

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

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Abstract

A detailed understanding of the effects of costimulatory signals on primary T cell expansion has been limited by experimental approaches that measure the bulk response of a cell population, without distinguishing responses of individual cells. Here, we have labeled live T cells *in vitro* with a stable, fluorescent dye that segregates equally between daughter cells upon cell division, allowing the proliferative history of any T cell present or generated during a response to be monitored over time. This system permits simultaneous evaluation of T cell surface markers, allowing concomitant assessment of cellular activation and quantitative determination of T cell receptor (TCR) occupancy on individual cells. Through this approach, we find that TCR engagement primarily regulates the frequency of T cells that enter the proliferative pool, but has relatively little effect on the number of times these cells will ultimately divide. In contrast, CD28-costimulation regulates both the frequency of responding cells (particularly at sub-maximal levels of TCR engagement), and more prominently, the number of mitotic events that responding cells undergo. When CD28-stimulation is blocked, provision of IL-2 restores the frequency of responding cells and the normal pattern of mitotic progression, indicating that the other CD28-induced genes are not required for this effect. An unexpected finding was that even at maximal levels of TCR engagement and CD28-mediated costimulation, only 50–60% of the original T cells in culture can be induced to divide. The nondividing cells are heterogeneous for naive versus memory markers, suggesting a more complex relationship between expression of memory markers and the ability to be recruited into the dividing pool. From these studies, we conclude that a stringent checkpoint regulates the participation of activated T cells in clonal expansion, with TCR and CD28 signals having both overlapping and differential effects on the induction and maintenance of T cell responses. (*J. Clin. Invest.* 1997. 100: 3173–3183.) Key words: cell division • precursor frequency • costimulation • antigen receptor • interleukin-2

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Introduction

T cells which encounter specific antigen require additional signals in order to mount a functional immune response (1–3). Several molecules expressed on the surface of T cells have been shown to provide these additional, costimulatory signals when engaged (4), however, the CD28 glycoprotein appears to be the most prominent costimulatory ligand (5). A crucial role for CD28 in the costimulation of T cell responses *in vivo* has been revealed in several models of T cell-dependent immunity. Forced expression of B7-1 or B7-2, the natural ligands of CD28, can restore the immunogenicity and induce T cell-mediated rejection of many otherwise weakly immunogenic tumors (6). Conversely, abrogation of B7-CD28 interactions *in vivo* has been shown to block the development of several autoimmune diseases (7–9), and to induce donor-specific tolerance to allogeneic organ transplants (10–12).

The mechanism by which CD28 signals interact with antigen-specific T cell receptor (TCR)¹ signals has been defined further by measuring DNA synthesis or cytokine production by populations of T cells stimulated *in vitro*. CD28 costimulation leads to the induction or augmentation of proliferation and greatly enhanced cytokine secretion (13, 14), as assessed in studies of bulk populations of T cells. However, it remains undetermined as to how this effect is exerted at the level of individual cells. For example, it has been shown that costimulation lowers the threshold for TCR signals to initiate a response as defined by tritiated thymidine incorporation or cytokine secretion by a population of heterogeneous naive T cells (15). This important study, however, was limited by the inability to differentiate between population effects and single cell effects. For example, one means by which increased response of a population could be exerted is through increasing the frequency of responding T cells in the population, such that the more vigorous observed readout is a result of the recruitment of more precursor cells into the response. This would occur if CD28-mediated costimulation lowers the threshold for T cell signaling to induce an individual T cell to initiate its activation program. A second possibility is that costimulation accelerates and/or sustains the response of already-recruited T cells, which is reflected as a greater response by the population as a whole. These two possibilities are not mutually exclusive and could each contribute to the ability of CD28-costimulation to augment immune responses.

In an attempt to further understand how signals from TCR and CD28 cooperate to regulate T cell responses, we have characterized and used an experimental system in which individual T cells are tagged with a fluorescent dye that binds irre-

1. Abbreviations used in this paper: CFDASE, carboxy-fluorescein diacetate succinimidyl ester; CFSE, the deacetylated form of CFDASE; TCR, T cell receptor.

versibly to the cell surface. As cells divide, their fluorescence halves sequentially with each generation, allowing the proliferative history of any single T cell present or generated during a response to be monitored over time. This system also permits simultaneous evaluation of T cell surface markers, allowing concomitant assessment of cellular activation and quantitative determination of TCR occupancy. This approach has enabled us to analyze T cell responses to TCR- and CD28-mediated signals on a single cell level, and to determine the requirements for, and limitations of, the participation of individual T cells in clonal expansion.

Methods

Mice and antibodies. Splenocytes from female BALB/c mice (6–10 wk of age) or DO11.10 mice (16) (provided by D. Loh, Washington University, St. Louis, MO) were used for all experiments. Purified monoclonal antibodies against CD3 (145-2C11, University of California, Berkeley, CA; provided by J. Bluestone, University of Chicago, Chicago, IL) and CD28 (37.51; provided by J. Allison, University of California, Berkeley, CA), as well as the fusion protein CTLA4Ig (17) (provided by P. Linsley, Bristol-Myers Squibb Pharmaceuticals, Princeton, NJ) were used during *in vitro* culture. Purified, fluorochrome- or biotin-conjugated monoclonal antibodies against CD16/CD32 (F_c-Block), Thy1.2, $\alpha\beta$ TCR, and CD25 (PharMingen, San Diego, CA) were used during flow cytometric analysis. The DO11.10 clonotype-specific antibody KJ1-26 (16) (provided by D. Loh) was purified from ascites and conjugated with sulfo-NHS-biotin (Pierce Chemical Co., Rockford, IL).

Cell labeling and culture conditions. Fluorescent labeling of splenocytes was achieved as described (18). Briefly, spleens were harvested and single-cell suspensions were prepared with the aid of Nitex fabric (100 μ ; Tetko, Inc., Elmsford, NY). Erythrocytes were lysed by hypotonic shock, and splenocytes were washed and resuspended at a density of 2×10^7 cells per ml in PBS. An equal volume of 5 μ M carboxy-fluorescein diacetate succinimidyl ester (CFDASE; Molecular Probes, Inc., Eugene, OR) in PBS was added, and the cells were gently mixed for 5–10 min at room temperature. Unbound CFDASE, or the deacetylated form, CFSE, was quenched by the addition of an equal volume of FCS. Analysis of cells immediately following CFSE labeling indicates a labeling efficiency that exceeds 99%, and all cells remain labeled for at least a 10-d period during tissue culture.

The labeled cells were washed two times in PBS and resuspended at 1×10^6 cells per ml in RPMI 1640 medium containing 10% FCS (HyClone, Logan, UT), 25 mM Hepes, 2 mM L-glutamine, 100 IU/ml of penicillin, 100 μ g/ml streptomycin, and 5 μ M 2-mercaptoethanol. Labeled splenocytes were plated at 1×10^5 cells per well in round-bottom 96-well microtiter plates, and T cell activation was achieved by the addition of soluble anti-CD3 antibody. In addition, cultures received either soluble antibody against CD28 (2 μ g/ml), CTLA4Ig (10 μ g/ml), or control hamster or human Ig (2 or 10 μ g/ml, respectively; The Jackson Laboratory, Bar Harbor, ME). Some cultures also received recombinant murine IL-2 (5–20 U/ml; Genzyme Corp., Cambridge, MA).

Measurement of DNA synthesis. DNA synthesis by CFSE-labeled splenocytes was measured at various time points by the incorporation of [³H]thymidine (1 μ Ci per well; ICN Biomedicals, Inc., Irvine, CA) over an 8-h pulse period.

Flow cytometric analysis. At the time of harvest, CFSE-labeled splenocytes were washed in cold PBS containing 2% FCS and 0.01% sodium azide, and at least 2×10^5 cells per sample were stained with a combination of PE-conjugated monoclonal antibody specific for Thy1.2 and biotin-conjugated monoclonal antibody specific for CD25, followed by cy-chrome-conjugated streptavidin (PharMingen). Unlabeled antibodies against CD16 (Fc γ III) and CD32 (Fc γ II) were included in all samples to block binding of antibodies to cells expressing F_c receptors. The vital dye TOPRO-3 (Molecular Probes, Inc.)

was added to each sample (1 nM final concentration) before acquisition to distinguish live and dead cells. Four-color flow cytometry was performed on a Becton Dickinson Immunocytometry Systems (San Jose, CA) FACSCalibur[®] dual-laser cytometer using standard Cell Quest[™] acquisition/analysis and ModFit[™] software, and fluorescence compensation was achieved using the appropriate single fluorochrome-labeled samples. 20,000 events were collected, and absolute cell counts were determined by dividing the total events by the product of the flow rate (0.001 ml/s) and the elapsed time (in seconds). Analysis of CD25 expression and cell division (CFSE-fluorescence profile) was restricted to the Thy1.2⁺ subset of CFSE-labeled splenocytes.

