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Prolonged TCR/CD28 Engagement Drives IL-2-Independent T Cell Clonal Expansion through Signaling Mediated by the Mammalian Target of Rapamycin[1]

Sara Colombetti,* Veronica Basso,* Daniel L. Mueller,† and Anna Mondino[2]*

Proliferation of Ag-specific T cells is central to the development of protective immunity. The concomitant stimulation of the TCR and CD28 programs resting T cells to IL-2-driven clonal expansion. We report that a prolonged occupancy of the TCR and CD28 bypasses the need for autocrine IL-2 secretion and sustains IL-2-independent lymphocyte proliferation. In contrast, a short engagement of the TCR and CD28 only drives the expansion of cells capable of IL-2 production. TCR/CD28- and IL-2-driven proliferation revealed a different requirement for PI3K and for the mammalian target of rapamycin (mTOR). Thus, both PI3K and mTOR activities were needed for T cells to proliferate to TCR/CD28-initiated stimuli and for optimal cyclin E expression. In contrast, either PI3K or mTOR were sufficient for IL-2-driven cell proliferation as they independently mediated cyclin E induction. Interestingly, rapamycin delayed cell cycle entry of IL-2-sufficient T cells, but did not prevent their expansion. Together, our findings indicate that the TCR, CD28, and IL-2 independently control T cell proliferation via distinct signaling pathways involving PI3K and mTOR. These data suggest that Ag persistence and the availability of costimulatory signals and of autocrine and paracrine growth factors individually shape T lymphocyte expansion in vivo. ***The Journal of Immunology,*** **2006, 176: 2730–2738.**

Proliferation of CD4 T lymphocytes is initiated by the engagement of the TCR and of costimulatory receptors, such as CD28. These receptors elicit the expression and secretion of the T cell growth factor IL-2 and the expression of its high-affinity receptor IL-2R, rendering the cells competent for IL-2-driven proliferation. Upon interacting with its receptor, IL-2 mediates the coordinated activation of several intracellular signaling events, which culminate with cell cycle entry and clonal expansion (1–4). In vivo, other costimulatory molecules and cytokines may then synergize with Ag and sustain proliferation of activated T cells (5–9).

The entry of resting T cells into the cell cycle is not only determined by IL-2, but also by the TCR and CD28. The degree and the length of TCR and CD28 occupancy are both critical for T cells to leave the G_0 stage, for the regulation of the frequency of cells entering the proliferative pool, and for determining the number of cycles that each cell completes (10–14). The role of TCR and CD28 receptors in the regulation of the cell cycle was initially attributed to their ability to elicit IL-2 secretion. Studies performed in vivo in IL-2-, CD28-, and CTLA-4-deficient mice later supported the existence of IL-2-independent T cell expansion and a possible direct role for the TCR and CD28 in cell division. Indeed, T cells derived from IL-2-deficient mice showed proliferative responses that, although reduced, were sensitive to CD28-mediated costimulation (15–17) and restored by the addition of exogenous IL-2 (18). In contrast, T cells derived from CD28-deficient mice had severely impaired proliferative responses, only partially increased by the addition of exogenous IL-2 (19). Furthermore, the massive proliferation of T cells derived from CTLA-4-deficient mice did not correlate with increased IL-2 production, but with unlimited CD28-mediated costimulation (20, 21). Together, these data suggested that, rather than controlling cell cycle progression exclusively through the regulation of autocrine IL-2 production and the up-regulation of the IL-2R, the TCR and CD28 could be directly capable of eliciting cell proliferation. In vitro studies also support the ability of TCR/CD28 to control cell cycle progression independently of IL-2 (4, 16, 22, 23). For instance, TCR and CD28 elicit activation of the PI3K/PKB pathway and phosphorylation the of 70-kDa S6 kinase ($p70^{S6k}$) (24, 25), which elicit E2F transcriptional activity and thus the transcription of genes required for S phase entry such as cyclin E (26). CD28-induced PI3K/PKB activation also elicits the down-regulation of the cyclin kinase inhibitor $p27^{Kip}$, allowing cyclin-dependent kinase Cdk4/Cdk6 activation and rendering the cells competent for G_1 to S cell cycle progression (4, 23). Consistent with these findings is our previous work showing that the optimal engagement of CD3 and CD28 induces comparable up-regulation of cyclin D2, D3, and E, hyperphosphorylation of retinoblastoma protein, down-regulation of $p27^{Kip}$, and cell division in cells functionally (anergic A.E7 T cells) and genetically (IL-2-deficient DO11.10 T cells) impaired in IL-2 secretion (27).

Because conflicting data exist on the need of prolonged vs short TCR engagement (9, 13, 28–30) and on the relative contribution of TCR, CD28, and IL-2 to T cell division, we thought of comparing the requirements for CD3/CD28 engagement in the presence or absence of autocrine IL-2 secretion with the requirements for IL-2-driven T cell expansion. To this aim, we have used T cells secreting different amounts of IL-2 and T cells that are functionally and genetically incapable of IL-2 secretion. As comparison we also analyzed primary T cells derived from DO11.10 TCR transgenic

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mice, which allows the study of Ag-driven T cell responses. We found that a short engagement of CD3 and CD28 was sufficient to induce T cells to proliferate in the presence of autocrine IL-2 secretion. In contrast, a sustained CD3/CD28 occupancy was needed to maintain cell division of IL-2-deficient T cells. Furthermore, TCR/CD28-dependent IL-2-independent cell proliferation was mediated by PI3K and mammalian target of rapamycin (mTOR)[3]-dependent signaling, which were both required for cyclin E expression. At difference, proliferation driven by optimal amounts of IL-2 was only partially sensitive to the PI3K inhibitor LY294002 and delayed, but not prevented, by the mTOR/GβL/raptor inhibitor rapamycin (RAPA), and either PI3K or mTOR signaling per se was sufficient to drive cyclin E up-regulation.

Thus, this study demonstrates that CD3/CD28 and IL-2 independently control cell proliferation, and although CD3/CD28-driven proliferation relies on RAPA-sensitive signals, possibly via cyclin E regulation, IL-2-driven proliferation is independently sustained by PI3K and mTOR.

Materials and Methods

Cells

The A.E7 T cell clone (31) was maintained in RPMI medium supplemented with 2 mM L-glutamine, 100 mg/ml streptomycin, 100 U/ml penicillin, 20 mg/ml gentamicin, and 50 μM 2-ME (Invitrogen Life Technologies), and 5% heat-inactivated FBS (Euroclone) at 37°C in a 5% CO_2 atmosphere. Cells were maintained by periodic stimulation with a peptide derived from pigeon cytochrome *c* (peptide 81–104; Primm) and from irradiated B10.BR (Harlan Sprague Dawley) splenic APC as previously described (31). The chicken OVA-specific wild-type and IL-2$^{-/-}$ DO11.10 T cell lines (27) were stimulated weekly with irradiated syngeneic BALB/c spleen cells (APC; Charles River Breeding Laboratories) pulsed with the OVA-derived peptide (peptide 323–339; Primm), and expanded in exogenous rIL-2 (10 IU/ml; Roche). All the experiments were performed at least 10 days from the last Ag exposure. At this time, the cells appeared to be in the G_0-G_1 stage of the cell cycle. Primary DO11.10 T cells were recovered from the axillary, brachial, cervical, and mesenteric lymph nodes of DO11.0 TCR transgenic mice, which were bred in our specific pathogen-free facility according to institutional guidelines.

Anergy induction

T cell anergy was induced as previously described (32). Briefly, A.E7 cells were cultured for 16 h on plate-bound (25–30 $\times$ 10^6 cells in 150-mm dishes) anti-CD3-ϵ mAb (clone 145-2C11, 4 μg/ml) (33). The cells were then removed from the mAb, and rested for an additional 5 days in fresh medium. At the same time, a similar number of A.E7 cells was harvested and rested in fresh medium. Both untreated and CD3-treated (anergic) cells appeared to be in the G_0-G_1 phase of the cell cycle. Thereafter, viable cells were separated on a Lympholyte-M (Cederlane Laboratories) density gradient and restimulated as indicated in the figures.

