CD28 Costimulation Mediates Down-Regulation of p27<sup>kip1</sup> and Cell Cycle Progression by Activation of the PI3K/PKB Signaling Pathway in Primary Human T Cells


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CD28 Costimulation Mediates Down-Regulation of p27kip1 and Cell Cycle Progression by Activation of the PI3K/PKB Signaling Pathway in Primary Human T Cells


CD28 provides a costimulatory signal that cooperates with the TCR/CD3 complex to induce T cell activation, cytokine production, and clonal expansion. We have recently shown that CD28 directly regulates progression of T lymphocytes through the cell cycle. Although a number of signaling pathways have been linked to the TCR/CD3 and to CD28, it is not known how these two receptors cooperate to induce cell cycle progression. Here, using cell-permeable pharmacologic inhibitors of phosphatidylinositol 3-hydroxykinase (PI3K) and mitogen-activated protein kinase kinase (MEK1/2), we show that cell cycle progression of primary T lymphocytes requires simultaneous activation of PI3K- and MEK1/2-dependent pathways. Decreased abundance of cyclin-dependent kinase inhibitor p27kip1, which requires simultaneous TCR/CD3 and CD28 ligation, was dependent upon both MEK and PI3K activity. Ligation of TCR/CD3, but not CD28 alone, resulted in activation of MEK targets extracellular signal-related kinase 1/2, whereas ligation of CD28 alone was sufficient for activation of PI3K target protein kinase B (PKB; c-Akt). CD28 ligation alone was also sufficient to mediate inactivating phosphorylation of PKB target glycogen synthase kinase-3 (GSK-3). Moreover, direct inactivation of GSK-3 by LiCl in the presence of anti-CD3, but not in the presence of anti-CD28, resulted in down-regulation of p27kip1. Hyperphosphorylation of retinoblastoma tumor suppressor gene product, and cellular proliferation. Thus, inactivation of the PI3K-PKB target GSK-3 could substitute for CD28 but not for CD3 signals. These results show that the PI3K-PKB pathway links CD28 to cell cycle progression and suggest that p27kip1 integrates mitogenic MEK- and PI3K-dependent signals from TCR and CD28 in primary T lymphocytes. The Journal of Immunology, 2002, 168: 2729–2736.

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L.J.A. and A.A.F.L.v.P. contributed equally to the present work and are listed alphabetically.

Abbreviations used in this paper: PI3K, phosphatidylinositol 3-hydroxykinase; PH, pleckstrin homology; PKB, protein kinase B; GSK, glycogen synthase kinase; cdk, cyclin-dependent kinase; RB, retinoblastoma tumor suppressor gene product; ERK, extracellular signal-related kinase; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase kinase; PI, propidium iodide.

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p27\textsuperscript{kip1} protein are present in resting T cells (27). Mitogenic signals increase levels of cyclin D protein and cyclin D/cdk4,6 kinase activity on its primary substrate, the retinoblastoma tumor suppressor gene product (Rb). Hyperphosphorylation of Rb by cyclin D/cdk4,6 prevents Rb binding to E2F family transcription factors. E2F heterodimers are subsequently released to activate transcription of genes such as cyclin E that are necessary for S phase entry. As levels of cyclin E rise, it is thought that p27\textsuperscript{kip1} is titrated away from cyclin E/cdk2 heterodimers. This allows cyclin E/cdk2 to phosphorylate p27\textsuperscript{kip1} on Thr\textsuperscript{187}, leading to its ubiquitin-targeted degradation (28, 29). As molecules of p27\textsuperscript{kip1} are degraded, increasing numbers of cyclin E/cdk2 heterodimers are released in a positive feedback autoregulatory loop.

Evidence in non-T cell models supports involvement of the Ras-Raf-mitogen-activated protein kinase kinase (MEK)-extracellular signal-related kinase (ERK)p42,44 signaling pathway in down-regulation of genes such as cyclin E that are necessary for S phase entry. The cascade also has an active role in cell cycle regulation and down-regulation of p27\textsuperscript{kip1} (32, 33). Phosphatase and tensin homolog deleted on chromosome 10, a lipid phosphatase that antagonizes PI3K, induces cell cycle arrest via up-regulation of p27\textsuperscript{kip1} (34). Taken together with the critical role of CD28 costimulation in cell cycle progression and down-regulation of p27\textsuperscript{kip1} in T cells, these results suggest that PI3K and mitogen-activated protein kinase (MAPK) pathways may have an active role in TCR/CD3 plus CD28-mediated cell cycle progression.