Determination of mitotic events and precursor frequencies. Division peaks (as determined by CFSE-intensity) were labeled from 0 to *n*. Since a single T cell dividing *n* times will generate 2^{*n*} daughter cells, if the total number of T cells which have divided three times (*n* = 3) is eight, then exactly one precursor had to divide three times to generate these eight cells (2³ = 8). Making use of this mathematical relationship, the number of division precursors was extrapolated from the number of daughters under each division peak (see Fig. 3 for further details).

Quantitative flow cytometric assessment of TCR expression. The quantitative assessment of $\alpha\beta$ TCR expression has been described elsewhere (15). Briefly, CFSE-labeled splenocytes were harvested 24 h after stimulation and stained with PE-conjugated anti- $\alpha\beta$ TCR antibody paired with biotinylated anti-Thy1.2 antibody, followed by cy-chrome-conjugated streptavidin. The anti- $\alpha\beta$ TCR antibody is conjugated with a mean of 1 PE-molecule per antibody molecule. It therefore could be used to derive the mean number of bound antibody molecules (equal to the estimated number of T cell receptors) per T cell by comparison to a standard curve generated by PE-conjugated bead standards (Flow Cytometry Standards, Inc., San Juan, Puerto Rico). The estimated number of TCR on T cells from anti-CD3-stimulated cultures was then subtracted from the estimated number of TCR on unstimulated T cells to give the number of TCR engaged.

Results

Visualizing T cell division in cultures of activated, CFSE-labeled splenocytes. To follow the fate of individual T cells throughout activation and clonal expansion, we have employed the fluorescent dye carboxyfluorescein succinimidyl ester. The diacetate form of CFDASE is a nontoxic, nonfluorescent molecule that can passively diffuse into cells. Once inside the plasma membrane of live cells, cellular esterases cleave the acetyl groups from CFDASE to form the active fluorophore (CFSE) that absorbs and emits light at wavelengths characteristic to its fluorescein moiety. The highly amine-reactive succinimidyl ester then forms dye-protein adducts which are retained within or at the surface of the cell. The end result is the very bright and homogeneous labeling of cell populations (Fig. 1A) which is stable over a span of weeks (19). This labeling is uniform within the T cell compartment, as all Thy1.2⁺ cells fluoresce brightly (Fig. 1 and data not shown).

CFSE segregates equally between daughter cells upon cell division, resulting in the sequential halving of cellular fluorescence intensity with each successive generation (18). When analyzed by flow cytometry, this sequential halving of fluorescence is visualized as distinct peaks or populations of cells, and can be used to track cell division in populations of proliferating cells (Fig. 1, B–D). T cell division after polyclonal activation with soluble anti-CD3 begins at \sim 48 h after stimulation. There is marked asynchrony in the kinetics of cell division exhibited by this population, with some T cells having divided as many as eight times by 96 h after stimulation, while other T cells remain

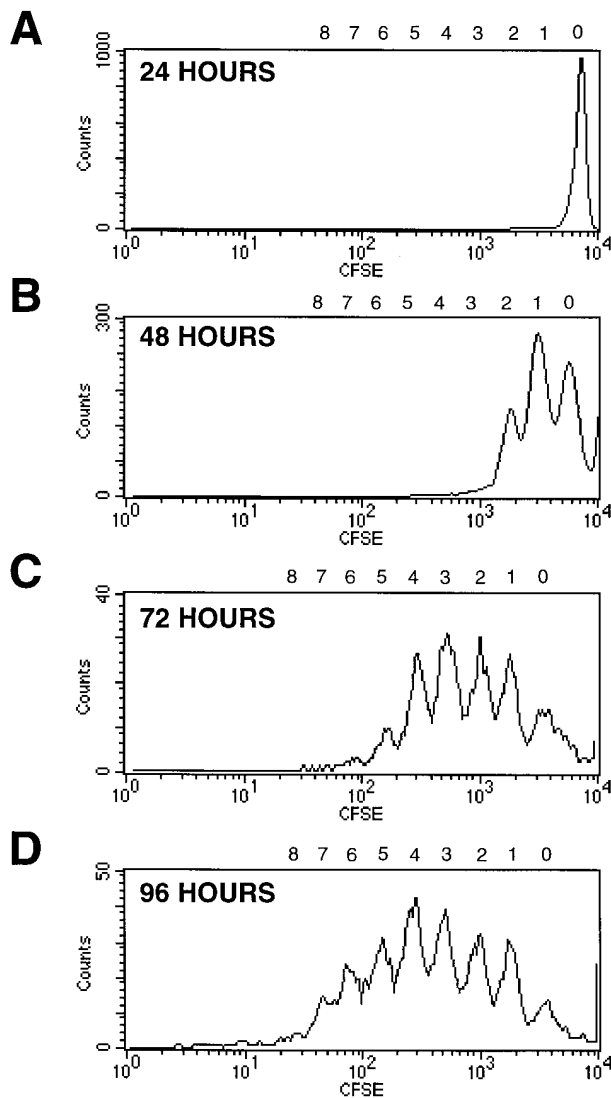


Figure 1. Kinetic analysis T cell division in populations of CFSE-labeled splenocytes. Splenocytes were labeled with CFSE and cultured in the presence of 1 $\mu\text{g/ml}$ soluble anti-CD3 for 24 (A), 48 (B), 72 (C), or 96 (D) h, at which time the cells from individual wells were harvested, stained with PE-conjugated anti-Thy1.2, and analyzed by flow cytometry. Histograms show the CFSE fluorescence profile of the live, Thy1.2⁺ subset of the CFSE-labeled splenocytes. The numbers appearing above each histogram (0–8) denote each division population, with the undivided T cells residing in the rightmost peak, and the T cells that have divided eight times residing in the leftmost peak. The experiment depicted here is representative of over 20 separate experiments.

undivided throughout this same time period. It should be noted that the undivided T cells continue to fluoresce brightly, confirming dye stability over the culture time period.

We have also observed that T cells retain their characteristic CFSE fluorescence for several days after their death. Dead T cells from anti-CD3 stimulated, CFSE-labeled splenocytes (Fig. 2 A) show a pattern of sequential halving of fluorescence intensity similar to that of live T cells from the same culture (Fig. 2 B). The fluorescence intensity of dead T cells from CFSE-labeled splenocytes does not decrease if anti-CD3 is omitted from the culture (Fig. 2 B), suggesting that this de-

crease in CFSE fluorescence is due to cell division occurring before death. Moreover, if cell division is blocked at the time of anti-CD3 stimulation by the addition of sodium azide, neither the live, nor the dead, T cells exhibit a decrease in CFSE fluorescence intensity over a 5-d period (data not shown). Finally, the binding of PE-conjugated anti-Thy1.2 to these dead T cells is specific because it can be effectively competed by unlabeled anti-Thy1.2 antibody, with identical kinetics as the competition for binding of PE-conjugated anti-Thy1.2 to live T cells (data not shown). Dead T cells from anti-CD3 stimulated cultures exhibit less distinct CFSE profiles than that of the live T cells (Fig. 2 B). This is due to a higher degree of variability in the CFSE fluorescence exhibited by dead cells which have divided a given number of times. The increased variability in fluorescence can be attributed to the higher degree of variability in cell size exhibited by dead cells. Nonetheless, accurate analysis of these CFSE profiles can still be achieved (particularly with concurrent use of ModFit software, not shown), and the degree of cell division having occurred in populations of dead T cells can still be assessed. In this way, the proliferative history of essentially every T cell present or generated during a short-term response can be assessed using CFSE.