T cell proliferation

To trace single cell proliferation, we adopted the Lyons-Parish technique (34). T cells were washed twice with PBS and resuspended at a density of 2×10^7 cells/ml in PBS. An equal volume of a PBS solution containing 1.25 μM fluorescent dye CFDASE (Molecular Probes) was added, and the cells were gently mixed for 8 min at room temperature. In the case of A.E7 T cells, the final concentration of CFDASE used was 2.5 μM. Unbound CFDASE, or the deacetylated form CFSE, was quenched by the addition of an equal volume of FBS. The labeled cells were washed twice in complete medium and stimulated as indicated in the figures. When indicated, at the time of harvest, CFSE-labeled cells were washed twice in PBS. Cell division analysis was performed on a BD Biosciences FACSCalibur dual-laser cytometer using standard CellQuest acquisition-analysis software. CFSE-labeling remained stable for up to 3 wk in our cell cultures.

Western blot analysis

Control and anergic A.E7 cells were stimulated with anti-CD3 and anti-CD28 mAbs (2 and 5 μg/ml, respectively) or with IL-2 (10 IU/ml) for the times indicated in the experiments. When indicated the cells were pretreated with RAPA (1 μM; Calbiochem), or with LY294002 (20 μM; Sigma-Aldrich) for 30 min at 37°C, and then stimulated in the presence of the drug. The cells were then harvested, washed twice with ice-cold PBS, and lysed for 20 min on ice in lysis buffer containing 50 mM Tris-HCl (pH 7.4), 1% Triton X-100, 150 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, 1 μg/ml PMSF, 1 μg/ml aprotinin, 5 μg/ml leupeptin, 1 mM NaF, 1 mM $NaOV_3$ (Sigma-Aldrich). Protein extracts were quantified by the Bradford assay. Samples containing an equal amount of protein (15 μg) were mixed with an equal volume of 2$\times$ Laemmli buffer, boiled, and separated on standard 10–15% SDS-PAGE.

Results

Prolonged TCR/CD28 engagement sustains IL-2-independent T cell proliferation via mTOR

A short engagement of the TCR and of the costimulatory CD28 receptor programs T cells to proliferate in response to autocrine IL-2 (11, 12, 29). Optimal stimulation of the TCR and CD28, however, can also sustain IL-2-independent cell division (27). It was of interest to investigate whether the TCR/CD28 engagement needed to be at a different length to sustain proliferation in the presence or in the absence of IL-2. To this aim, we analyzed CD3/CD28-driven proliferation of A.E7 T cells and of cells induced into clonal anergy by chronic CD3 engagement as previously described (27) and depicted in Fig. 1*A*. Although control A.E7 cells proliferated to Ag (Fig. 1*B*) and produced IL-2 upon optimal CD3/CD28 stimulation (Fig. 1*C*), anergic T cells had impaired Ag responsiveness and defective autocrine IL-2 secretion (Fig. 1, *B* and *C*). CFSE-labeled control and anergic cells were thus cultured on immobilized anti-CD3 and anti-CD28 mAbs for 5 days or cultured on the mAbs for 1 day, and then in their conditioned medium in the absence of the mAbs for an additional 4 days (Fig. 1*D*). Control and anergic T cells were also cultured with exogenous rIL-2 as control. Although control cells proliferated to similar extents in response to a prolonged (5 days) or a short (24 h) CD3/CD28 engagement (Fig. 1, *E* or *F*, respectively) and to IL-2 (Fig. 1*G*), anergic T cells proliferated to continuous (5 days) CD3/CD28 engagement (Fig. 1*H*) and to IL-2 (Fig. 1*L*), but not to a transient (24 h) CD3/CD28 engagement (Fig. 1*I*). These data indicate that in the absence of autocrine IL-2 production, a prolonged TCR/CD28 occupancy is required to sustain T cell proliferation.

To further investigate the relative contribution of TCR/CD28 and IL-2 to cell division, we compared CD3/CD28-driven proliferation of wild-type DO11.10 T cells with proliferation of IL-2$^{-/-}$ DO11.10 T cells (27), as well as each cells sensitivity to the immunosuppressive agent RAPA. RAPA is known to prevent signaling via the mTOR/GβL/raptor complex (35–37) and IL-2-driven cell division (2). Most of the wild-type DO11.10 T cells proliferated in response to CD3/CD28 stimulation and completed several rounds of cell division (Fig. 2*A*). Although to a reduced extent, IL-2$^{-/-}$ DO11.10 T cells also responded to optimal CD3/CD28 stimulation (Fig. 2*C*), supporting the existence of IL-2-independent T cell division. Surprisingly, RAPA only partially inhibited proliferation of wild-type T cells (Fig. 2*B*), and instead completely abrogated CD3/CD28-driven proliferation of IL-2$^{-/-}$ T cells (Fig. 2*D*). In the case of A.E7 T cells, both control and anergic T cells proliferated to anti-CD3/CD28 mAbs, and proliferation was severely hampered in the presence of RAPA (Fig. 2, *E–H*).

The RAPA-sensitive mTOR activity is dispensable for T cell proliferation in the presence of optimal IL-2 amounts

IL-2-sufficient DO11.10 T cells produce 5–10 times more IL-2 per cell when compared with A.E7 T cells (data not shown). The

[3] Abbreviations used in this paper: mTOR, mammalian target of rapamycin; RAPA, rapamycin.

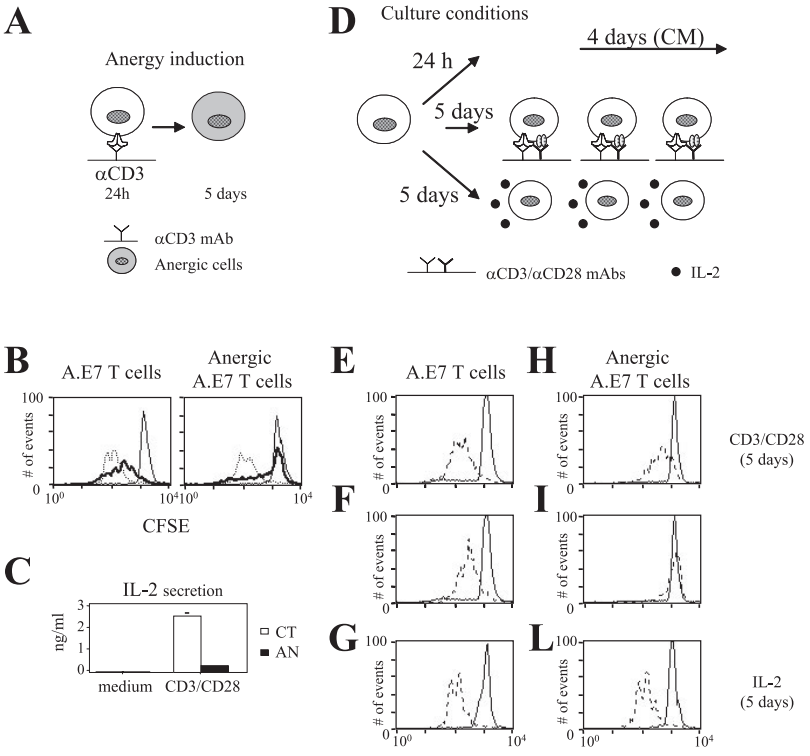

FIGURE 1. Prolonged CD3/CD28 engagement is needed to drive IL-2-independent T cell proliferation. *A–C*, A.E7 T cells were either left untreated or rendered anergic by chronic CD3 engagement as described in *Materials and Methods*. Resting control (CT) and anergic (AN) A.E7 T cells were labeled with the fluorescent dye CFSE and stimulated with Ag and irradiated splenocytes or with exogenous IL-2 (10 IU/ml) for 5 days (*B*) or with immobilized anti-CD3 and anti-CD28 mAbs for 24 h (*C*). *B*, Representative flow cytometry analysis of viable lymphocytes is shown. *C*, Supernatants were analyzed for IL-2 contents. *D–L*, CFSE-labeled control (*E–G*) and anergic (*H–L*) T cells were stimulated on immobilized anti-CD3 and anti-CD28 mAbs for 5 days (*E* and *H*) or for 24 h and then cultured in their conditioned medium (CM) for an additional 4 days (*F* and *I*). As control, the cells were cultured in exogenous IL-2 for 5 days (*G* and *L*). *D*, Schematic representation of the experiment is provided. *E–L*, Representative FACS histograms of replicate cultures are depicted. Unstimulated control cells (solid line histogram) and CD3/CD28 or IL-2-stimulated cells (dotted line histogram) are shown. The experiment was repeated three times with similar results.

