CD3 on $itk^{-/-}$ cells was found to be consistently lower (less than twofold)

than on $itk^{+/-}$ cells from control littermates. This difference in CD3 levels is unlikely to account for the increased proliferation of $itk^{-/-}$ cells induced with anti-CD3 and anti-CD28, because in the presence of anti-CD28 $itk^{-/-}$ cells showed a stronger proliferative response than $itk^{+/-}$ cells over a range of anti-CD3 concentrations. We showed previously that in the absence of Itk the efficiency of TCR signaling is reduced (27). However, it remains possible that the increased proliferation of $itk^{-/-}$ cells upon costimulation is due to an indirect consequence of reduced TCR signaling.

To address this issue, we attempted to reveal the effect of Itk on CD28 costimulation in the context of a minimal and defined contribution from the TCR signaling pathway. Previous studies using highly purified human or mouse T cells have shown that anti-CD28 augments proliferation in cells stimulated with the phorbol ester PMA, whereas PMA alone does not induce proliferation (31, 38). Stimulation of freshly isolated $CD4^+$ lymph node cells with anti-CD28 in the presence of PMA greatly augmented cell proliferation (Fig. 3 *A*). In this assay, cells from the Itk-deficient mice displayed 5–10-fold greater thymidine incorporation than cells from control littermates expressing Itk (Fig. 3 *A*). In addition, titration of anti-CD28 antibodies suggests that the loss of Itk does not simply result in a shift of sensitivity to CD28 stimulation, because the proliferative response of the $itk^{+/+}$ cells to high concentrations of Ab remains low when compared with $itk^{-/-}$ cells stimulated with much lower concentrations of anti-CD28 (Fig. 3 *B*). As a control, $CD4^+$ T cells were treated with purified anti-CD3 Ab and PMA. Consistent with previous studies (27), cells from the Itk-deficient mice had severely impaired proliferative responses (Fig. 3, *A* and *C*).

A hallmark of CD28-mediated signal transduction is its resistance to immunosuppressive agents such as cyclosporin A (CsA) and FK506 (31, 39). The proliferative responses of wild-type and $itk^{-/-}$ cells to anti-CD28 with PMA were relatively resistant to FK506 (Fig. 3, *A* and *B*). This is in contrast with proliferation in response to anti-CD3, which was completely abolished by FK506 (Fig. 3, *A* and *C*). Together, these results indicate that augmentation of proliferation with anti-CD28 in the absence of Itk is occurring uniquely through the CD28 costimulation pathway. Remarkably, in the absence of Itk, the calcineurin-independent CD28 signaling pathway is enhanced, whereas calcineurin-dependent TCR signaling is severely compromised.

Activation of the TCR signaling pathway can be effectively mimicked by treating T cells with PMA and ionomycin. Cells from $itk^{+/+}$ and $itk^{-/-}$ mice proliferate equally well in the presence of PMA and ionomycin (Fig. 3 *D*; reference 27), indicating that the TCR signaling deficit in the mutant mice lies proximal to the effects of these agents. Because CD28 costimulation is also observed in the presence of PMA and ionomycin (31, 40), we sought to use this regimen to substitute for the TCR signal, thus bypassing the involvement of Itk in this pathway. In the presence of PMA and ionomycin, anti-CD28 treatment resulted in enhanced proliferation of T cells from both $itk^{-/-}$ and $itk^{+/+}$ mice (Fig. 3 *D*). Proliferation of cells from the mutant mice was potentiated to a greater extent in comparison to negative littermates, but the effect was modest when compared with that observed with PMA and anti-CD28 stimulation.

Roles of IL-2 in Cell Proliferation Induced with Anti-TCR and Anti-CD28. CD28 costimulation has been shown to result in increased cytokine levels through increased transcription as well as mRNA stabilization (2, 41, 42). Production of cytokines such as IL-2, as a consequence of T cell activation, further stimulates T cell proliferation by way of an autocrine mechanism. In the experiments described above, we were unable to identify a correlation between the extent of cell proliferation and of IL-2 production in response to costimulation (see Fig. 1 *B*; data not shown). It is possible that IL-2 produced in these cultures was consumed during cell proliferation. To address whether enhanced CD28 costimulation of $itk^{-/-}$ cells may be dependent on IL-2, we studied cell proliferation in the presence of blocking antibodies.