Estimation of mitotic events and division precursor frequencies in proliferating T cell populations using CFSE. Flow cytometric analysis of the distinct division populations generated by cell division, as defined by CFSE fluorescence (Figs. 1 and 2), allows the total number of T cells (both live and dead) under each division peak to be determined. Given these values, the absolute number of mitotic events that have occurred in the T cell subset can now be estimated. The process of calculating mitotic events from the number of T cells under each division peak is illustrated in Fig. 3. An equation that gives the mitotic events (M) from the experimentally obtained values of the proportion of T cells under each division peak n (X_n), and the total T cell yield (T), is shown below:

$$M = \sum_{n=0}^8 \left(X_n(T) - \frac{X_n(T)}{2^n} \right).$$

As shown in Fig. 3, calculation of the total number of mitotic events involves extrapolation of precursor numbers for each division peak. A particularly important consequence of this precursor extrapolation is that the frequency of the original T cells which responded to a stimulus by proliferating can be determined directly from a complex mixture of cell populations; i.e., without subjecting the cells to limiting dilution at the beginning of culture.

The precision of this method is dependent on the ability to accurately enumerate the live and dead T cells present at any given time in culture. Accuracy of the mitotic event estimation can be assessed by comparing the number of precursor T cells predicted by the expression $X_n(T)/2^n$ with the known input of T cells at day 0. In the experiment illustrated in Fig. 3, the actual input was 11,040 T cells, therefore the figure of 10,052 precursor T cells, as predicted by extrapolation from the daughter cell division profile at day 3, is accurate within 9%. We have observed that an accurate (< 15% error) estimation of mitotic events can be achieved up to at least 72–96 h after stimulation with anti-CD3. After this time point, cell fragmentation in the terminal phases of apoptosis precludes accurate quantification of mitoses.

Relationship between the avidity of TCR engagement and T

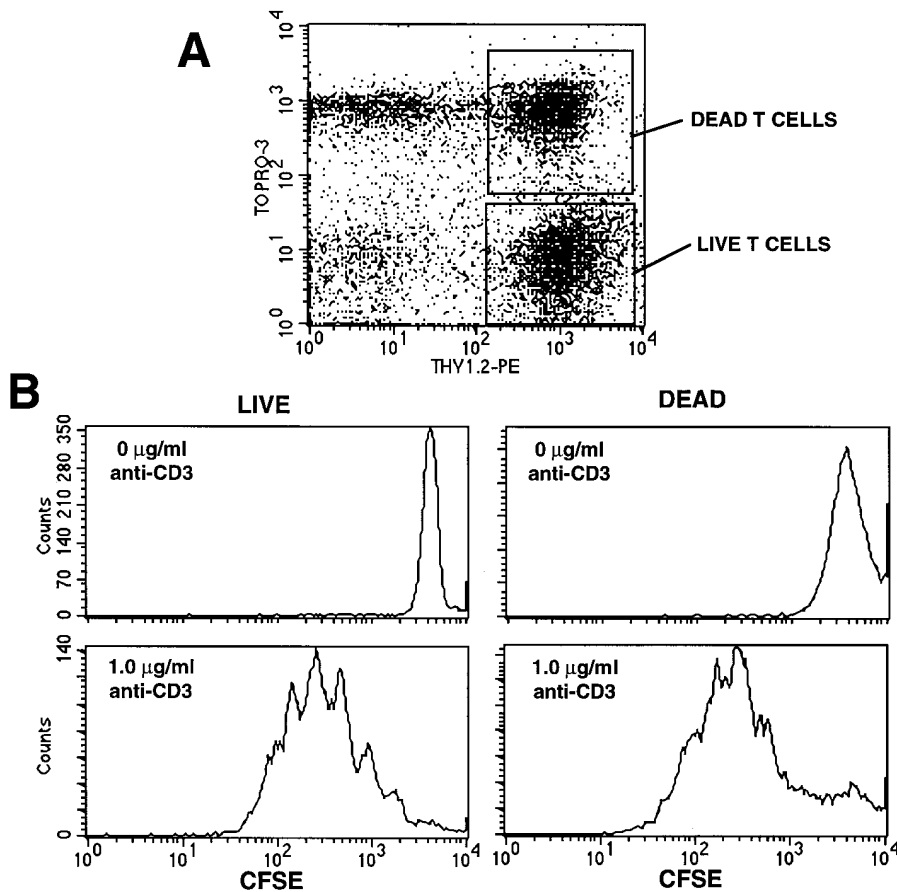


Figure 2. Dose response and proliferative history of both live and dead T cells. (A) Live and dead T cells were distinguished from other cell subsets within CFSE-labeled, anti-CD3-stimulated splenocytes by the combination of anti-Thy1.2 antibody and the vital dye TOPRO-3. (B) CFSE profiles of Thy1.2⁺/TOPRO-3⁻ (LIVE) and Thy1.2⁺/TOPRO-3⁺ (DEAD) CFSE-labeled splenocytes cultured for 72 h in the presence of 0, or 1.0 µg/ml soluble anti-CD3. The patterns of sequential fluorescence halving exhibited by live and dead T cells are quite similar, and it should be noted that the same markers which define the distinct CFSE fluorescence peaks in histograms of live T cells are used for the analysis of CFSE profiles in dead T cell populations.

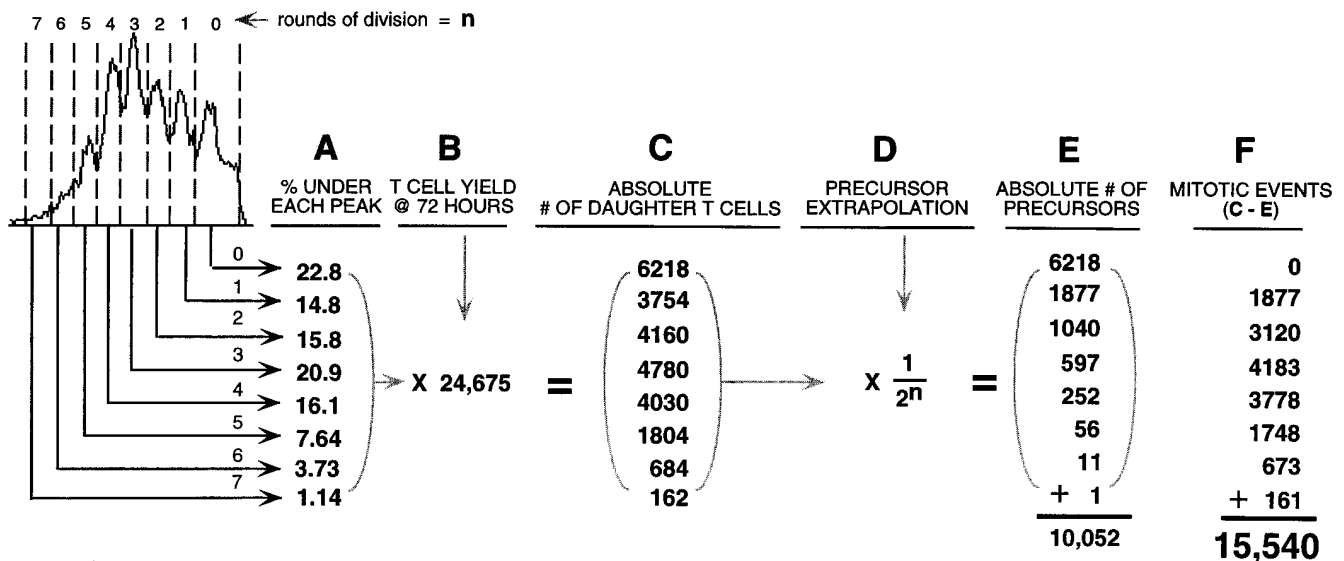


Figure 3. Quantitative estimation of mitotic events. CFSE-labeled splenocytes were cultured for 72 h in the presence of 18.5 µg/ml anti-CD3. From the experimentally determined values of the percentage of total Thy1.2⁺ events under each division peak *n* (A), and the absolute T cell count at the time of harvest (B), the absolute number of T cells (live and dead) that have divided *n* times can be calculated (C). The absolute number of original, or precursor, T cells required to have generated these daughters is extrapolated by dividing the number of daughters at *n* divisions by 2^{*n*} (D). The sum of the precursors from each division peak should equal the known input number of T cells at day 0 (E), and functions as an internal control for error in the determination of T cell yield. The proportion of the original T cell input that responded by dividing one or more times, or the division precursor frequency, can now be determined (in this experiment, 3,834 T cells out of the original 10,052 have divided at least once, while 6,218 never divided, giving a precursor frequency of 38%). The absolute number of mitotic events is derived by subtracting the number of division precursors from the number of daughters generated by each precursor population (C minus E). The sum of these events (F) represents the total number of cell divisions having occurred in the T cell subset by the time of harvest.