results presented in Fig. 2 suggest that RAPA-sensitive mTOR-dependent signaling might be required when autocrine IL-2 production is limited (A.E7 cells) or absent (IL-$2^{-/-}$ DO11.10 T cells), but only transiently needed in the presence of optimal IL-2 amounts. To verify this possibility, CFSE-labeled A.E7 T cells were cultured with different doses of exogenous IL-2 in the absence or in the presence of RAPA (Fig. 3*A*) and analyzed on different days (Fig. 3*B*). The percentage of cells diluting the CFSE content and the number of cell divisions performed by individual cells increased with the dose of IL-2. Up to 20, 60, and 90% of the cells showed a $CFSE^{dim}$ profile in 5 days after stimulation with 0.4, 2, and 10 IU/ml IL-2, respectively. Furthermore, although the cells performed only one division cycle in the presence of 0.4 IU/ml, they completed two and up to four cell divisions in response to 2 and 10 IU/ml IL-2, respectively. In the presence of RAPA, the cells failed to divide in response to IL-2 at the dose of 0.4 IU/ml and only 30% of the cells completed one division cycle at the dose of 2 IU/ml. At difference, in the presence of 10 IU/ml IL-2, only ~10% of the cells treated with RAPA were still undivided and the dividing cells had performed a number of cell cycles comparable to the number observed in cells stimulated in the absence of RAPA (Fig. 3*A*). When analyzed at earlier times, however, RAPA had an antiproliferative effect. Indeed, although most of the cells stimulated by optimal IL-2 amounts (10 IU/ml) had divided by day 3 in the absence of the drug, cells failed to proliferate in the presence of RAPA and retained their original CFSE content (Fig. 3*B*). By day 4, although control cells continued to proliferate as demonstrated by further CFSE dilution, RAPA-treated cells started to divide. By day 10 the CFSE profile of the cells stimulated with IL-2 in the absence or presence of RAPA was mostly indistinguishable. Increasing the concentration of RAPA, or providing new drug every other day, did not increase the antiproliferative activity of the drug (data not shown). Thus, rather than by drug inefficacy, cell division is best explained by RAPA-insensitive proliferation.

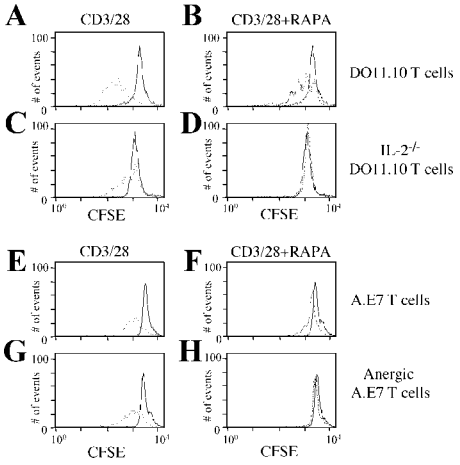

FIGURE 2. mTOR activity is required for CD3/CD28-induced, IL-2-independent T cell proliferation. Wild-type (*A* and *B*) and IL-$2^{-/-}$ (*C* and *D*) DO11.10 TCR transgenic T cells, and control (*E* and *F*) and anergic (*G* and *H*) A.E7 T cells were labeled with CFSE and left unstimulated (solid line histogram) on immobilized anti-CD3 and anti-CD28 mAbs (dotted line histogram) in the absence (*A*, *C*, *E*, and *G*) or presence (*B*, *D*, *F*, and *H*) of RAPA. Representative FACS histograms of CFSE content of viable cells are depicted.

Together, these findings indicate that mTOR is required for CD3/CD28-driven proliferation and in the presence of limiting amounts of IL-2, whereas it is dispensable at optimal concentrations of IL-2.

Signaling via PI3K and mTOR regulates CD3/CD28-dependent, IL-2-independent T cell proliferation

In addition to mTOR, PI3K also plays a crucial role in cell proliferation and cell growth (38, 39). To investigate whether the RAPA-insensitive IL-2-driven proliferation was sensitive to PI3K inhibitors, cells were activated in the presence of LY294002, RAPA, and a combination of the two drugs. CFSE-labeled A.E7 T

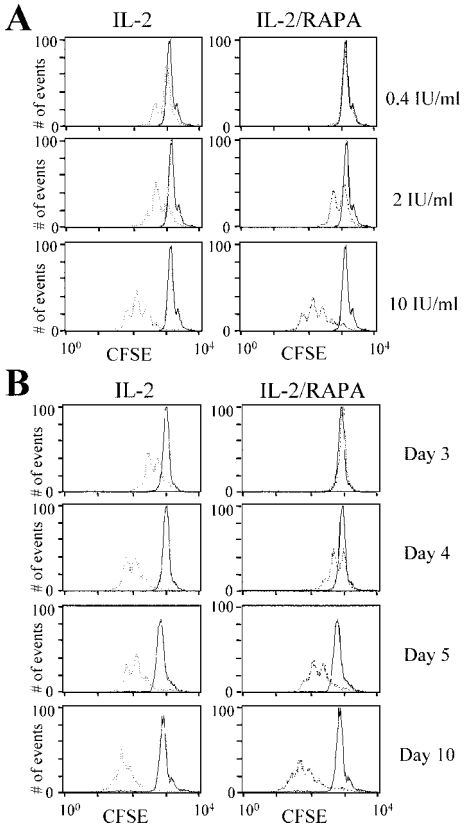

FIGURE 3. Signaling via mTOR is dispensable for T cell proliferation in the presence of optimal IL-2 amounts. CFSE-labeled A.E7 cells were cultured in plain medium (solid line histogram) or with IL-2 (dotted line histogram) at 0.4, 2, and 10 IU/ml in the absence or presence of RAPA. *B*, IL-2 was used at 10 IU/ml. Cells were harvested after 5 days (*A*) or after 3, 4, 5, and 10 days (*B*) as depicted. Thereafter, the CFSE cellular content was analyzed by flow cytometry. Representative FACS histograms of viable cells from replicate cultures are shown. The experiment was repeated four times with comparable results.

cells were stimulated for 5 days with anti-CD3/CD28 mAbs or with suboptimal (0.4 IU/ml) and optimal (10 IU/ml) IL-2 amounts in the absence or presence of LY294002 and/or RAPA at concentrations able to inhibit CD3/CD28- and IL-2-driven phosphorylation of Akt and $p70^{S6k}$ (data not shown). In the presence of either LY294002 or RAPA, CD3/CD28 stimulation failed to elicit cell division, as most of the cells maintained the original CFSE content (Fig. 4*A*). Comparable results were obtained in the presence of suboptimal IL-2 amounts, which induced limited cell division requiring both PI3K and mTOR activities (Fig. 4*B*). At difference, proliferation induced by optimal IL-2 amounts was partially sensitive to LY294002 or RAPA when provided alone, and only completely abolished when the drugs were simultaneously provided (Fig. 4*C*). Interestingly, when T cells were stimulated by immobilized anti-CD3/CD28 mAb and optimal amounts of exogenous IL-2, A.E7 T cell division was more resistant to inhibition of PI3K, and residual proliferation was detected even in the presence of both LY294002 and RAPA (Fig. 4*D*). Residual phosphorylation was not due to drug inefficacy, as LY294002 and RAPA prevented $p70^{S6k}$ phosphorylation in these cultures (data not shown). The antiproliferative activity of LY294002 and RAPA could not be explained by cell death because by the end of the culture, up to 40% of the cells resulted from TO-PRO-3^+ in the absence or the presence of the drugs and regardless of the CFSE dilution profile (Fig. 4*E*).