In the studies reported below, we show that simultaneous activation of both MEK and PI3K is required for cell cycle progression of primary T lymphocytes. Ligation of TCR/CD3/CD alone, but not CD28 alone, resulted in activation of MEK targets ERK1/2, whereas ligation of CD28 alone was sufficient for activation of PI3K target PKB. CD28 ligation alone is sufficient to mediate inactivating phosphorylation of PKB target GSK-3. Moreover, direct inactivation of GSK-3 by LiCl in the presence of anti-CD3, but not in the presence of anti-CD28, resulted in down-regulation of p27\textsuperscript{kip1}, hyperphosphorylation of Rb, and cellular proliferation. These results show that the PI3K-PKB pathway links CD28 costimulation in cell cycle progression and suggest that pharmacologic regulation of PKB activation may be used to modulate T cell clonal expansion. Moreover, these results show that p27\textsuperscript{kip1} integrates mitogenic MEK- and PI3K-dependent signals from TCR and CD28 to regulate cell cycle progression in primary T lymphocytes.

Materials and Methods
Isolation of primary peripheral blood T cells
Leukopacks (platelet apheresis by-product) were obtained from the blood banks of the Dana-Farber Cancer Institute and Brigham & Women’s Hospital. Mononuclear cells were isolated by Ficoll/paque (Amersham Pharmacia Biotech, Uppsala, Sweden) gradient centrifugation for 20 min at 2000 rpm at room temperature. T cells were enriched by depletion of plastic-adherent mononuclear cells and positive selection by E-rosetting using sheep RBC. Cells were washed twice with ice-cold PBS and lysed in lysis buffer (0.5% Nonidet P-40, 50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 1 mM PMSF, 1 mM sodium orthovanadate, 10 μg/ml leupeptin, and 10 μg/ml aprotinin) for 20–30 min on ice. Protein concentration of whole cell lysates was determined using the DC Protein Assay (Bio-Rad, Hercules, CA) according to the manufacturer’s protocol. The lysates were mixed 1:2 with Laemml sample buffer (Bio-Rad) followed by heating for 5 min at 95°C. Equal amounts of lysates were resolved on SDS-PAGE. Proteins were transferred to Immobilon-P membranes (Millipore, Bedford, MA) and incubated with blocking buffer (TBST/5% BSA (Sigma-Aldrich)) or TBST/3% nonfat dry milk overnight at 4°C. The membranes were then immunoblotted with the indicated primary Ab for 1–2 h at room temperature followed by the appropriate HRP-conjugated goat anti-mouse (1/5000) or goat anti-rabbit (1/5000) IgG for 1 h at room temperature. mAb s or antiserum specific for cyclin D2, cyclin D3, cyclin E, cyclin A, p27\textsuperscript{kip1}, phospho-ERK1/2, and ERK2 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The Rb mAb was obtained from BD Pharmingen (San Diego, CA). Phospho-ERK1/2 and phospho-p38 MAPK rabbit polyclonal IgG Abs and the phospho-GSK-3α,β-specific mAb were purchased from Cell Signaling Technologies. HRP-conjugated goat anti-mouse (1/5000) and goat anti-rabbit (1/5000) IgG were from Promega (Madison, WI). Immunodetection was performed with ECL (NEL Life Science Products, Boston, MA). Stripping and reprobing of the blots was performed as described before. Immunoprecipitations for in vitro kinase reactions were performed with antisera specific for cdk4 or cdk2 (Santa Cruz Biotechnology) using 500 μg of protein per sample followed by kinase reactions using Rb-GST as substrate for cdk4 and histone H1 as substrate for cdk2.

Cell cycle
Cells were maintained in RPMI 1640 (Cellgro/Mediatech, Herndon, VA) medium supplemented with 10% (v/v) heat-inactivated FBS (Harlan, Indianapolis, IN) supplemented with HEPES-buffered (10 mM; Cellgro) MEM sodium pyruvate (1 mM; Life Technologies/Invitrogen, Carlsbad, CA), and penicillin (50 IU/ml), streptomycin (50 μg/ml) (Cellgro/Mediatech) in a 5% CO2 humidified atmosphere at 37°C.

Agents and Abs
PMA, ionomycin, PI3K inhibitor LY294002, and p38\textsuperscript{MAPK} inhibitor SB203580 were purchased from Sigma-Aldrich (St. Louis, MO). MEK1/2 inhibitor U0126 was purchased from Cell Signaling Technologies (Beverly, MA). Recombinant human IL-2 was a gift from the National Cancer Institute. The T cell-activating anti-CD3 (CLB-T3/4.E, 1X; IgE) and anti-CD28 (CLB-CD28/1, 15E8; IgG1) mAbs were from Research Diagnostics (Franklin Lakes, NJ).