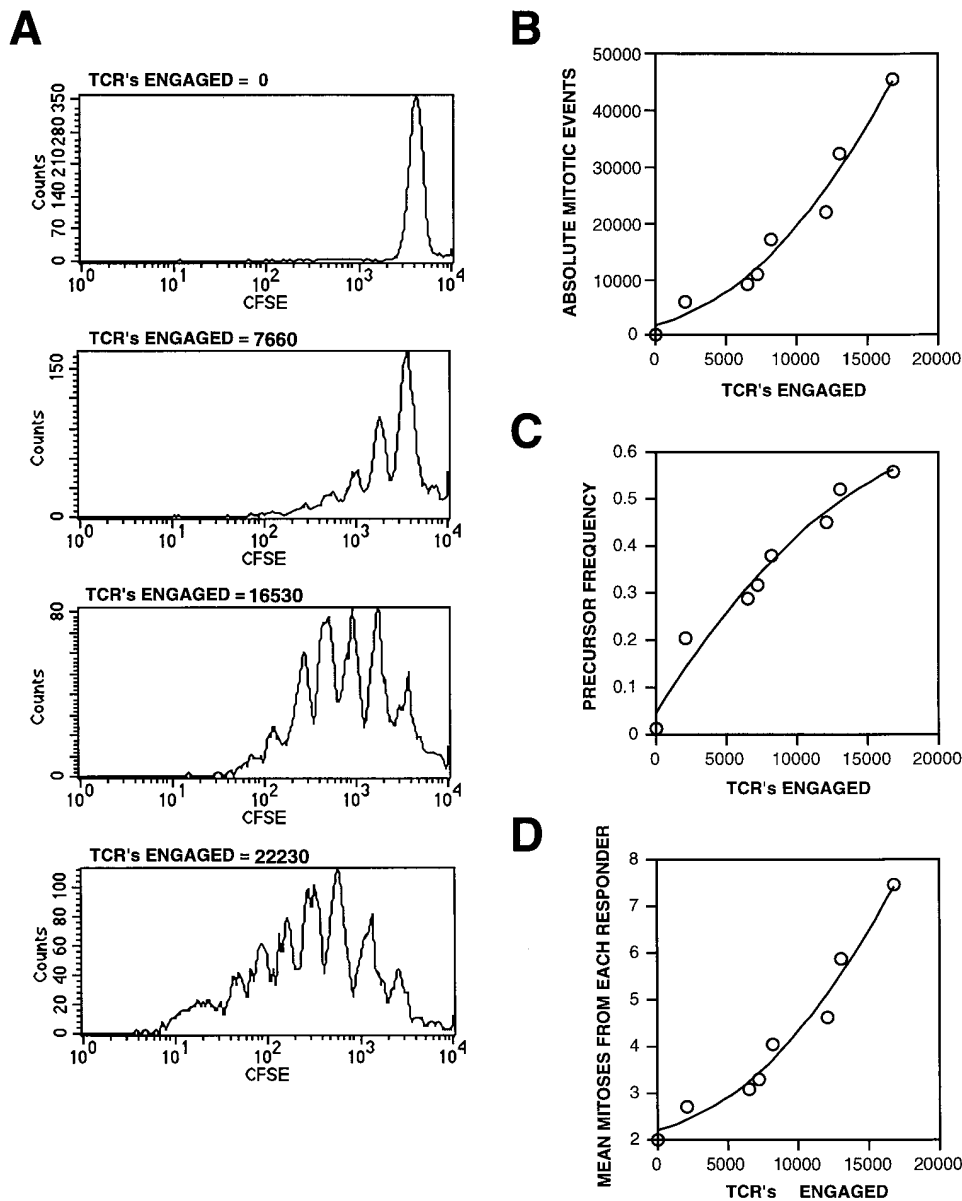


Figure 4. The avidity of TCR ligation controls both the frequency and the proliferative capacity of T cells that respond to stimulation by dividing. (A) Stimulation of CFSE-labeled splenocytes with 0, 0.01, 0.05, or 1.0 $\mu\text{g/ml}$ anti-CD3 resulted in the engagement of 0, 7660, 16530, and 22230 TCR, respectively. The CFSE fluorescence profiles of the T cells at each dose are plotted. (B–D) In a separate experiment, stimulation of CFSE-labeled splenocytes with 0, 0.00069, 0.0021, 0.0062, 0.0185, 0.0556, 0.167, or 0.5 $\mu\text{g/ml}$ anti-CD3 resulted in the engagement of 0, 2,094, 6,519, 7,224, 8,148, 12,093, 13,044, and 16,800 TCR, respectively. The average number of TCR on T cells from unstimulated splenocyte cultures ranged from 25,000 to 30,000. The absolute number of mitoses (B) and the precursor frequencies (C) at 96 h after stimulation were determined for each dose as described in Fig. 3, and are plotted against the number of TCR engaged 24 h after stimulation. The mean number of mitotic events generated by each responding precursor (D) was determined by dividing the number of mitoses at each dose by the corresponding number of precursors that responded to stimulation by dividing. Note that a non-zero mitoses per responder value is obtained under nonstimulatory conditions. This is because we consistently detect a very small population of responding T cells ($\sim 1\%$) in the absence of any overt stimulation. Second-order polynomial curves are shown fitted to the data points in B, C, and D.

cell division. A physiological consequence of the interaction of a T cell with antigen is the internalization of TCR-CD3 complexes from the cell surface (20). The degree of TCR internalization is dependent on the dose of TCR ligand (i.e., MHC-peptide complexes, superantigens, or antibodies directed against the TCR-CD3 complex), and T cell activation appears to be linked quantitatively to the number of TCR internalized (15). Therefore, the downregulation of TCR from the cell surface in response to antigen exposure can serve as a biologically relevant, quantitative indicator of TCR occupancy.

A dose-dependent relationship exists between the number of TCR engaged within 24 h and the cell division exhibited over 3–5 d by splenic T cells stimulated with anti-CD3 (Fig. 4A). Specifically, a shallow rise in the number of mitoses is observed as the number of TCRs engaged approaches 7,500, followed by a cooperative increase in the number of mitotic events occurring with additional TCR ligation (Fig. 4B). This relationship between the absolute number of cell divisions and

the number of TCR's engaged has two components. First, an increase in the frequency of T cells which respond to stimulation by dividing is observed as the avidity of TCR engagement increases (Fig. 4C). Specifically, the responder frequency appears to increase linearly from the lowest TCR engagement measured through $\sim 12,000$ TCR's engaged, and begins to saturate with the engagement of $\sim 15,000$ TCRs. Secondly, the proliferative capacity of each responder increases as a function of the number of TCR's engaged, such that T cells that respond to a relatively high level of antigen receptor ligation progress through more cell divisions than T cells which respond to low-level receptor stimulation (Fig. 4D). Because the absolute number of mitotic events is a product of two components (the frequency and the proliferative capacity of the responding T cells) which vary as a linear function of the number of TCR's engaged, a cooperative, geometric increase in mitoses with increasing TCR engagement is observed (Fig. 4B).