It was important to determine whether CD3-, CD28-, and IL-2-induced PI3K and mTOR activities could also independently contribute to the proliferation of primary lymphocytes. To this aim, DO11.10 lymph node cells were cultured on immobilized anti-CD3 mAb, on anti-CD3 and exogenous IL-2, and on anti-CD3/CD28 mAb. Cells were also stimulated with Ag and irradiated syngeneic splenocytes (Fig. 5). CD3-induced T cell proliferation was inhibited by LY294002 and, although to a reduced extent, by RAPA and severely hampered by the combination of the two drugs (Fig. 5, *A* and *E*). In the presence of rIL-2, CD3-activated cells had a reduced sensitivity to RAPA, but were still highly dependent on

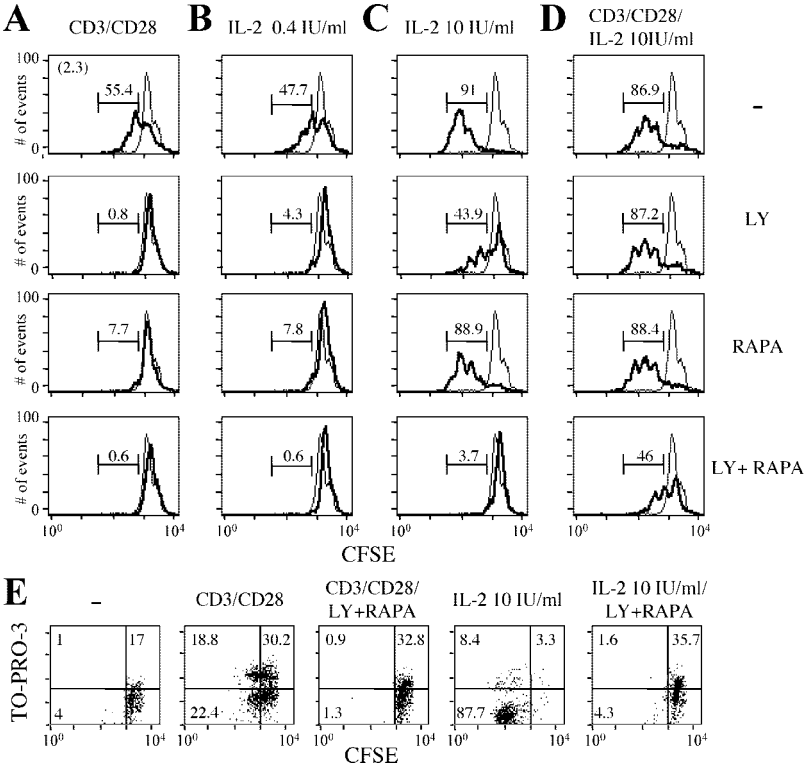

FIGURE 4. PI3K and mTOR signals differentially regulate CD3/CD28- and IL-2-driven T cell proliferation. CFSE-labeled A.E7 cells were cultured in plain medium (thin line histogram) or stimulated (thick line histogram) with immobilized anti-CD3 and anti-CD28 mAbs (CD3/CD28) (*A*), IL-2 (0.4 IU/ml) (*B*), IL-2 (10 IU/ml) (*C*), or with anti-CD3 and anti-CD28 mAbs and IL-2 (10 IU/ml) (*D*) in the absence (−) or the presence of LY294002 (LY), RAPA, and a combination of the drugs (LY+RAPA). After 5 days cells were analyzed by flow cytometry. TO-PRO-3 was added at the time of flow cytometry analysis. *A–D*, Histograms depict the CFSE content of TO-PRO-3^- viable cells. The percentage of $CFSE^{dim}$ cells is indicated in each plot. In the absence of stimulation, 2.3% (*inset top left*) of the cells appeared as $CFSE^{dim}$ cells. *E*, Dot plots of total events are shown. The frequency of cells within each quadrant is indicated. The experiment is representative of four independent experiments.

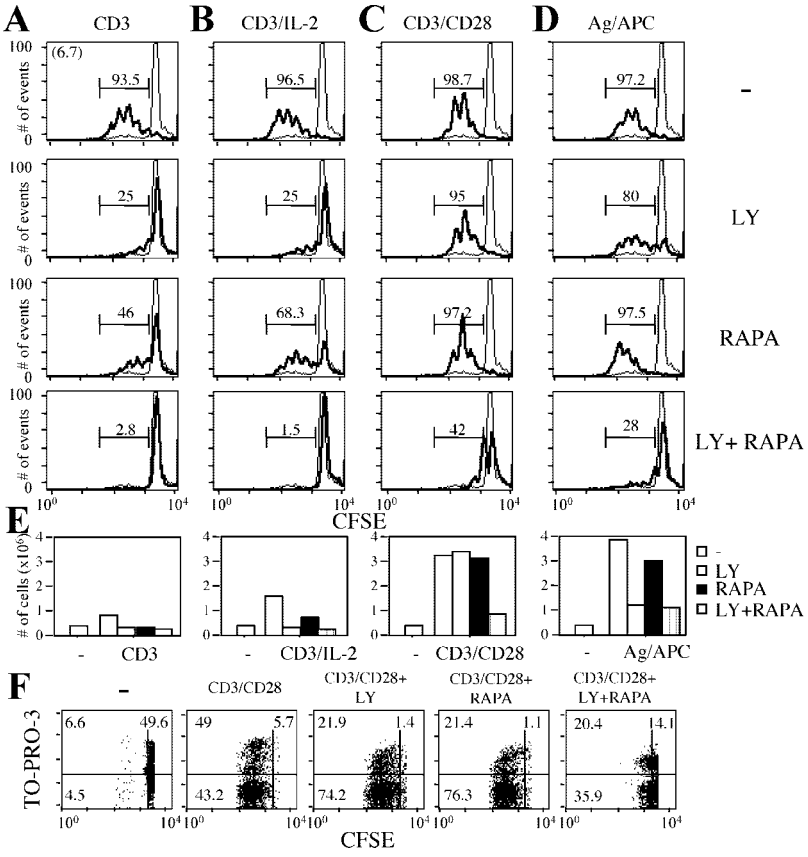

FIGURE 5. CD3, CD28, and IL-2 control proliferation of primary T cells via PI3K and mTOR. CFSE-labeled primary DO11.10 lymph node cells were cultured in plain medium (thin line histogram) or stimulated (thick line histogram) with immobilized anti-CD3 mAb (*A*), with anti-CD3 and IL-2 (10 IU/ml) (*B*), with anti-CD3 and anti-CD28 mAbs (*C*), or with $OVA_{323–339}$-pulsed irradiated splenocytes (Ag/APC) (*D*) in the absence (−) or presence of LY294002 (LY), RAPA, or a combination of the drugs (LY+RAPA). After 5 days cells were analyzed by flow cytometry. TO-PRO-3 was added at the time of flow cytometry analysis. *A–D*, Histograms depict the CFSE content of TO-PRO-3$^-$ viable cells. The percentage of $CFSE^{dim}$ cells is indicated in each plot. In the absence of stimulation, 6.7% (*inset top left*) of cells appeared as $CFSE^{dim}$ cells. *E*, The total number of trypan blue-negative viable cells is shown. *F*, Dot plots of total events are shown. The frequency of cells is shown in each quadrant. The experiment is representative of four independent experiments.

PI3K activity to proliferate (Fig. 5, *B* and *E*). Again, cells failed to divide in the absence of both PI3K and mTOR activities (Fig. 5, *B* and *E*). Stimulation by anti-CD3 and anti-CD28 mAb and by Ag and irradiated syngeneic splenocytes induced cell division in the presence of either LY294002 or RAPA (Fig. 5, *C–E*), suggesting PI3K and the mTOR independently regulate proliferation of optimally activated T cells. Interestingly, CD3/CD28-activated, and to a lesser extent Ag-activated T cells, showed residual proliferation in the absence of both PI3K and RAPA-sensitive mTOR-dependent signaling (Fig. 5, *C–E*), as was also found in the case of A.E7 T cells stimulated by CD3/CD28 mAb and rIL-2 (Fig. 4*D*). Again, LY294002 and RAPA inhibited proliferation, and did not preferentially induce cell death in the cultures as shown by the CFSE to TO-PRO-3 profile (Fig. 5*F*).

The finding that CD3/IL-2- and CD3/CD28-induced proliferation had different sensitivity to LY294002 and RAPA suggested that CD3, CD28, and IL-2 may independently drive proliferation of primary cells. To investigate this possibility, primary DO11.10 T cells were cultured on immobilized anti-CD3/CD28 mAbs for 5 days or for 24 h on the mAbs and an additional 4 days in their conditioned medium. To a set of cultures the conditioned medium was changed after 24 h, and the cells were cultured in fresh medium for an additional 4 days (Fig. 6). T cells diluted their CFSE content and increased in numbers to similar extents when cultured for the entire time on the CD3/CD28 mAbs in the presence or in the absence of the conditioned medium (Fig. 6, *A* and *B*, respectively, and Fig. 6*E*). Comparable results were obtained by culturing the cells on immobilized mAbs and changing the culture medium every 24 h (data not shown). DO11.10 T cells also proliferated to similar extent when activated for 24 h on the anti-CD3/CD28 mAb, and were then cultured for an additional 4 days in their conditioned medium (Fig. 5, *C* and *E*). In contrast, DO11.10 T cells failed to proliferate when deprived of TCR/CD28 engagement and conditioned medium (Fig. 6, *D* and *E*). Similar results were obtained by analyzing Ag-driven T cell responses. In these experiments, only the simultaneous addition of anti-B7 and anti-IL-2R Abs efficiently prevented proliferation of Ag-stimulated DO11.10 T cells (J. L. Bonnevier, C. A. Yarke, and D. L. Mueller, submitted for publication). Together, these data indicate that primary T cells need either the prolonged occupancy of the TCR and CD28 or the presence of autocrine IL-2 to expand optimally.