Proliferation (DNA synthesis) assay
Cells were seeded in triplicate at a concentration of 1 × 10^5 cells/well in 200 μl RPMI 1640/10% FBS in 96-well flat-bottom plates (Costar, Corning, NY). Kinase inhibitors were added at the appropriate concentrations and the cells were preincubated for 30 min before initiation of the cultures. Cells were pulsed with 0.5 μCi/well [3H]Tdr for the last 16–18 h of a 72-h incubation period. Cells were then harvested onto membranes by using a cell harvester (Tomtec, Hamden, CT), and the incorporated [3H]Tdr was measured using a liquid scintillation counter (Wallac Trilux, Turku, Finland).

Cell cycle analysis by flow cytometry
Enriched human primary T cells were seeded at a concentration of 2 × 10^5 cells/well in 4 ml RPMI 1640/10% FBS in 6-well plates (Costar) and left untreated or treated for 48 h with stimulating mAb. Before the addition of mAbs, cells were preincubated for 30 min with kinase inhibitors. After incubation at 37°C, cells were washed once with PBS and then fixed with ice-cold PBS-ethanol (40%) for at least 30 min at −20°C. Fixed cells were washed once with PBS and incubated in PBS containing 2.5 μg/ml propidium iodide (PI, Sigma-Aldrich) and 50 μg/ml RNase A (Boehringer Mannheim, Indianapolis, IN) for 30 min at 37°C. Samples were subjected to FACS (BD Biosciences, Mountain View, CA) analysis using Lysis II software.

T cell activation, Western blotting, and in vitro kinase reactions
For T cell stimulation by TCR/CD3 and CD28 cross-linking, T-cells (10^7) were seeded in Eppendorf tubes in 1 ml HEPES-buffered serum-free RPMI 1640 medium and left untreated or treated with anti-CD3 (1 μg/ml) and/or anti-CD28 (1 μg/ml) for 30 min on ice. Cells were washed twice with plain medium and then stimulated by cross-linking with rabbit anti-mouse Iggs (DAKO, Carpintere, CA; 20 μg/ml) in 25-μl prewarmed medium for the indicated times. For mitogenic stimulation that bypasses the TCR, T cells were stimulated with the combination of PMA (50 μg/ml) and calcium ionophore (ionomycin; 0.5 μM) for 5 min at 37°C. Subsequently, cells were washed twice with ice-cold PBS and lysed in lysis buffer (0.5% Nonidet P-40, 50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 1 mM PMSF, 1 mM sodium orthovanadate, 10 μg/ml leupeptin, and 10 μg/ml aprotinin) for 20–30 min on ice. Protein concentration of whole cell lysates was determined using the DC Protein Assay (Bio-Rad, Hercules, CA) according to the manufacturer’s protocol. The lysates were mixed 1:2 with Laemml sample buffer (Bio-Rad) followed by heating for 5 min at 95°C. Equal amounts of lysates were resolved on SDS-PAGE. Proteins were transferred to Immobilon-P membranes (Millipore, Bedford, MA) and incubated with blocking buffer (TBST/5% BSA (Sigma-Aldrich)) or TBST/3% nonfat dry milk overnight at 4°C. The membranes were then immunoblotted with the indicated primary Ab for 1–2 h at room temperature followed by the appropriate HRP-conjugated goat anti-mouse (1/5000) or goat anti-rabbit (1/5000) IgG for 1 h at room temperature. mAbs or antiserum specific for cyclin D2, cyclin D3, cyclin E, cyclin A, p27\textsuperscript{kip1}, phospho-ERK1/2, and ERK2 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The Rb mAb was obtained from BD Pharmingen (San Diego, CA). Phospho-ERK1/2 and phospho-p38 MAPK rabbit polyclonal IgG Abs and the phospho-GSK-3α,β-specific mAb were purchased from Cell Signaling Technologies. HRP-conjugated goat anti-mouse (1/5000) and goat anti-rabbit (1/5000) IgG were from Promega (Madison, WI). Immunodetection was performed with ECL (NEL Life Science Products, Boston, MA). Stripping and reprobing of the blots was performed as described before. Immunoprecipitations for in vitro kinase reactions were performed with antisera specific for cdk4 or cdk2 (Santa Cruz Biotechnology) using 500 μg of protein per sample followed by kinase reactions using Rb-GST as substrate for cdk4 and histone H1 as substrate for cdk2.
Results