Kinetic studies reveal that the vast majority (i.e., $> 90\%$) of

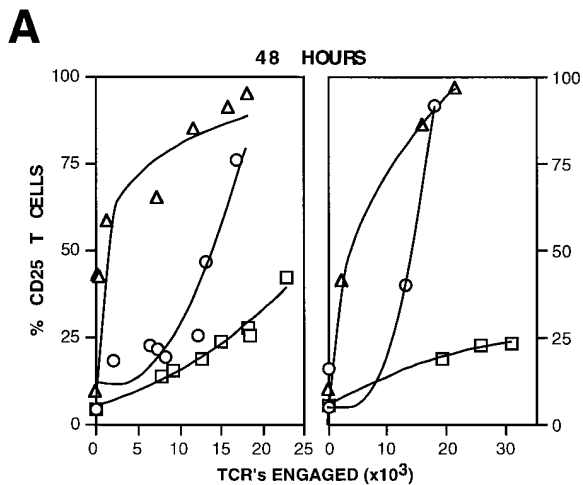
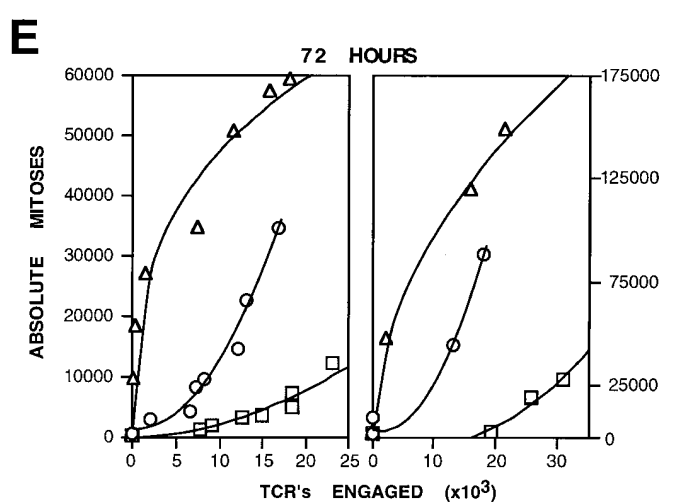
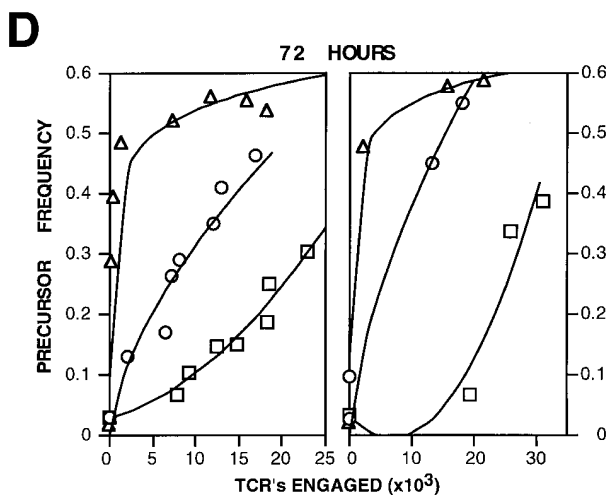
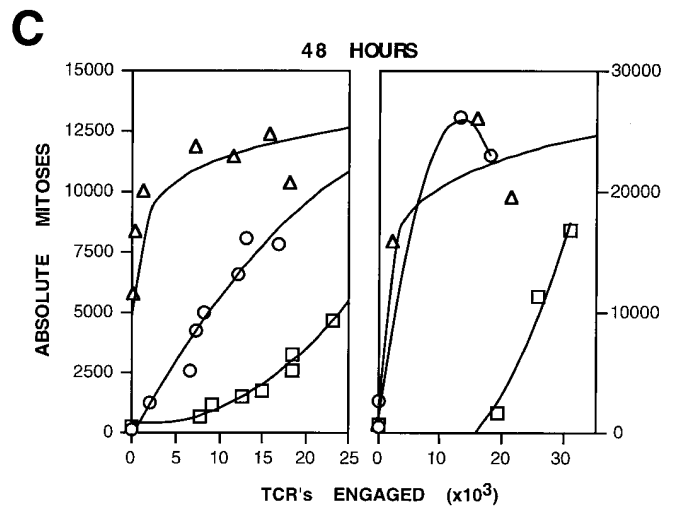
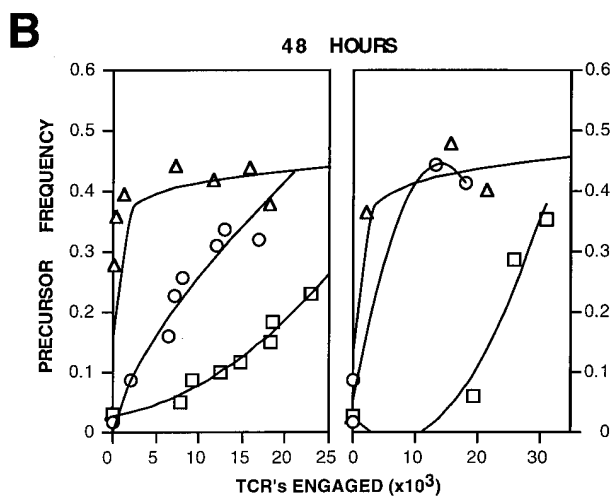


Figure 5. CD28 signals synergize with suboptimal TCR signals to enhance the participation of T cells in clonal expansion. The data shown in *A–E* represent two separate experiments in which CFSE-labeled splenocytes were stimulated by the addition of either 0, 0.00069, 0.0021, 0.0062, 0.0185, 0.0556, 0.167, or 0.5 $\mu\text{g/ml}$ anti-CD28 (*left panels*), or 0, 0.01, 0.05, or 1.0 $\mu\text{g/ml}$ anti-CD3 (*right panels*). The cells were then cultured in the presence of either anti-CD28 (*triangles*), CTLA4Ig (*squares*) or control hamster or human Ig (*circles*). Expression of CD25 (*A*), precursor frequencies (*B* and *D*) and absolute mitotic events (*C* and *E*) were determined for each culture condition at 48 h (*A*, *B*, and *C*) and 72 h (*D* and *E*) after stimulation. These values are shown plotted against the corresponding number of TCR engaged at 24 h after stimulation. The average number of TCR on T cells from unstimulated splenocyte cultures ranged from 25,000 to 30,000. The data shown here are representative of five separate experiments in which detailed mitotic and precursor frequency analyses have been performed.



T cells that respond to polyclonal stimulation by dividing have undergone at least one cell division by 72 h (data not shown). Thus, it is somewhat surprising that the frequency of T cells that respond by dividing consistently saturates at $\sim 60\%$, even when measured as late as 4 d after stimulation. This means that $\sim 40\%$ of the T cells fail to respond by proliferating. It is important to note that this limit in the frequency of responders is

not due to the failure to activate a portion of the original T cells. Up to 95% of the T cells show the hallmarks of early activation, such as CD25 expression (Fig. 5 *A*, *open circles*) and CD69 expression (data not shown).

Effect of CD28 costimulation on T cell responsiveness and cell division. In the experiments described above, accessory cells present in the bulk spleen cell preparations serve to cross-

link anti-CD3 antibodies bound to T cells, and provide a physiological level of CD28 ligation via the expression of B7 molecules (21). In order to differentiate the effects of TCR signals from costimulatory signals on the dynamics of T cell division, we sought to modulate the costimulatory environment facing T cells during anti-CD3 stimulation. Agonistic anti-CD28 antibody was used to provide maximal CD28 signaling, whereas CTLA4Ig was used to block endogenous B7-CD28 interactions and effectively uncouple TCR signaling from CD28 costimulatory signals.

The first point to be made is that physiological CD28 engagement is, in one sense, limiting in that further CD28 ligation with mAb increases the responsiveness of T cells to submaximal TCR ligation. The proportion of T cells which express CD25 (Fig. 5 A) and which go on to divide under physiological CD28 costimulation (Fig. 5 B) becomes maximal after the engagement of $\sim 18,000$ TCR. In the presence of maximal CD28 ligation, $< 1,000$ TCR must be engaged in order to attain optimal CD25 expression (Fig. 5 A) and maximal participation in clonal expansion (Fig. 5 B). The same trends are reflected in the absolute number of mitotic events exhibited by T cells stimulated for 48 h under varying costimulatory conditions (Fig. 5 C). It should also be noted that although signaling through CD28 can drastically increase the sensitivity of T cells to TCR ligation, even with maximal CD28 stimulation the proportion of T cells that respond by dividing still never exceeds 60%.

Under conditions of CD28 blockade (i.e., in the presence of CTLA4Ig), T cells are relatively insensitive to TCR ligation, as measured by the proportion of the original T cells that respond to a given level of stimulation by either expressing IL-2 receptor (CD25), or by dividing (Fig. 5). Under these conditions, the engagement of nearly every available TCR (from 25,000 to 30,000 per unstimulated splenic T cell; data not shown) is required in order to induce CD25 expression (Fig. 5 A, *squares*) and to reach a responder frequency at the 48 h time point that approaches that achieved in cultures stimulated in the presence of CD28 costimulation (Fig. 5 B).