When a mixture of antibodies against murine IL-2 and murine IL-2 receptor were added to $CD4^+$ T cells stimulated with anti-CD28 and increasing amounts of plate-bound anti-CD3, proliferation of both $itk^{+/-}$ and $itk^{-/-}$ cells was abolished (Fig. 4). This result suggests that proliferation in

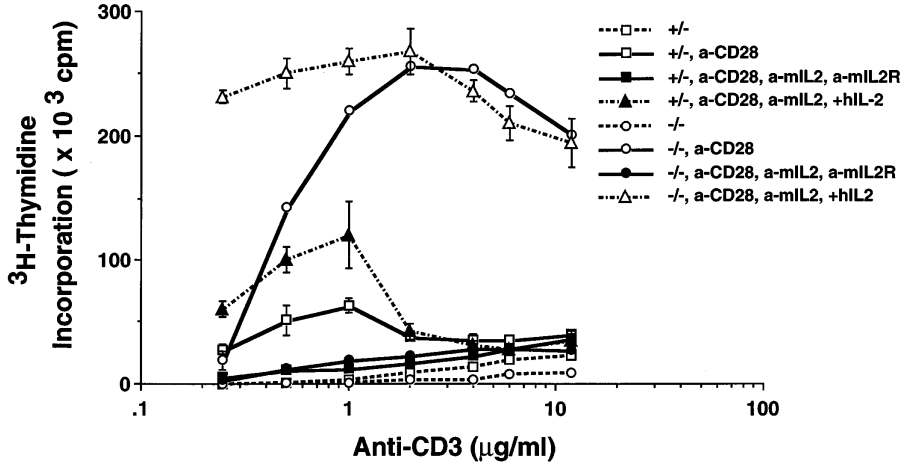

Figure 4. Cell proliferation induced by TCR and CD28 is dependent on IL-2. $CD4^+$ T cells from $itk^{-/-}$ mice and $itk^{+/-}$ littermates were stimulated with plate-bound anti-CD3 at different concentrations as indicated, in the presence or absence of anti-CD28 ascites (1:500). Anti-CD28 and anti-murine IL-2 were added with anti-murine IL-2R or with recombinant human IL-2 at the beginning of culture. Cell proliferation was measured 72 h after stimulation. a-CD28, anti-CD28 ascites; a-mIL2, anti-murine IL-2 mAb; a-mIL2R, anti-murine IL-2Rα mAb; and hIL-2, recombinant human IL-2.

response to costimulation is dependent on IL-2 and that the contribution of other cytokines to cell proliferation is minimal in this system.

To determine whether $itk^{+/-}$ and $itk^{-/-}$ cells have intrinsic differences in their response to IL-2 after stimulation with anti-CD3 and anti-CD28, recombinant human IL-2 was added to the culture together with antibody against murine IL-2. Both $itk^{+/-}$ and $itk^{-/-}$ cells responded to the addition of recombinant IL-2 with enhanced proliferation, especially at low anti-CD3 concentrations (Fig. 4). The difference in proliferation between $itk^{-/-}$ and $itk^{+/-}$ cells still persisted in the presence of human IL-2, suggesting that $itk^{-/-}$ cells have a better response to IL-2 compared with $itk^{+/-}$ cells under these conditions. T cell activation increased CD25 expression on both $itk^{-/-}$ and $itk^{+/-}$ cells. However, we did not observe any consistent difference of CD25 expression between $itk^{-/-}$ and $itk^{+/-}$ cells (data not shown).

Discussion

In this study, we examined the role of the nonreceptor protein tyrosine kinase Itk in regulating costimulatory signaling through CD28. Under several conditions investigated, mature T cells from Itk-deficient mice had a much stronger proliferative response upon CD28 costimulation than cells from control littermates expressing Itk or from age-matched wild-type mice. However, the level of IL-2 was consistently lower in Itk^- cells compared with Itk^+ cells under these culture conditions. In cells stimulated with limiting amounts of anti-CD3 plus anti-CD28, proliferation was dependent on IL-2 and IL-2 receptor. However, the differential proliferation between mutant and wild-type cells was retained when an equivalent amount of human IL-2 was added to cells treated with anti-mouse IL-2. This suggests that the Itk^- cells have increased sensitivity to IL-2 receptor–mediated signaling. Taken together, these results suggest that, in the absence of Itk, there is an enhancement in CD28-dependent processing of signals that result in cell cycle progression. The nature of the signals involved remains unclear. We have shown that it is not due to a lack of the inhibitory signal mediated by CTLA-4 (Fig. 2), nor to a difference in activation-induced cell death (data not shown).