Activation of PI3K and MEK1/2 is required for TCR/CD3 plus CD28-mediated proliferation and cell cycle progression of primary human T lymphocytes

To study the signaling pathways that link TCR/CD3 and CD28 to cell cycle progression in primary T lymphocytes, we used cell-permeable small molecule inhibitors specific for PI3K (LY294002) (34), MEK1/2 (UO126) (35), and p38MAPK (SB203580) (36). The effect of the inhibitors on proliferation induced by activating mAbs against CD3 and CD28 was assessed by [3H]thymidine incorporation. Consistent with previous observations, neither anti-CD3 nor anti-CD28 alone induced significant DNA synthesis, whereas anti-CD3 plus anti-CD28 together resulted in robust T cell proliferation (Fig. 1A). This proliferative response was abrogated by pretreatment of primary T cells with LY294002 or UO126 (Fig. 1A). Titration of these compounds showed that both LY294002 and UO126 inhibited proliferation in a dose-dependent manner (Fig. 1B). SB203580 did not affect proliferation at concentrations shown to inhibit p38MAPK (Fig. 1A, B and C) but had a modest effect at higher concentrations, at which phosphorylation of PKB by phosphatidylinositol-dependent kinase-1 is blocked (data not shown) (37).

To determine the effects of the inhibitors on cell cycle progression, we performed FACS analysis on PI-labeled cells. LY294002 and UO126 inhibited cell cycle progression before DNA synthesis, whereas SB203580 had no effect (Fig. 1C). Simultaneous assessment of apoptosis by analysis of DNA content showed that none of these inhibitors affected cell survival (data not shown). These results show that TCR/CD3 plus CD28-mediated cell cycle entry and proliferation of human primary T cells is dependent on activation of PI3K and MEK1/2 but not on activation of p38MAPK.

Activation of PI3K and MEK1/2 is required for TCR/CD3 plus CD28-mediated production of Th1- and Th2-type cytokines in primary human T lymphocytes

It was recently reported that PI3K regulates transcription of Th1- but not Th2-type cytokines via its downstream target PKB (c-Akt) and that activated PKB can substitute for CD28 costimulation for the induction of Th1 but not Th2 cytokine gene transcription (38). These observations suggest that inhibition of the PI3K-PKB pathway in primary human T cells may result in selective inhibition of Th1- but not Th2-type cytokine production. Our results described above showed that inhibitors of PI3K and MEK had a comparable inhibitory effect on cellular proliferation of primary T lymphocytes stimulated via TCR/CD3 plus CD28. Therefore, we examined whether these inhibitors might have a distinct effect on cytokine production under the same culture conditions. As shown in Table I, culture of T cells in the presence of CD3 and CD28 Abs resulted in a dramatic augmentation of IL-2, IFN-γ, IL-4, and IL-10 compared with the amounts of these cytokines produced in the presence of CD3 alone. However, we were surprised to find that addition of either the PI3K inhibitor LY294002 or the MEK inhibitor UO126 equivalently diminished production of all types of cytokines. These results indicate that PI3K-PKB- and MEK1/2-mediated pathways are required for production of both Th1- and Th2-type cytokines in primary human T cells.

**FIGURE 1.** Proliferation and cell cycle progression of human primary T cells is dependent on PI3K and MEK1/2 activation. A. Effect of the pharmacologic inhibitors LY294002, UO126, and SB203580 on the proliferation of human primary T cells. Enriched human primary T cells were cultured with medium, anti-CD3, or anti-CD28, or the combination of anti-CD3 plus anti-CD28 mAbs for 72 h. Separate aliquots of cells were pretreated with the PI3K inhibitor LY294002 (10 μM), MEK1/2 inhibitor UO126 (10 μM), or p38MAPK inhibitor SB203580 (10 μM) before anti-CD3 plus anti-CD28 culture. Proliferation was determined by [3H]thymidine incorporation during the last 16–18 h of the incubation period. Data are expressed as the mean of triplicate measurements ± SEM. Results are representative of more than a dozen independent experiments using different donors. B. Dose-dependent inhibition of proliferation by LY294002 and UO126, but not by SB203580. T cells were prepared and treated as in A, except that a range of concentrations of the PI3K inhibitor LY294002, MEK1/2 inhibitor UO126, and p38MAPK inhibitor SB203580 were used during pretreatment. C. Cells were treated and cultured as in A. After a 48-h incubation period, cell cycle analysis was performed by flow cytometry on PI-labeled cells. Data are expressed as the mean of triplicate measurements ± SEM. Results are representative of over a dozen independent experiments using different donors.