A comparison of the responses seen at 48 and 72 h, during which time interval a majority of the total mitotic events appear to occur (Fig. 5, compare panels B and D, C and E), provides additional insights. First, in both the experiments shown, the proportion of the original T cells that go on to divide in cultures stimulated in the presence of CD28 costimulation increases from 40% at 48 h to $\sim 55\%$ by 72 h (Fig. 5 D). In contrast, essentially no newly-dividing precursor T cells can be observed during this same period in cultures stimulated in the absence of costimulation (Fig. 5 D; *squares*). Thus, in contrast to the 48 h time point, at 72 h even maximal TCR ligation by itself can no longer support continued entry of T cells into the dividing pool. Second, while there is a large accumulation of mitotic events during this second 24-h period in cultures stimulated in the presence of CD28 costimulation (Fig. 5 E), representing a five- to sixfold increase over the number of divisions having occurred by 48 h, mitotic events do not accumulate during this period in cultures stimulated in the absence of costimulation (Fig. 5 E). The absence of cell division in CTLA4Ig-treated cultures at later time points suggests that the early, CD28-independent proliferation that occurs at very high TCR engagement (Fig. 5, B and C; *squares*) cannot be sustained throughout the response. During the next 24 h (96 h after stimulation), T cells stimulated under physiological costimulatory

conditions did not divide considerably, nor did T cells stimulated in the absence of CD28 costimulation (data not shown). However, mitotic events continued to accumulate in anti-CD28-treated cultures, even though the responder frequency did not increase significantly from that observed at 72 h after stimulation (data not shown). Taken together, these results suggest that while very strong TCR engagement in the absence of costimulation can induce early proliferation, signaling through CD28 serves two separate and distinct functions: the recruitment of new T cells into a proliferative phase at later time points (Fig. 5 D), and the maintenance of continued cell division in the responding population (Fig. 5 E; see Fig. 6 below).

Effect of CD28 costimulation on mitotic progression and clonal expansion. As shown in Fig. 5, CD28 signaling greatly increases the number of mitotic events while exerting at best a small effect on the frequency of responding cells, suggesting that those T cells that respond in anti-CD28-treated cultures have an enhanced proliferative capacity. Indeed, analysis of proliferation at the single cell level reveals that the average T cell which responds in the absence of CD28 costimulation gives rise to 4.2 mitotic events, while the average T cell which responds in the presence of maximal CD28 costimulation gives rise to 15.3 mitotic events (Fig. 6 A).

A comparison of CD25 expression versus CFSE fluorescence shows that the enhanced mitotic progression exhibited by T cells stimulated in the presence of CD28 costimulation is associated with prolonged IL-2 receptor expression (Fig. 6 B). IL-2 receptor expression here is dependent on CD28 signals, and does not vary as a function of cell division, as T cells with the same proliferative history can exhibit drastically different CD25 expression (Fig. 6 B; compare middle right and bottom right panels).

The effect of CD28 on the proliferative capacity of responding T cells is most likely mediated by IL-2, as exogenous IL-2 can restore both IL-2 receptor expression (Fig. 7 A) and efficient mitotic progression (Fig. 7 B) by T cells stimulated in the absence of CD28 costimulation. Calculation of the precursor frequency of viable dividing cells in this experiments reveal the following: CD3 alone (not shown), 53%; CD3 + CTLA4Ig, 40%; CD3 + CTLA4Ig + IL-2, 62%; and CD3 + IL-2, 62%. Thus, we find that addition of IL-2 restores the precursor frequency of dividing cells that is otherwise reduced by blocking CD28-mediated costimulation with CTLA4Ig, indicating that neither other cytokines induced by, nor signals transduced by, CD28 are required for this effect.

Mitotic analysis of an MHC-restricted T cell response to nominal antigen. We have also used this CFSE technique for the mitotic and precursor frequency analysis of antigen-specific T cells stimulated with nominal peptide in the context of major histocompatibility complex (MHC) molecules. Fig. 8 shows the proliferative history of ovalbumin-specific, TCR transgenic T cells from splenocytes cultured in vitro with an optimal concentration of ovalbumin peptide. This analysis shows that, similar to polyclonal stimulation of T cells with anti-CD3, the maximal responder frequency in a monoclonal population of T cells after stimulation with specific MHC-peptide complexes is $\sim 65\%$.

Discussion

A detailed understanding of the dynamics of T cell activation and clonal expansion has been limited by experimental ap-

proaches that measure the bulk response of a cell population, without distinguishing the responses of individual cells. As a consequence, it has been difficult to determine whether modulations in T cell responses with changing TCR or costimulatory signals were due to recruitment of more cells into the response, a more vigorous output by responding cells, or both. It has also not been clear if this balance would vary in a kinetic fashion. We have developed an experimental system in which the fate of individual T cells can be followed throughout activation and clonal expansion. By monitoring the generation of daughter cells during a T cell response using the fluorescent dye CFSE (18), we are able to ascertain the proportion of the original T cells that have responded to stimulation by dividing, and to provide an accurate estimate of the absolute number of cell divisions which have occurred. Furthermore, because this method of monitoring cell division employs flow cytometry, we are able to examine multiple parameters of an immune response on a single cell basis, such as the expression of CD25 or CD69 as a function of mitotic number in a mixed population. This approach allows for an in-depth analysis of the dynamics of a T cell response.

Recent studies by Viola and Lanzavecchia (15) have suggested that T cells can count TCR-antigen interactions and respond after a threshold number of TCR have been engaged. These studies also suggested that the threshold for activation can be lowered by costimulatory signals through CD28, a finding that is confirmed by our studies. However, the conclusions of Viola and Lanzavecchia were based on analysis of T cell responses at the bulk population level, through measuring either proliferation or cytokine production, and therefore it is unclear whether this observation reflected the uniform behavior of all cells or enhanced responses by a small subset of cells. Our method of analysis reveals that there is an element of both. The degree or size of clonal expansion is a product of two factors: the proportion of T cells that respond to stimulation by dividing, and the proliferative capacity of each responder. We find that CD28-mediated costimulation enhances both elements, yet a sizable fraction (up to 40%) of T cells fails to respond by dividing even once, despite the nearly uniform T cell activation as measured by CD25 and CD69 expression.

The data in Fig. 5 also show that T cells stimulated with anti-CD3 antibody in the presence of agonistic CD28 antibody express more T cell receptor at 24 h than T cells stimulated with the same concentration of anti-CD3 but in the presence of CTLA4Ig. These results imply that the efficiency of TCR downregulation may depend on the level of costimulation present at the time of anti-CD3 stimulation. This result does not alter the interpretation of the data, because if the absolute mitotic values and responder frequencies shown in Fig. 5 are graphed as a function of the dose of anti-CD3 added to each culture (instead of the number of TCR's engaged as a consequence), the individual response curves have the same slope and plateau at the same values. When comparing the data graphed against anti-CD3 dose versus against TCRs engaged, only the relative dose responses (i.e., the positioning of the curves along the X axis) shift slightly. Experiments are currently in progress in our laboratory to address whether these differences in TCR downregulation reflect actual differences in signal strength, or are the result of some other indirect or unrelated process(es).