Itk is a member of a small family of protein tyrosine kinases, which also includes Btk and Tec. In the absence of Btk, B cell development is impaired, as is proximal signal transduction through the B cell antigen receptor (43, 44). Itk is similarly required for effective signaling and proliferation following antibody cross-linking of the TCR complex (27). Thus, Itk and Btk appear to contribute to similar signaling mechanisms after ligation of the antigen receptor complexes on T and B cells, respectively. The signaling defects of the antigen receptors in cells lacking Itk or Btk can be overcome by stimulation with phorbol esters and calcium ionophore, indicating that these protein tyrosine kinases function at an early juncture in the signal transduction pathways.

Itk has been previously implicated in CD28 costimulation because it can be coprecipitated with CD28 (28, 45). The results of this study suggest that association of Itk with CD28 is not required for signal transduction, but may, instead, downregulate CD28-derived signals. The mechanism of such downregulation remains unclear. It does not involve regulation of cell surface expression of CD28, which is similar in $itk^{+/+}$, $itk^{+/-}$, and $itk^{-/-}$ cells (data not shown). It is possible that association of Itk with CD28 interferes with signal transduction by changing the conformation of CD28 or by preventing other signaling molecules from interacting with CD28. Alternatively, Itk could activate or inhibit a downstream effector of CD28 signaling.

The interplay between the TCR and CD28 signaling pathways may provide some clues regarding the mechanism of action of Itk. Itk appears to regulate the TCR and CD28 signaling pathways in opposite fashion. A major distinction between these pathways is their relative dependence on calcineurin, exhibited as sensitivity to immunosuppressive agents such as CsA and FK506 (31, 38, 40). CD28-induced cell proliferation persists in the presence of FK506, whereas proliferation in response to anti-CD3 is completely abolished (Fig. 3; references 31, 39). The enhanced CD28-mediated proliferation of $itk^{-/-}$ cells remains resistant to FK506, suggesting that Itk regulates a specific step in CD28 signaling, before it converges with the TCR signaling pathway.

The intersection of the TCR and CD28 signaling pathways remains poorly understood. One recent study (46) suggests that integration of the CD28 costimulatory signal and the TCR signaling pathway occurs at the level of activation of the c-Jun NH_2 terminus kinase (JNK). JNK belongs to the mitogen-activated protein kinase family of serine/threonine kinases. Stimulation of T cells with PMA and anti-CD28 activates JNK, whereas either alone has no effect (46). Despite the differences in proliferation of mutant and wild-type T cells after treatment with PMA and anti-CD28, we failed to observe any difference in activation of JNK between $itk^{-/-}$ and $itk^{+/+}$ cells (data not shown).

CD28 and T Cell Maturation. CD28 is highly expressed on developing thymocytes (31). In addition, there appears to be tissue-restricted expression of B7 family members on the thymic epithelia and dendritic cells within the corticomedullary and medullary regions of the thymus (47, 48). The importance of CD28 in T cell development has been suggested from experiments which showed that negative selection of $CD4^+CD8^+$ thymocytes may involve CD28 costimulation (49). This issue has been further addressed using mice defective in CD28 expression. Although these mice did not display any obvious developmental phenotype, $CD28^{-/-}$ T cells showed a selective disadvantage compared with $CD28^+$ T cells in mixed bone marrow chimeras (50).

Itk-deficient mice exhibit defects in T cell maturation (27). There are reduced numbers of T cells, particularly $CD4^+$ cells. Studies in mice expressing transgenic TCRs in the Itk-deficient background suggest that Itk may be important for positive selection of thymocytes. The developmental phenotype of Itk-deficient mice thus may be ex-

plained by a reduction in TCR signaling in the absence of Itk. However, there are intriguing observations suggesting that Itk may reduce signaling in potentially autoreactive cells. In mice expressing the transgenic AND TCR, lack of Itk resulted in accumulation of $CD4^-CD8^-$ mature T cells expressing the transgenic TCR (27). Double-negative ($CD4^-$ $CD8^-$) T cells have been previously observed in transgenic mice that express a self-reactive transgenic TCR (51). These cells may be under selective pressure to reduce the avidity of TCR interaction with MHC by turning off expression of the CD4 coreceptor. This suggests that, at a certain stage in T cell maturation, lack of Itk may actually increase the strength of the signal through the TCR complex and CD28. The inhibitory function of Itk in CD28 costimulation thus may provide a mechanistic explanation for the abnormal T cell development observed in the Itk^- mice.

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