TCR/CD28 drives cyclin D3 and cyclin E expression via mTOR-dependent signaling

Hleb et al. (40) reported that the up-regulation of cyclin D3 in PMA and ionomycin-stimulated human T lymphocytes cells is sensitive to RAPA, providing a link between early signaling, mTOR, and cell cycle progression. In a previous report, we showed that optimal CD3/CD28 stimulation induced the expression of cyclin D2, D3, and E, the down-regulation of $p27^{Kip}$, and the hyperphosphorylation of retinoblastoma protein in IL-2-sufficient as well as IL-2-deficient $CD4^+$ T cells (27). We thus investigated the relative contribution of PI3K and mTOR to TCR/CD28 and IL-2-driven cell cycle protein expression. To this aim, we analyzed A.E7 T cells because these cells produce suboptimal autocrine IL-2 amounts and are capable of CD3/CD28 and IL-2-driven proliferation (Fig. 1). Cells were stimulated with immobilized anti-CD3/CD28 mAbs or with IL-2 for 1, 3, and 5 days in the absence or presence of the PI3K and mTOR/GβL/raptor inhibitors (LY294002 and RAPA, respectively) (Fig. 7). Cells were lysed and proteins analyzed by Western blot with Abs specific for cyclin D3, cyclin E, and $p27^{Kip}$. Twenty-four hours after stimulation, cyclin D3 expression was induced, while $p27^{Kip}$ levels were decreased (Fig. 7*A*, day 1). At this time, cyclin E remained within background detection (data not shown). The addition of RAPA to

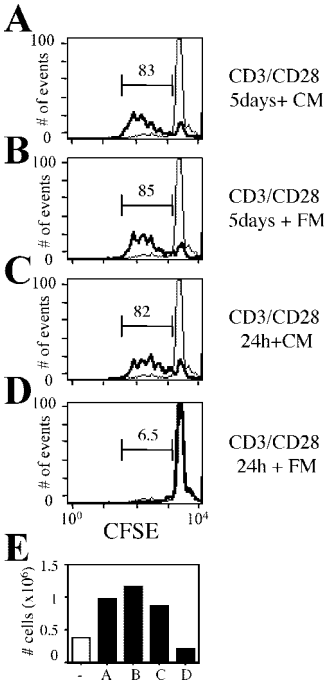

FIGURE 6. CD3, CD28, and IL-2 independently control proliferation of primary T cells. DO11.10 primary cells were left untreated (thin line histogram) or stimulated on immobilized anti-CD3 and anti-CD28 mAbs (thick line histogram) for 5 days (*A* and *B*) or for 24 h (*C* and *D*) and then cultured in their conditioned medium (CM) (*A* and *C*) or in fresh medium (FM) (*B* and *D*) for an additional 4 days. *B* and *D*, The conditioned medium was removed after 24 h and replenished with fresh medium. Representative FACS histograms of $CD4^+$ viable cells are depicted. *E*, The total number of trypan blue-negative viable cells is shown. One representative experiment of two independent determinations is reported.

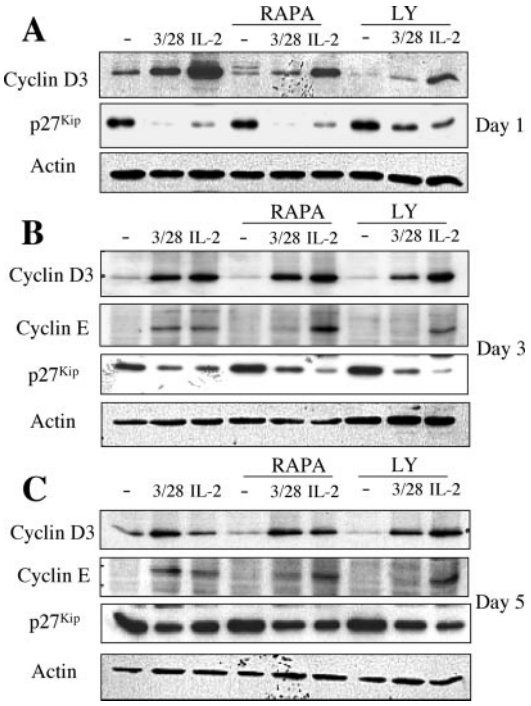

FIGURE 7. mTOR-dependent signaling is required for CD3/CD28-induced cyclin D3 and cyclin E expression. A.E7 cells were either left untreated (−) or stimulated with immobilized anti-CD3 and anti-CD28 mAbs (CD3/CD28) or IL-2 (10 IU/ml) in the absence or presence of RAPA or LY294002 (LY). *A–C*, After 1, 3, and 5 days, cells were recovered and protein extracts were analyzed by SDS-PAGE with anti-cyclin D3, anti-$p27^{Kip}$, anti-cyclin E, and anti-actin Abs as indicated. The experiment reported is representative of at least three independent determinations.

CD3/CD28- and IL-2-stimulated cells prevented the optimal induction of cyclin D3, whereas it allowed comparable down-regulation of $p27^{Kip}$ (Fig. 7*A*). In the presence of LY294002, both the up-regulation of cyclin D3 and the down-regulation of $p27^{Kip}$ were partially diminished. After 3 and 5 days of culture, increased expression of cyclin D3 and cyclin E and decreased levels of $p27^{Kip}$ were detected in both CD3/CD28- and IL-2-stimulated cells (Fig. 7, *B* and *C*). However, although the up-regulation of cyclin D3 and the down-regulation of $p27^{Kip}$ levels appeared to be insensitive to RAPA and LY294002 in both CD3/CD28- and IL-2-stimulated cells, the up-regulation of cyclin E induced by CD3/CD28, but not by IL-2, was completely abolished by the two drugs.

The role of the RAPA-sensitive, mTOR-dependent signaling event responsible for cyclin E expression in CD3/CD28-stimulated, but not in IL-2-stimulated, T cells was also analyzed in anergic A.E7 T cells (Fig. 8), which have severely impaired IL-2 secretion. As in the case of control cells, cyclin E expression was also induced in anergic cells upon CD3/CD28 and IL-2R engagement and was prevented by RAPA in CD3/CD28-stimulated cells, but not IL-2-stimulated cells (Fig. 8).

Together, these results indicate that TCR and CD28 mediate cyclin E expression and T cell proliferation independently from IL-2 via PI3K and mTOR-dependent signaling.

Discussion

These data presented demonstrate that the TCR, CD28, and IL-2 can independently control T cell proliferation via intracellular events requiring PI3K and mTOR.

The requirements for TCR, costimulatory, and cytokine receptor engagement during cell proliferation were previously analyzed and debated and more recently directly investigated in vivo (9, 13, 28–30). Although informative, these studies did not directly address the individual role of TCR, CD28, and IL-2. In this study, we took advantage of IL-2-sufficient and IL-2-deficient T cells, and dissected the need for TCR/CD28 and IL-2R occupancy, as well as for intracellular signals that are evoked by these receptors and are responsible for cell division. We found that a prolonged engagement of the TCR and CD28 bypasses the need for autocrine IL-2 secretion and sustains IL-2-independent cell proliferation. In contrast, a short (24-h) TCR/CD28 engagement is unable to sustain proliferation of T cells incapable of autocrine IL-2 secretion, but in

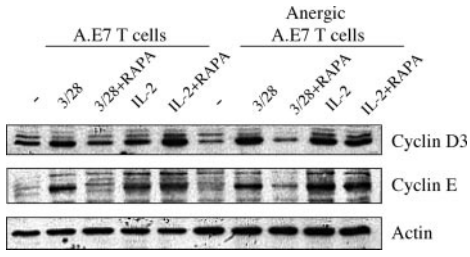

FIGURE 8. mTOR signaling is needed for CD3/CD28-induced cyclin D and cyclin E expression in anergic T cells. Control and anergic A.E7 cells were either left untreated (−) or stimulated with immobilized anti-CD3 and anti-CD28 mAbs (3/28) or IL-2 (10 IU/ml) in the absence or in the presence of RAPA. After 4 days, the cells were recovered and protein extracts were analyzed by SDS-PAGE with anti-cyclin D3, anti-cyclin E, and anti-actin Abs. The experiment shown was repeated once with similar results.

accordance with previous reports (11, 12, 29) is sufficient to commit T cells to proliferate to autocrine IL-2. Thus, TCR and CD28 and IL-2 independently regulate $CD4^+$ T cell division, which is best induced upon optimal and sustained occupancy of either TCR/CD28 or the IL-2R.