At optimal TCR signaling levels, CD28-stimulation no longer enhances recruitment of T cells into the dividing pool, and its

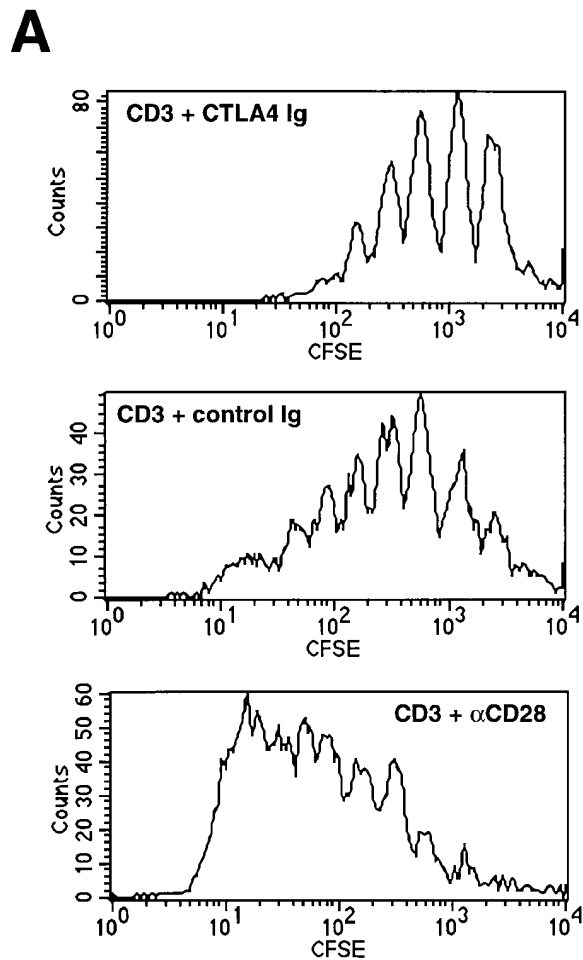


Figure 6. Signaling through CD28 results in prolonged IL-2 receptor expression and efficient mitotic progression. (A) CFSE-labeled splenocytes were stimulated with 1 μ g/ml anti-CD3 in the presence of either CTLA4Ig, control Ig or anti-CD28. The CFSE fluorescence profiles of the live T cells from each culture at 96 h are shown. The absolute number of mitotic events represented by each histogram are 27,500 in the presence of CTLA4Ig, 93,540 in the presence of control Ig and 248,100 in the presence of α CD28. The mean number of mitotic events generated per responding T cell is 4.2 in the presence of CTLA4Ig, 7.3 in the presence of control Ig, and 15.3 in the presence of anti-CD28. (B) CFSE-labeled splenocytes were stimulated and cultured in the presence of anti-CD28, CTLA4Ig, or control Ig. Bivariate dot plots show the expression of CD25 at 48 and 72 h after stimulation as a function of cell division on the T cell subset of CFSE-labeled splenocytes stimulated as in A.

ability to enhance clonal expansion predominates. Thus, a high degree of TCR ligation in the absence of CD28-stimulation yields a pattern where a significant proportion of the input T cells are induced to divide, however, the proliferative capacity of each responding T cell is very low (the average responder generates only five daughter cells). This pattern of high responder frequency, but poor mitotic progression, explains previous observations in which both CD4⁺ (22, 23) and CD8⁺ (24, 25) T cells were shown to proliferate early to high doses of antigen in the absence of CD28 costimulatory signals, but failed to sustain this proliferative response over time. Conversely,

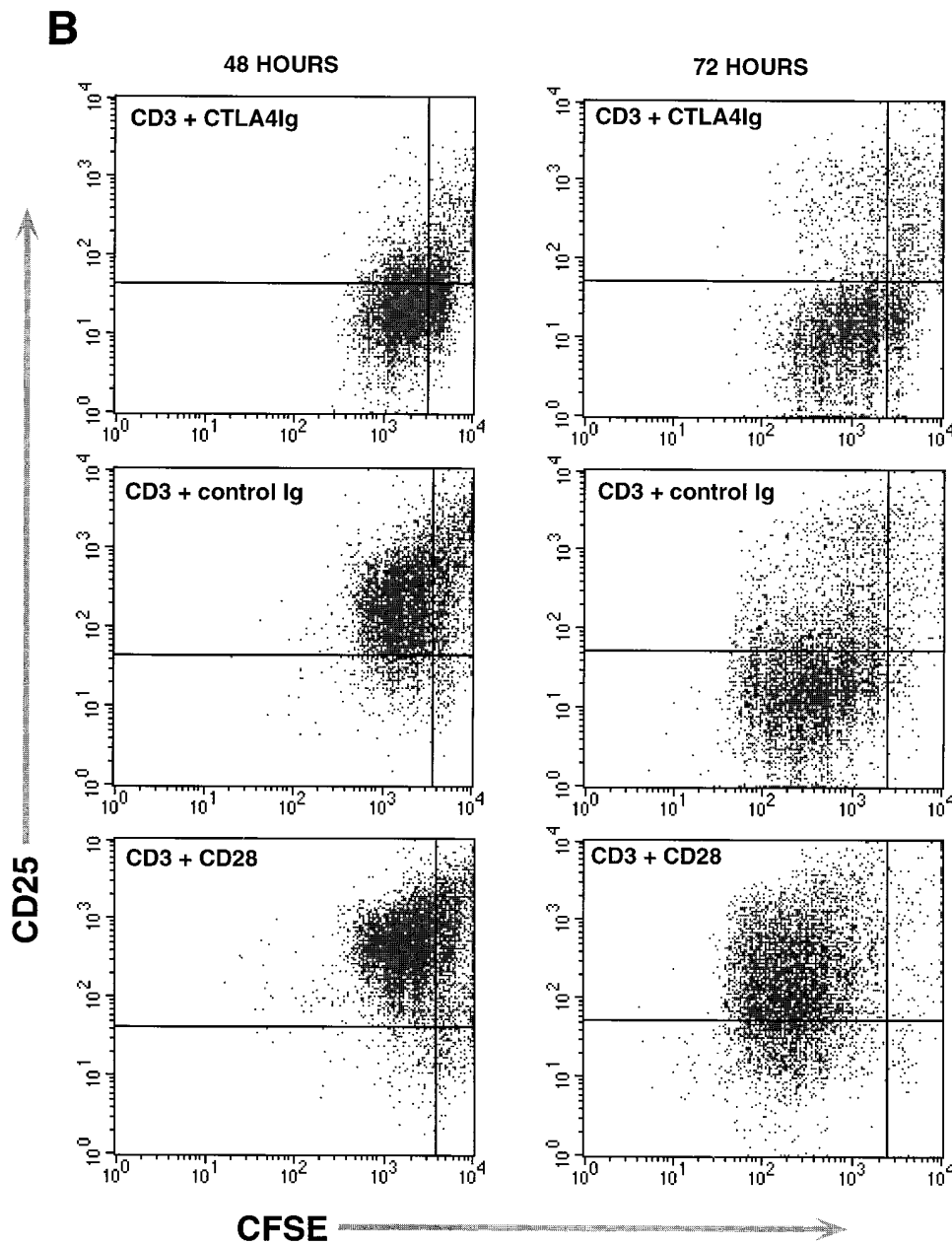


Figure 6 (Continued)

under conditions of avid CD28 costimulation, even very low levels of TCR engagement can induce a maximal number of individual T cells to participate in clonal expansion, and each responding T cell exhibits a very high proliferative capacity (the average responder can generate 16 or more daughter cells).

Particularly striking is the observation that only 50–60% of the input T cells ever respond to polyclonal stimulation by dividing, even under culture conditions in which essentially every T cell is activated. For example, with the addition of high doses of anti-CD3 and anti-CD28 antibodies (i.e., a situation in which neither TCR or CD28 ligands are limiting), 100% of the T cells have fully downregulated their TCR and 98% of the T cells express CD69 and CD25; however, only 60% of these cells can be observed to divide over the next 5 d. This finding is

consistent with recent studies by Cai et al. (26) in a different system, showing that TCR downregulation can occur without consequent activation and proliferation. However, our results suggest that even activated T cells may fail to proliferate, and therefore this failure to participate in clonal expansion is likely due to a distinct mechanism.