To determine the effects of the inhibitors on cell cycle progression, we performed FACS analysis on PI-labeled cells. LY294002 and UO126 inhibited cell cycle progression before DNA synthesis, whereas SB203580 had no effect (Fig. 1C). Simultaneous assessment of apoptosis by analysis of DNA content showed that none of these inhibitors affected cell survival (data not shown). These results show that TCR/CD3 plus CD28-mediated cell cycle entry and proliferation of human primary T cells is dependent on activation of PI3K and MEK1/2 but not on activation of p38MAPK.
Separate aliquots of cells were pretreated with the PI3K inhibitor LY294002 (10 μM) before anti-CD3, anti-CD28, or the combination of anti-CD3 plus anti-CD28 mAbs. We have previously shown that decreased abundance of p27kip1 required CD28 costimulation and is dependent on PI3K and MEK1/2. A key event in CD28-mediated cell cycle progression. Therefore, we examined the role of PI3K and MEK1/2 in p27kip1 down-regulation after TCR/CD3 plus CD28-mediated activation of primary T cells. Consistent with previous observations, culture with anti-CD3 or anti-CD28 alone did not affect the expression of p27kip1 (Fig. 2D, lanes 1–3). Coglition of TCR/CD3 and CD28 resulted in a dramatic down-regulation of p27kip1, which was significantly inhibited by either LY294002 or UO126, indicating that both PI3K and MEK had an active role in the regulation of p27kip1 (Fig. 2D, lanes 3–5). In contrast to LY294002 and UO126, SB203580 did not inhibit down-regulation of p27kip1 induced by TCR/CD3 plus CD28. Among the cell cycle regulatory molecules, including cdks and cyclins, only expression of p27kip1 remained completely unaltered by TCR/CD3 signals alone and required CD28 costimulation to be down-regulated (Fig. 2, A, B, and D). Moreover, although SB203580 partially inhibited enzymatic activation of cdks (Fig. 2A, lane 7) and expression of cyclin E (Fig. 2B, lane 7), it did not affect down-regulation of p27kip1 (Fig. 2D, lane 7) or inhibit TCR/CD3 plus CD28-mediated proliferation (Fig. 1A).

**Table I. Activation of PI3K and MEK1/2 is required for TCR/CD3 plus CD28-mediated production of Th1 and Th2 type cytokines**

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<tr>
<th>Culture Conditions</th>
<th>IL-2</th>
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<td>25</td>
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</table>

* Purified human T cells were cultured as indicated in Materials and Methods. The concentration of cytokines was assessed by ELISA in supernatants collected at 24 h of culture. Lowest limits of ELISA detection are: IL-2, 6 pg/ml; IFN-γ, 8 pg/ml; IL-4, 0.13 pg/ml; IL-10, 3.9 pg/ml.

**FIGURE 2.** CD28 costimulation-mediated expression and enzymatic activation of cdks and cyclins and down-regulation of p27kip1 are dependent on PI3K and MEK1/2. A, Primary human T cells were cultured with medium, anti (α)-CD3, anti-CD28, or the combination of anti-CD3 plus anti-CD28 mAbs. Separate aliquots of cells were pretreated with the PI3K inhibitor LY294002 (10 μM), MEK1/2 inhibitor UO126 (10 μM), or p38MAPK inhibitor SB203580 (10 μM) before anti-CD3 plus anti-CD28 culture. Cell lysates were prepared at 48 h of culture, and equal amounts of protein (75 μg) were resolved by SDS-PAGE followed by immunoblotting with cyclin D3-specific antiserum. Blots were stripped and rebotted with antisera specific for cdk4 and cdk6. The cdk4 kinase activity was determined in cdk4 immunoprecipitates followed by an in vitro kinase assay using GST-Rb as a substrate. B, Immunoblots from A were stripped and subsequently blotted with antisera or mAbs specific for cyclin A, cyclin E, cdk2, and p27kip1 (D) followed by incubation with the indicated secondary Ab and immunodetection by ECL. The cdk2 kinase activity was determined in cdk2 immunoprecipitates followed by in vitro kinase assay using histone H1 as a substrate. C, After culture as indicated, samples were analyzed by 6% SDS-PAGE followed by immunoblot with Rb-specific mAb.
The combination of anti-CD3 plus anti-CD28 mAbs followed by cross-linking with rabbit anti-mouse Ig for the indicated time periods. Stimulation with the combination PMA-ionomycin (Iono.) was included as control. Cell lysates were prepared, and equal amounts of protein (75 µg) were resolved by 10% SDS-PAGE followed by immunoblotting with phospho-ERK2-specific antiserum. Detection was with ECL.