These data reinforce the idea that rather than being programmed to divide, $CD4^+$ T lymphocytes require a prolonged engagement of surface receptors, such as the TCR and CD28 or the IL-2R, to remain in the cell cycle. Benoist and colleagues (9) recently reported that the optimal proliferation of Ag-specific $CD4^+$ T cells in vivo is obtained if Ag persists throughout T cell expansion. Together with CD28 or additional costimulatory molecules, such as members of TNFR superfamily (41–43), TCR-derived signals might directly support cell cycle progression or sustain autocrine IL-2 secretion and by that proliferation. In either case the prolonged occupancy of these surface receptors is likely to develop sequential waves of intracellular signaling events able to sustain optimal cell division. This effect was originally proven to occur in response to growth factors such as PDGF (44, 45), and recently proposed for IL-2 and IL-7 (46).

The finding that both TCR/CD28 and IL-2/IL-2R independently control T cell proliferation could have been predicted by the fact that they activate a number of common intracellular signaling pathways. We found that PI3K and mTOR, known to regulate lymphocyte activation, growth, and proliferation (38, 39, 47), controlled, although to a different extent, CD3/CD28-driven as well as IL-2-driven proliferation. Indeed, either LY294002 or RAPA prevented CD3/CD28-induced proliferation of T cells capable of limited autocrine IL-2 production (A.E7 T cells and IL-$2^{-/-}$ DO11.10 T cells). This finding indicates that the activity of both PI3K and of the RAPA-sensitive mTOR/GβL/raptor complex is necessary to sustain TCR/CD28-driven, IL-2-independent cell proliferation. In contrast, neither LY294002 nor RAPA completely abolished CD3/CD28-induced proliferation of T cells capable of autocrine IL-2 secretion (DO11.10 T cell lines and primary DO11.10 T cells) or proliferation induced by optimal amounts of rIL-2 (A.E7 T cells). This indicates that although TCR/CD28-driven, IL-2-independent T cell proliferation relies on both PI3K and mTOR signaling, IL-2-induced proliferation is independently regulated by PI3K and mTOR (see schematic Fig. 9).

The observation that LY294002 inhibited CD3/CD28-induced proliferation of A.E7 T cells is in apparent contrast with the finding that $CD4^+$ T cells expressing a mutant CD28 receptor incapable of PI3K recruitment had normal proliferative responses (48, 49). It should be noted, however, that these T cells were capable of optimal autocrine IL-2 secretion, and thus proliferation of the cells most likely reflected cell division in response to IL-2, which in our hands is less susceptible to LY294002 than CD3/CD28-driven cell division. Also, the finding that RAPA prevented CD3/CD28-driven proliferation was unexpected as mTOR was originally described as a downstream effector of growth factor and cytokine receptors (39) and was shown to regulate IL-2-induced Cdk2 and Cdc2 kinase activation (50) and $p27^{Kip}$ down-regulation (2). More recent evidence, however, proposed mTOR as a target of CD28-dependent signaling (24, 51). Accordingly, we found that the engagement of TCR and CD28 induces the rapid and RAPA-sensitive phosphorylation of $p70^{S6k}$ and 4EBP-1, which are known substrates of the mTOR/GβL/raptor complex (35–37). In A.E7 T cells, the CD3/CD28-induced phosphorylation of $p70^{S6k}$ and 4EBP-1 as well the phosphorylation of the Akt kinase were sensitive to LY294002, indicating that the TCR and CD28 activate mTOR via PI3K/Akt (S. Colombetti, V. Basso, S. Caserta, A. Conti, M. Alessio, D. L. Mueller, and A. Mondino, submitted for publication). In nonlymphoid cells, mTOR complex with GβL and raptor, which is sensitive to RAPA complex (35–37), but also binds to rictor (52). The mTOR/rictor complex is insensitive to RAPA and regulates the organization of the actin cytoskeleton (52). Whether such complex is present in T cells, and whether TCR/CD28 (or IL-2) controls it, remains to be determined.

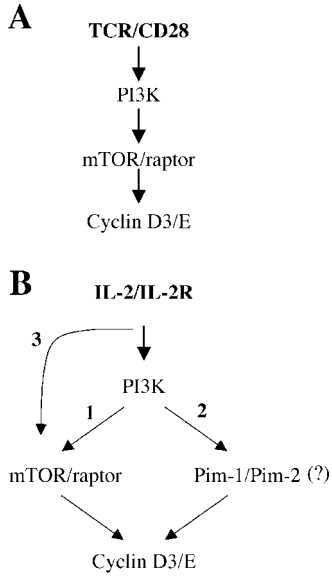

FIGURE 9. Pathways leading to T cell proliferation. *A*, Schematic representation of the intracellular events induced by the engagement of the TCR and CD28 or by the IL-2R leading to cell division. *B*, The numbers labeled refer to the possible pathways: 1, PI3K-dependent, mTOR/raptor-dependent; 2, PI3K-dependent, mTOR/raptor-independent; 3, PI3K-independent, mTOR/raptor-dependent. Pathways are induced by the optimal occupancy of the IL-2R and revealed by the selective sensitivity to the PI3K and mTOR/raptor inhibitors.

Although mTOR activity was necessary for TCR/CD28-driven proliferation, it was only transiently needed in the presence of optimal IL-2 amounts and for proliferation of cells capable of autocrine IL-2 secretion. This finding seems inconsistent with the proposed role for mTOR in T cell proliferation (47). Notably, however, most of the experiments reporting the anti-proliferative activity of RAPA measured proliferation by standard [^{3}H]thymidine incorporation assays at 48–72 h of culture. Using this assay (data not shown) and analyzing the CFSE dilution profile of the cells by 72 h of culture, we also found that T cells failed to proliferate at this early time. However, by 4 days of culture cells started to proliferate and by later times showed a CFSE content comparable to the one obtained in control cultures. Thus, rather than blocking IL-2-driven cell division, RAPA possibly only delays cell cycle entry as suggested by Terada et al. (53), and once the cells start to divide, they do so via a RAPA-insensitive pathway as observed for CD8 T cell clones (54, 55). In addition to mTOR, also PI3K appeared to be needed for cell cycle entry, as LY294002 prevented IL-2-driven cell proliferation in the first 3 days of culture (data not shown). This result indicates that PI3K might directly regulate mTOR function as proposed elsewhere (26) or that both PI3K and mTOR-dependent signaling are needed to enter the cell cycle.

In our experiments, RAPA and LY294002 prevented the optimal early expression of cyclin D3 induced by CD3/CD28 or IL-2. $p27^{Kip}$ down-regulation instead appeared to be insensitive to RAPA and rely on PI3K and MAPK activities (Fig. 7 and data not shown, respectively) as also reported elsewhere (23, 55, 56). Thus, the early inhibition of cyclin D3 expression possibly explains the failure of T cells to proliferate to CD3/CD28 and IL-2 by day 3 in

the presence of the drugs. By 72 h of activation (day 3), the intracellular signaling initiated by TCR/CD28 and IL-2R and controlling cell cycle protein expression appear to have diverged. Although the levels of cyclin D3, cyclin E, and $p27^{Kip}$ were comparable in either CD3/CD28- or IL-2-stimulated T cells, PI3K and mTOR activities were differentially required. Both RAPA and LY294002 inhibited cyclin E expression induced in response to CD3/CD28 activation. In contrast, RAPA and LY294002 had no and partial inhibitory effect on cyclin E induction in response to IL-2. Accordingly, although CD3/CD28-stimulated T cells failed to divide in the presence of RAPA (or LY294002), they proliferated to IL-2 in the presence of the drug, provided that PI3K was active. It is possible that CD3/CD28 activation drives cyclin E expression through the proposed PI3K/mTOR/E2F pathway (26, 57). Instead, IL-2 possibly regulates cell cycle protein expression and T cell proliferation by parallel pathways, possibly involving the RAPA-insensitive mTOR/rictor complex (52), the RAPA-insensitive E2F activation pathway (26), or pathways parallel to the conventional E2F-driven G_1-to-S phase transition (58, 59) (model in Fig. 9). In this respect, Thompson and colleagues (60) recently reported that the Pim-1 and Pim-2 kinases lead to RAPA-resistant cell division. It is tempting to speculate that the expression of Pim-1 and Pim-2, and to some extent cyclin E, is controlled by PI3K and that the mTOR and Pim pathways synergize to drive IL-2-dependent T cell expansion.