The inability to observe cell division in a significant proportion of T cells after polyclonal stimulation may reflect differential requirements of distinct T cell populations (e.g., naive versus memory T cells (27, 28)). However, preliminary experiments using multicolor staining for CD45RB and CD62L fail to support a simple model in which the nondividing cells have a naive phenotype, and suggest a more complex relationship between expression of memory markers and the ability of an individual T cell to be recruited into the dividing pool (unpub-

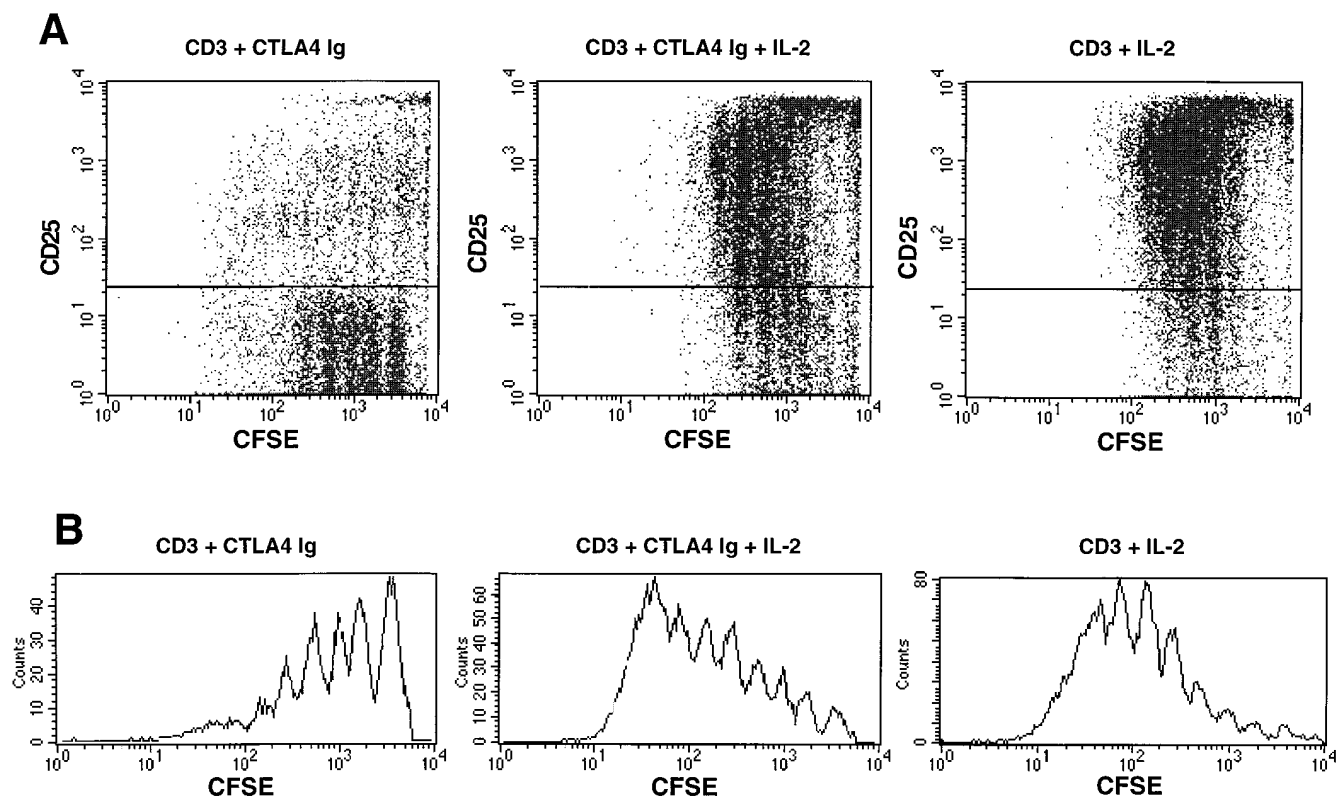


Figure 7. IL-2 restores IL-2 receptor expression and mitotic progression by T cells stimulated in the absence of CD28 costimulation. CFSE-labeled splenocytes were stimulated with 1 $\mu\text{g/ml}$ anti-CD3 with or without 10 $\mu\text{g/ml}$ CTLA4Ig in the absence or presence of 20 U/ml recombinant murine IL-2. (A) Bivariate plots of CD25 expression as a function of cell division at 72 h after stimulation, and (B) histograms depicting the CFSE fluorescence of live T cells at 96 h after stimulation are shown.

lished observations). Alternatively, the failure of a significant portion of stimulated T cells to enter the dividing pool may reflect a physiological checkpoint characteristic to the activation program of individual T cells. This interpretation is consistent with studies that used altered peptide ligands to demonstrate that activation and proliferation exhibit differential signaling requirements in T cell clones (29).

It remains formally possible that the failure of > 60% of T cells to proliferate is an artifact of the use of anti-CD3 antibody for mitogenic stimulation. However, we have observed similar results in preliminary experiments where splenocytes from ovalbumin-specific TCR transgenic mice were stimulated with ovalbumin-derived peptide (Fig. 8). Furthermore, mitogenic stimulation with PMA and ionomycin, which activate T cells by bypassing receptor ligation and initiating distal signal transduction cascades, resulted in maximal responder frequencies of 75–85%, compared with 60% with anti-CD3 (data not shown). This suggests that a portion of the missing response reflects cells in which the signal transduction pathway leading to proliferation is intact, but are impaired in the response to receptor ligation. However, since PMA and Ionomycin could not bring the responder frequency to 100%, there remains a checkpoint that functions at a more distal level in the signal transduction pathway or in the execution of the mitotic program. Experiments are ongoing in our laboratory to determine whether this limit in the ability to induce proliferation in all ac-

tivated T cells reflects the absence of a factor(s) from our *in vitro* culture system which normally promotes the entry of T cells into a proliferative phase, or whether this limit represents an actual hallmark of a natural T cell response, particularly in situations with a high frequency of potentially responsive T cells.

In summary, we conclude that TCR signal strength directly regulates the recruitment of individual T cells into the proliferating pool, and that the degree of signaling which must occur before a maximal responder frequency is reached can be regulated by the costimulatory environment of the T cell. In this system, there is a linear relationship between TCR engagement and responder frequency. Furthermore, a stringent checkpoint appears to limit the participation of individual T cells in clonal expansion, such that only 50–60% of the T cells which become activated after polyclonal stimulation actually go on to divide. Consistent with previous reports (15), we observe that costimulatory signals through CD28 render T cells more responsive to suboptimal TCR ligation. Our studies reveal that this is due to both an enhanced response of dividing cells and the recruitment of additional T cells into a proliferative phase, and that these effects of CD28 costimulation on cell recruitment and cell division appear to be mediated by IL-2. Additional effects of CD28 signaling, such as induction of Bcl-x expression (30, 31), do not appear to play a role in the enhanced primary response.

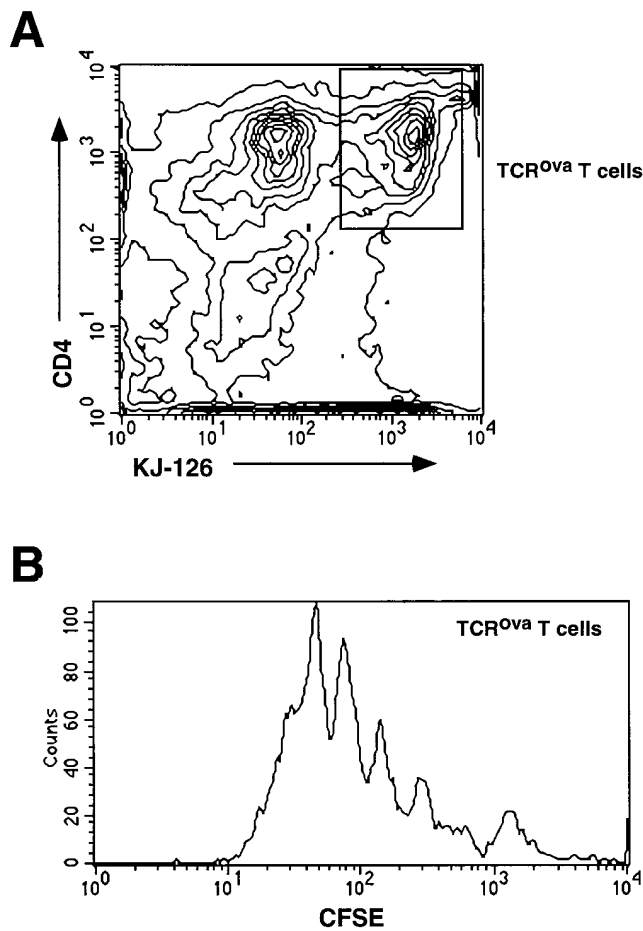


Figure 8. Analysis of cell division in TCR-transgenic T cells stimulated with nominal peptide antigen. CFSE-labeled splenocytes from D011.10 mice were stimulated with 50 $\mu\text{g/ml}$ peptide corresponding to amino acids 323-339 of ovalbumin. (A) Bivariate plot of CD4 and clonotypic TCR expression (identified by the clonotype-specific antibody KJ1-26) on D011.10 splenocytes cultured as described above. (B) Histogram depicting the CFSE fluorescence of the live TCR^{OVA} clonotype T cells at 72 h after stimulation. The frequency of TCR^{OVA} T cells which responded to stimulation with specific peptide is 66%.

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