**FIGURE 3.** Ligation of TCR/CD3 induces activation of ERK1/2, whereas ligation of CD28 induces activation of PI3K/PKB in primary human T cells. A, T cells were stimulated with anti-(α)-CD3, anti-CD28, or the combination of anti-CD3 plus anti-CD28 mAbs followed by cross-linking with rabbit anti-mouse Ig. TCR/CD3 cross-linking resulted in the activation of ERK1/2, as determined by immunoblot with an Ab specific for the phosphorylated form of ERK1 and ERK2 (Fig. 3A, lanes 1–4). In contrast, cross-linking of CD28 did not induce phosphorylation of ERK1 and ERK2 (Fig. 3A, lanes 5–7). Moreover, simultaneous cross-linking of both TCR/CD3 and CD28 did not alter the degree of ERK1 and ERK2 activation (Fig. 3A, compare lanes 2–4 with lanes 8–10).

Cross-linking of CD28 alone resulted in the activation of PKB (c-Akt; Fig. 3B, lanes 5–7). Moreover, simultaneous cross-linking of both CD28 and TCR/CD3 did not augment the degree of PKB phosphorylation (Fig. 3B, compare lanes 5–7 with lanes 8–10). Whereas CD28 ligation activated PKB in every experiment performed, the effect of TCR/CD3 activation varied from donor to donor. In most individuals, anti-CD3 did not activate PKB to any detectable degree (Fig. 3B, lanes 2–4), whereas in other donors the activation was equivalent to that seen with anti-CD28 (data not shown). Taken together, our results suggest that TCR/CD3 plus CD28-induced cell cycle progression of primary T cells requires activation of both MEK1/2 and PI3K. The MEK substrate ERK1/2 proteins were activated exclusively by TCR/CD3, whereas the PI3K target PKB was activated consistently by CD28 and only occasionally by TCR/CD3. Thus, although TCR/CD3 may also contribute to PKB activation, our data suggest that CD28 plays the most important role in this process.

TCR/CD3 and CD28 have unique roles in the activation of MEK1/2- and PI3K-PKB-dependent pathways

To determine whether distinct signaling events evoked by TCR/CD3 and by CD28 resulted in the concomitant activation of PI3K and MEK1/2, we examined the biochemical events initiated by independent ligation of each receptor. Purified primary human T cells were left untreated or treated with anti-CD3, anti-CD28, or the combination of anti-CD3 and anti-CD28 mAbs followed by cross-linking with rabbit anti-mouse Ig. TCR/CD3 cross-linking resulted in the activation of ERK1/2, as determined by immunoblot with phospho-ERK2-specific antiserum. Detection was with ECL. A, After cell treatment and analysis of the cell lysates as in A, immunoblot was performed with phospho-PKB (Ser473)-specific antiserum. Immunoblots were stripped and incubated with a PKB-specific mAb.

Inactivation of GSK-3, a substrate of PKB, substitutes for CD28 but not for CD3 in down-regulation of p27kip1 and cell cycle progression

A number of PKB substrates have been identified, and several of these proteins are directly involved in the regulation of cell cycle progression (16, 18–20, 39–43). To determine whether activation of PKB by PI3K after CD28 costimulation was directly involved in cell cycle progression in primary human T cells, we examined whether the activation state of PKB substrate GSK-3 affected p27kip1 expression and clonal expansion of primary T cells. GSK-3α and GSK-3β are homologous serine/threonine kinases that are expressed in a number of cell types. In contrast to most other kinases, GSK-3 exists in an activated, unphosphorylated form in resting cells. Extracellular signals induce phosphorylation of GSK-3 and subsequent loss of enzymatic activity. Inactivating phosphorylation of GSK-3 (Ser 17, GSK-3α, Ser 19 in GSK-3β) is mediated by PKB in response to PI3K-dependent stimuli (19, 40, 43). Because our data suggested that CD28 rather than TCR/CD3 is the predominant activator of the PI3K/PKB pathway, we hypothesized that inactivation of GSK-3 by PKB might be an important consequence of CD28 signaling. Immunoblot with an Ab specific for the phosphorylated, inactivated form of GSK-3 showed that this form of the protein was not detected in unstimulated cells, but CD28 ligation alone was sufficient to induce GSK-3 phosphorylation (Fig. 4A). To determine the functional significance of GSK-3 inactivation, we examined the effect of direct inactivation of GSK-3 on T cell proliferation and cell cycle progression. We addressed this question by treating T cells with lithium chloride (LiCl), which inhibits GSK-3 by competing for the Mg2+-binding site within the kinase (44, 45). Addition of LiCl to T cells cultured with anti-CD3 mAb induced a proliferative response that was consistently greater than that seen with anti-CD3 alone (Fig. 4B). This event coincided with phosphorylation of Rb, synthesis of cyclin A, and down-regulation of p27kip1 (Fig. 4C). Lithium chloride alone or in combination with CD28 did not increase proliferation. These data show that direct inactivation of GSK-3 could substitute for CD28 signals in the presence of CD3 ligation but could not substitute for CD3 signals in the presence of CD28 ligation. Taken together, these results provide evidence that GSK-3 is a target of CD28. Consistent with the requirement for simultaneous activation of both MEK1/2 and PI3K-PKB signals for T cell cycle progression (Fig. 1, A and C), activation of this PKB downstream target results in functional effects only when a TCR/CD3 stimulus is also provided. Thus, these data support the idea that CD28 may play the dominant role in regulation of cell cycle progression via activation of the PI3K-PKB pathway.