Our findings were primarily obtained using T cell clones, and thus might be more relevant to the expansion of memory cells. However, primary T cells also benefited from the synergy of CD3-, CD28-, and IL-2-driven proliferation and showed a comparable sensitivity to PI3K and mTOR inhibition. Thus, together our data support the idea that clonal expansion in vivo is independently determined by the TCR/CD28 engagement and by IL-2 (15–17) and possibly shaped by several additional factors. These factors include the persistence of Ag, the local autocrine and paracrine growth factor production, and the presence of additional costimulatory signals that are able to overcome the requirement for prolonged TCR/CD28- or IL-2-driven signals. As susceptibility or resistance to a given drug might depend on the signals available to activated T cells at any give time, it is foreseeable that a different combination of inhibitors might have to be defined to prevent lymphocyte expansion in specific clinical settings.

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Disclosures

The authors have no financial conflict of interest.

References

1. Minami, Y., T. Kono, T. Miyazaki, and T. Taniguchi. 1993. The IL-2 receptor complex: its structure, function, and target genes. *Annu. Rev. Immunol.* 11: 245–268.
2. Nourse, J., E. Firpo, W. M. Flanagan, S. Coats, K. Polyak, M. H. Lee, J. Massague, G. R. Crabtree, and J. M. Roberts. 1994. Interleukin-2-mediated elimination of the $p27^{kip1}$ cyclin-dependent kinase inhibitor prevented by rapamycin. *Nature* 372: 570–573.
3. Brennan, P., J. W. Babbage, B. M. Burgering, B. Groner, K. Reif, and D. A. Cantrell. 1997. Phosphatidylinositol 3-kinase couples the interleukin-2 receptor to the cell cycle regulator E2F. *Immunity* 7: 679–689.
4. Appleman, L. J., A. Berezovskaya, I. Grass, and V. A. Boussiotis. 2000. CD28 costimulation mediates T cell expansion via IL-2-independent and IL-2-dependent regulation of cell cycle progression. *J. Immunol.* 164: 144–151.
5. Bevan, M. J., and P. J. Fink. 2001. The CD8 response on autopilot. *Nat. Immunol.* 2: 381–382.
6. Jenkins, M. K., A. Khoruts, E. Ingulli, D. L. Mueller, S. J. McSorley, R. L. Reinhardt, A. Itano, and K. A. Pape. 2001. In vivo activation of antigen-specific CD4 T cells. *Annu. Rev. Immunol.* 19: 23–45.
7. Storni, T., C. Ruedl, W. A. Renner, and M. F. Bachmann. 2003. Innate immunity together with duration of antigen persistence regulate effector T cell induction. *J. Immunol.* 171: 795–801.
8. Croft, M. 2003. Co-stimulatory members of the TNFR family: keys to effective T-cell immunity? *Nat. Rev. Immunol.* 3: 609–620.
9. Obst, R., H. M. van Santen, D. Mathis, and C. Benoist. 2005. Antigen persistence is required throughout the expansion phase of a $CD4^+$ T cell response. *J. Exp. Med.* 201: 1555–1565.
10. Wells, A. D., H. Gudmundsdottir, and L. A. Turka. 1997. Following the fate of individual T cells throughout activation and clonal expansion: signals from T cell receptor and CD28 differentially regulate the induction and duration of a proliferative response. *J. Clin. Invest.* 100: 3173–3183.
11. Iezzi, G., K. Karjalainen, and A. Lanzavecchia. 1998. The duration of antigenic stimulation determines the fate of naive and effector T cells. *Immunity* 8: 89–95.
12. Gett, A. V., and P. D. Hodgkin. 2000. A cellular calculus for signal integration by T cells. *Nat. Immunol.* 1: 239–244.
13. Schrum, A. G., and L. A. Turka. 2002. The proliferative capacity of individual naive $CD4^+$ T cells is amplified by prolonged T cell antigen receptor triggering. *J. Exp. Med.* 196: 793–803.
14. Bonnevier, J. L., and D. L. Mueller. 2002. Cutting edge: B7/CD28 interactions regulate cell cycle progression independent of the strength of TCR signaling. *J. Immunol.* 169: 6659–6663.
15. Razi-Wolf, Z., G. A. Hollander, and H. Reiser. 1996. Activation of $CD4^+$ T lymphocytes form interleukin 2-deficient mice by costimulatory B7 molecules. *Proc. Natl. Acad. Sci. USA* 93: 2903–2908.
16. Boulougouris, G., J. D. McLeod, Y. I. Patel, C. N. Ellwood, L. S. Walker, and D. M. Sansom. 1999. IL-2-independent activation and proliferation in human T cells induced by CD28. *J. Immunol.* 163: 1809–1816.
17. Khoruts, A., A. Mondino, K. A. Pape, S. L. Reiner, and M. K. Jenkins. 1998. A natural immunological adjuvant enhances T cell clonal expansion through a CD28-dependent, interleukin (IL)-2-independent mechanism. *J. Exp. Med.* 187: 225–236.
18. Schorle, H., T. Holtschke, T. Hunig, A. Schimpl, and I. Horak. 1991. Development and function of T cells in mice rendered interleukin-2 deficient by gene targeting. *Nature* 352: 621–624.
19. Shahinian, A., K. Pfeffer, K. P. Lee, T. M. Kundig, K. Kishihara, A. Wakeham, K. Kawai, P. S. Ohashi, C. B. Thompson, and T. W. Mak. 1993. Differential T cell costimulatory requirements in CD28-deficient mice. *Science* 261: 609–612.
20. Tivol, E. A., F. Borriello, A. N. Schweitzer, W. P. Lynch, J. A. Bluestone, and A. H. Sharpe. 1995. Loss of CTLA-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction, revealing a critical negative regulatory role of CTLA-4. *Immunity* 3: 541–547.
21. Waterhouse, P., J. M. Penninger, E. Timms, A. Wakeham, A. Shahinian, K. P. Lee, C. B. Thompson, H. Griesser, and T. W. Mak. 1995. Lymphoproliferative disorders with early lethality in mice deficient in Ctla-4. *Science* 270: 985–988.
22. Boonen, G. J., A. M. van Dijk, L. F. Verdonck, R. A. van Lier, G. Rijksen, and R. H. Medema. 1999. CD28 induces cell cycle progression by IL-2-independent down-regulation of $p27^{kip1}$ expression in human peripheral T lymphocytes. *Eur. J. Immunol.* 29: 789–798.
23. Appleman, L. J., A. A. van Puijenbroek, K. M. Shu, L. M. Nadler, and V. A. Boussiotis. 2002. CD28 costimulation mediates down-regulation of $p27^{kip1}$ and cell cycle progression by activation of the PI3K/PKB signaling pathway in primary human T cells. *J. Immunol.* 168: 2729–2736.
24. Pai, S. Y., V. Calvo, M. Wood, and B. E. Bierer. 1994. Cross-linking CD28 leads to activation of 70-kDa S6 kinase. *Eur. J. Immunol.* 24: 2364–2368.
25. Parry, R. V., K. Reif, G. Smith, D. M. Sansom, B. A. Hemmings, and S. G. Ward. 1997. Ligation of the T cell co-stimulatory receptor CD28 activates the serine-threonine protein kinase protein kinase B. *Eur. J. Immunol.* 27: 2495–2501.
26. Brennan, P., J. W. Babbage, G. Thomas, and D. Cantrell. 1999. $p70^{s6k}$ integrates phosphatidylinositol 3-kinase and rapamycin-regulated signals for E2F regulation in T lymphocytes. *Mol. Cell. Biol.* 19: 4729–4738.
27. Colombetti, S., F. Benigni, V. Basso, and A. Mondino. 2002. Clonal anergy is maintained independently of T cell proliferation. *J. Immunol.* 169: 6178–6186.
28. Lee, W. T., G. Pasos, L. Cecchini, and J. N. Mittler. 2002. Continued antigen stimulation is not required during $CD4^+$ T cell clonal expansion. *J. Immunol.* 168: 1682–1689.
29. Bajénoff, M., O. Wurtz, and S. Guerder. 2002. Repeated antigen exposure is necessary for the differentiation, but not the initial proliferation, of naive $CD4^+$ T cells. *J. Immunol.* 168: 1723–1729.
30. Gett, A. V., F. Sallusto, A. Lanzavecchia, and J. Geginat. 2003. T cell fitness determined by signal strength. *Nat. Immunol.* 4: 355–360.
31. Hecht, T. T., D. L. Longo, and L. A. Matis. 1983. The relationship between immune interferon production and proliferation in antigen-specific, MHC-restricted T cell lines and clones. *J. Immunol.* 131: 1049–1055.
32. DeSilva, D. R., K. B. Urdahl, and M. K. Jenkins. 1991. Clonal anergy is induced in vitro by T cell receptor occupancy in the absence of proliferation. *J. Immunol.* 147: 3261–3267.
33. Leo, O., M. Foo, D. H. Sachs, L. E. Samelson, and J. A. Bluestone. 1987. Identification of a monoclonal antibody specific for a murine T3 polypeptide. *Proc. Natl. Acad. Sci. USA* 84: 1374–1378.
34. Lyons, A. B., and C. R. Parish. 1994. Determination of lymphocyte division by flow cytometry. *J. Immunol. Methods* 171: 131–137.
35. Kim, D. H., D. D. Sarbassov, S. M. Ali, J. E. King, R. R. Latek, H. Erdjument-Bromage, P. Tempst, and D. M. Sabatini. 2002. mTOR interacts with raptor to form a nutrient-sensitive complex that signals to the cell growth machinery. *Cell* 110: 163–175.

36. Hara, K., Y. Maruki, X. Long, K. Yoshino, N. Oshiro, S. Hidayat, C. Tokunaga, J. Avruch, and K. Yonezawa. 2002. Raptor, a binding partner of target of rapamycin (TOR), mediates TOR action. *Cell* 110: 177–189.
37. Kim, D. H., D. D. Sarbassov, S. M. Ali, R. R. Latek, K. V. Guntur, H. Erdjument-Bromage, P. Tempst, and D. M. Sabatini. 2003. GβL, a positive regulator of the rapamycin-sensitive pathway required for the nutrient-sensitive interaction between raptor and mTOR. *Mol. Cell* 11: 895–904.
38. Kane, L. P., and A. Weiss. 2003. The PI-3 kinase/Akt pathway and T cell activation: pleiotropic pathways downstream of PIP3. *Immunol. Rev.* 192: 7–20.
39. Hay, N., and N. Sonenberg. 2004. Upstream and downstream of mTOR. *Genes Dev.* 18: 1926–1945.
40. Hleb, M., S. Murphy, E. F. Wagner, N. N. Hanna, N. Sharma, J. Park, X. C. Li, T. B. Strom, J. F. Padbury, Y. T. Tseng, and S. Sharma. 2004. Evidence for cyclin D3 as a novel target of rapamycin in human T lymphocytes. *J. Biol. Chem.* 279: 31948–31955.
41. Gramaglia, I., A. D. Weinberg, M. Lemon, and M. Croft. 1998. OX-40 ligand: a potent costimulatory molecule for sustaining primary CD4 T cell responses. *J. Immunol.* 161: 6510–6517.
42. Song, J., S. Salek-Ardakani, P. R. Rogers, M. Cheng, L. Van Parijs, and M. Croft. 2004. The costimulation-regulated duration of PKB activation controls T cell longevity. *Nat. Immunol.* 5: 150–158.
43. Song, J., T. So, M. Cheng, X. Tang, and M. Croft. 2005. Sustained survivin expression from OX40 costimulatory signals drives T cell clonal expansion. *Immunity* 22: 621–631.
44. Jones, S. M., R. Klinghoffer, G. D. Prestwich, A. Toker, and A. Kazlauskas. 1999. PDGF induces an early and a late wave of PI3-kinase activity, and only the late wave is required for progression through G_1. *Curr. Biol.* 9: 512–521.
45. Jones, S. M., and A. Kazlauskas. 2001. Growth-factor-dependent mitogenesis requires two distinct phases of signalling. *Nat. Cell Biol.* 3: 165–172.
46. Lali, F. V., J. Crawley, D. A. McCulloch, and B. M. Foxwell. 2004. A late, prolonged activation of the phosphatidylinositol 3-kinase pathway is required for T cell proliferation. *J. Immunol.* 172: 3527–3534.
47. Abraham, R. T., and G. J. Wiederrecht. 1996. Immunopharmacology of rapamycin. *Annu. Rev. Immunol.* 14: 483–510.
48. Burr, J. S., N. D. Savage, G. E. Messah, S. L. Kimzey, A. S. Shaw, R. H. Arch, and J. M. Green. 2001. Cutting edge: distinct motifs within CD28 regulate T cell proliferation and induction of Bcl-x_L. *J. Immunol.* 166: 5331–5335.
49. Okkenhaug, K., L. Wu, K. M. Garza, J. La Rose, W. Khoo, B. Odermatt, T. W. Mak, P. S. Ohashi, and R. Rottapel. 2001. A point mutation in CD28 distinguishes proliferative signals from survival signals. *Nat. Immunol.* 2: 325–332.
50. Morice, W. G., G. Wiederrecht, G. J. Brunn, J. J. Siekierka, and R. T. Abraham. 1993. Rapamycin inhibition of interleukin-2-dependent $p33^{cdk2}$ and $p34^{cdc2}$ kinase activation in T lymphocytes. *J. Biol. Chem.* 268: 22737–22745.
51. Wu, L. X., J. La Rose, L. Chen, C. Neale, T. Mak, K. Okkenhaug, R. Wange, and R. Rottapel. 2005. CD28 regulates the translation of Bcl-x_L via the phosphatidylinositol 3-kinase/mammalian target of rapamycin pathway. *J. Immunol.* 174: 180–194.
52. Sarbassov, D. D., S. M. Ali, D. H. Kim, D. A. Guertin, R. R. Latek, H. Erdjument-Bromage, P. Tempst, and D. M. Sabatini. 2004. Rictor, a novel binding partner of mTOR, defines a rapamycin-insensitive and raptor-independent pathway that regulates the cytoskeleton. *Curr. Biol.* 14: 1296–1302.
53. Terada, N., K. Takase, P. Papst, A. C. Nairn, and E. W. Gelfand. 1995. Rapamycin inhibits ribosomal protein synthesis and induces G_1 prolongation in mitogen-activated T lymphocytes. *J. Immunol.* 155: 3418–3426.
54. Slavik, J. M., D. G. Lim, S. J. Burakoff, and D. A. Hafler. 2001. Uncoupling $p70^{s6}$ kinase activation and proliferation: rapamycin-resistant proliferation of human $CD8^+$ T lymphocytes. *J. Immunol.* 166: 3201–3209.
55. Slavik, J. M., D. G. Lim, S. J. Burakoff, and D. A. Hafler. 2004. Rapamycin-resistant proliferation of $CD8^+$ T cells correlates with $p27^{kip1}$ down-regulation and bcl-x_L induction, and is prevented by an inhibitor of phosphoinositide 3-kinase activity. *J. Biol. Chem.* 279: 910–919.
56. Takuwa, N., and Y. Takuwa. 1997. Ras activity late in G_1 phase required for $p27^{kip1}$ downregulation, passage through the restriction point, and entry into S phase in growth factor-stimulated NIH 3T3 fibroblasts. *Mol. Cell. Biol.* 17: 5348–5358.
57. Pajalunga, D., and M. Crescenzi. 2004. Regulation of cyclin E protein levels through E2F-mediated inhibition of degradation. *Cell Cycle* 3: 1572–1578.
58. Alevizopoulos, K., J. Vlach, S. Hennecke, and B. Amati. 1997. Cyclin E and c-Myc promote cell proliferation in the presence of $p16^{INK4a}$ and of hypophosphorylated retinoblastoma family proteins. *EMBO J.* 16: 5322–5333.
59. Lukas, J., T. Herzinger, K. Hansen, M. C. Moroni, D. Resnitzky, K. Helin, S. I. Reed, and J. Bartek. 1997. Cyclin E-induced S phase without activation of the pRb/E2F pathway. *Genes Dev.* 11: 1479–1492.
60. Fox, C. J., P. S. Hammerman, and C. B. Thompson. 2005. The Pim kinases control rapamycin-resistant T cell survival and activation. *J. Exp. Med.* 201: 259–266.