Discussion

Despite the established biologic role of CD28 as a costimulatory receptor, the downstream signaling events activated upon CD28 triggering are still poorly defined. It remains unclear whether CD28 activates signaling pathways distinct from those triggered by the TCR complex or whether CD28 functions by augmenting TCR-induced signaling events. Our present studies found that TCR/CD3 and CD28 receptors had distinct roles in the regulation of MEK- and PI3K-dependent mitogenesis in primary T lymphocytes. Moreover, both MEK and PI3K-dependent pathways were indispensable for T cell cycle progression.

The role of PI3K and its targets in T cell activation has been a matter of ongoing study and debate. Much of the work on this subject has been performed using the Jurkat T leukemia cell line, which has been an important tool for studying for TCR- and
Cell lysates were prepared, and equal amounts of protein (75 μg) by cross-linking with rabbit anti-mouse Ig for the indicated time periods. Subsequently, blots were stripped and immunoblotted. For detection of Rb phosphorylation, aliquots of the same samples were analyzed by 6% SDS-PAGE followed by immunoblotting with Rb-specific mAb.

CD28-dependent signaling pathways. Various studies have reported a negative effect, a positive effect, or no role for PI3K in IL-2 gene transcription after CD28 ligation (22, 46). However, the relevance of these data to primary T cells has been called into question (47) because Jurkat cells do not express the PI3K-antagonizing lipid phosphatase and tensin homolog deleted on chromosome 10 and therefore contain elevated levels of 3'-phosphorylated inositol phospholipids (48). It follows that PI3K substrate PKB. For detection of Rb phosphorylation, aliquots of the same samples were analyzed by 6% SDS-PAGE followed by immunoblot with Rb-specific mAb.

PI3K-PKB COUPLES CD28 SIGNALING TO THE CELL CYCLE MACHINERY

FIGURE 4. PKB downstream effectors have a direct role in down-regulation of p27kip1 and cell cycle progression in primary human T cells. A, T cells were incubated with either medium or anti (α)-CD28 mAb followed by cross-linking with rabbit anti-mouse Ig for the indicated time periods. Cell lysates were prepared, and equal amounts of protein (75 μg) were resolved by 10% SDS-PAGE followed by immunoblotting with phospho-p27kip1-specific mAb. Detection was performed with ECL. B, Purified T cells were cultured with medium, anti-CD3, anti-CD28, or their combination. In the same experiment, separate aliquots of cells were treated with LiCl (10 mM) and subsequently cultured with medium, anti-CD3, or anti-CD28 mAbs. Proliferation was assessed by incorporation of [3H]thymidine for the last 16 h of a 72-h culture period. C, After 48 h of culture under the indicated conditions, cell lysates were prepared and equal amounts of protein were analyzed by 10% SDS-PAGE followed by immunoblot with an Ab specific for cyclin A. Subsequently, blots were stripped and immunoblotted with an Ab specific for p27kip1. For detection of Rb phosphorylation, aliquots of the same samples were analyzed by 6% SDS-PAGE followed by immunoblot with Rb-specific mAb.

ways in primary T cells. However, cell-permeable small molecule inhibitors of PI3K have demonstrated that PI3K activity is required for CD28-mediated proliferation and IL-2 synthesis in primary T cells (24).

The PI3K/PKB pathway has been found to be required for TCR/CD3-mediated and CD28-mediated cytokine expression in separate reports (38, 49). In the present study, we investigated the contribution of TCR/CD3 and CD28 signaling to cell cycle progression in primary T cells. Experiments were performed under conditions in which TCR/CD3 ligation alone was submitogenic, but the simultaneous ligation of both TCR/CD3 and CD28 induced robust proliferation. We observed substantial variation between blood donors in the degree to which TCR/CD3 ligation activates PI3K substrate PKB. However, CD28 ligation consistently induced phosphorylation of PKB on Ser473 and downstream events such as inactivating phosphorylation of GSK-3. These data suggest that CD28 is the most important activator of PI3K/PKB signaling under conditions of submitogenic TCR complex ligation in primary T cells. Consistent with this, direct inactivation of the PKB target GSK-3 by LiCl substituted for CD28 ligation in proliferation assays but did not induce cell cycle entry either alone or in combination with CD28 ligation. Lithium chloride failed to bypass PI3K blockade by LY294002 (data not shown), suggesting that additional effectors of PKB and/or PI3K are required for TCR/CD3 plus CD28-induced T cell proliferation. Failure of an activated allele of PKB to bypass the effect of PI3K inhibitor wortmannin on IL-2 production in murine T cell blasts similarly suggests that other effectors of PI3K may be required in T cell activation (50).

Whereas our data suggest that CD28 predominates over TCR/CD3 stimulation in activating the PI3K-PKB signaling pathway, the opposite was found for ERK-dependent signaling. Ligation of the TCR/CD3 complex consistently resulted in phosphorylation of ERK1/2 in vivo, whereas CD28 activation did not result in ERK phosphorylation in any of the blood donors tested. MEK is the immediate upstream activator of ERK1 and ERK2, which are the only known MEK1 and MEK2 substrates. Our studies found that TCR/CD3 signals alone can activate the MEK-ERK1/2 pathway but that they are not sufficient to induce cell cycle progression. Consistent with this finding, it has been shown that activated MEK stimulates expression of AP-1 components in quiescent 3T3 cells but that it is not capable of inducing cellular proliferation. Interestingly, that report showed that PI3K signals are corequired to stimulate DNA synthesis (51).

Previous work from our group and others has shown that down-regulation of cdk inhibitor p27kip1 is a critical biochemical event in TCR/CD3 plus CD28-induced cell cycle progression (25, 33). Simultaneous ligation of the TCR/CD3 complex and CD28 is required for down-regulation of p27kip1, and anergizing ligation of the TCR in the absence of costimulation actually increased the abundance of p27kip1 (52). In this study, we used cell-permeable, small-molecule inhibitors to investigate the roles of PI3K, MEK, and p38MAPK in the down-regulation of p27kip1 and other biochemical events associated with cell cycle entry. The previously reported requirement for PI3K (24) and MEK (35) in primary T cell proliferation was confirmed by thymidine uptake and PI staining. Whereas either PI3K inhibitor LY294002 or MEK inhibitor U0126 caused near-total G0 arrest in these experiments, neither agent increased the apoptotic fraction of T cells. The relative importance of PI3K for proliferative rather than survival signals in primary T cells is supported by the recent finding that LY294002 actually abrogated cytarabine-induced apoptosis of PHA-stimulated peripheral blood lymphocytes (53).

In studying molecular mechanisms of cell cycle progression, we found that G1 phase events such as expression of cyclin D and
associated kinases cdk4 and cdk6, as well as phosphorylation of Rb, can be induced by ligation of TCR/CD3 alone and that this stimulus also results in MEK activation. Coligation of CD28 with TCR/CD3 augments these G1 phase effects, which are blocked by MEK inhibitor U0126 to a greater extent than by PI3K inhibitor LY294002. In contrast, p27kip1 expression remains totally unaltered by TCR/CD3 ligation alone, and both MEK and PI3K are equally required for its down-regulation. There is a tight quantititative and temporal correlation between inhibition of cell cycle entry, inhibition of p27kip1 down-regulation, and lack of S-phase proteins such as cyclin A. Because CD28 but not TCR/CD3 consistently activated PI3K/PKB in primary T cells from all individuals tested, our results suggest that PI3K and its downstream effector PKB link CD28 to the down-regulation of p27kip1 and cell cycle progression. Finally, our data demonstrate that p27kip1 integrates MEK- and PI3K-dependent mitogenic signals at a critical control point in the G1 phase to regulate cell cycle progression and clonal expansion of primary human T lymphocytes.

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References


