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Proliferating CD4⁺ T Cells Undergo Immediate Growth Arrest upon Cessation of TCR Signaling In Vivo

Cory A. Yarke,* Stacy L. Dalheimer,* Na Zhang,* Drew M. Catron, † Marc K. Jenkins, † and Daniel L. Mueller2*

To investigate the role of TCR signaling in the exit of CD4⁺ T cells from cell cycle, we took advantage of a low frequency TEa T cell adoptive transfer technique as well as the Y-Ae mAb to interrupt Ag/MHC recognition before the completion of clonal expansion. Termination of TCR signaling after 36 h of Ag exposure caused an immediate reduction in cell size and deceleration of G₁→S phase cell cycle progression. As a consequence, clonal expansion in the absence of durable TCR signaling decreased by two-thirds. Thus, CD4⁺ T cells scan for the presence Ag throughout their clonal expansion response, and continuously adjust their rate of cell growth and G₁→S phase transition to match their intensity of TCR signaling. The Journal of Immunology, 2008, 180: 156–162.

Recognition of Ag by low frequency naive T cells can occur incidentally, as in the case of a self Ag, or can follow an infection. Accordingly, the outcome of an Ag recognition event is not predetermined. T cells stimulated by self Ag generally fail to proliferate effectively and instead are induced to undergo apoptosis or to develop clonal anergy. In contrast, T cells activated in the setting of infection undergo a robust clonal expansion that leads to the generation of a large effector T cell pool and clearance of the Ag, as well as the development of memory.

For the case of CD8⁺ T cells, the recognition of TCR ligands on pathogen-infected APC for a relatively brief period of time (<10 h) appears sufficient to elicit robust clonal expansion over the course of several days (1–3). Such “programming” of CD8⁺ T cells is thought to be protective, because cytotoxic T effector cells are then free to quickly destroy the Ag-infected APC even before the culmination of their clonal expansion response. In contrast, overwhelming infection associated with chronic Ag stimulation can be associated with CD8⁺ T cell exhaustion, a combination of clonal anergy and cell death, to protect against progressive T cell-mediated immunopathology (4, 5). Nevertheless, there are additional, and at times conflicting, in vivo data to suggest that persistent TCR signaling may augment the capacity of daughter CD8⁺ T cells to proliferate to their full extent, to differentiate into a fully functional effector or memory cell phenotype, or to survive (6–8).

The activation requirements for the clonal expansion of naive CD4⁺ T cells are also controversial. It was originally shown that a 4–6 h period of in vitro Ag stimulation was sufficient to program CD4⁺ T cells to proliferate, even after their separation from Ag-bearing APC (9, 10). This development of competence to proliferate after just 4–6 h of activation reflects the time needed to up-regulate high affinity IL-2 receptors and to secrete IL-2, thus facilitating IL-2-dependent autocrine growth in vitro (9, 11). Under such conditions, continued TCR ligation can, in fact, interfere with this autocrine proliferative pathway (12). Our own investigations of CD4⁺ T cell proliferation have extended this work, but further indicate that sustained TCR and CD28 signaling can be both necessary and sufficient to maintain an optimal rate of cell division when access to IL-2 in the in vitro cultivation system is limited (13–15). IL-2 has not been observed to play a major role in Ag-dependent naive CD4⁺ T cell clonal expansion in vivo (16). Therefore, persistent stimulation through the TCR and CD28 may be more important to ensure optimal in vivo cell cycle progression.

Consistent with this hypothesis, Obst et al. (17) have recently demonstrated using a tetracycline-regulatable transgenic Ag (moth cytochrome c) that as Ag availability wanes, a premature cessation of cell division and clonal expansion occurs for CD4⁺ T cells. Thus, CD4⁺ T cells remain aware of the availability of Ag even at late times during the course of their clonal expansion response, and stop proliferating as Ag becomes cleared. The design of this study, however, relied on the turnover of the transgenic protein and relevant peptide Ag/MHC complexes to abrogate TCR ligation, and did not allow for a kinetic analysis of T cell activation events that followed a loss of TCR signaling. Furthermore, their use of Ag-specific transgenic T cells at relatively high adoptive transfer frequency (2–4 × 10⁶ per animal) could be expected to shorten the duration of a clonal expansion response and reduce the opportunity to see positive effects of late TCR occupancy (18). Therefore, it has remained uncertain whether the clearance of Ag and premature cessation of peptide Ag/MHC presentation can be quickly sensed by a rapidly dividing CD4⁺ T cell and lead to an immediate slowing of the rate of cell cycle progression, or whether a CD4⁺ T cell continues to proliferate at a high rate until a defined proliferative program is completed. We have now investigated further this control of in vivo CD4⁺ T cell clonal expansion by ongoing peptide Ag/MHC complex recognition, and find no evidence for programming; rather, CD4⁺ T cells demonstrate a continuous regulation of blastogenesis and their rate of G₁→S phase progression by TCR-mediated signaling events.

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Materials and Methods

Mice and reagents

TEa CD4+ T cells express Thy1.1 (CD90.1) and Vβ6 and recognize the I-E\d{d} α-chain peptide 52-68 (pE\d{d})3 presented by I-A\d{B} (19). Thy1.1–

C57BL/6NCr (B6) (H-2d) mice were purchased from Charles River Laboratories through a contract with the National Cancer Institute of the National Institutes of Health, and were used as syngeneic recipient mice for adoptive transfer studies. Congenic Thy1.1+ B6.PL-Thy1.1cyt (PL) (H-2d) mice were purchased from The Jackson Laboratory. All mice were housed under specific pathogen-free conditions and used in accordance with guidelines put forth by the National Institutes of Health and the University of Minnesota Institutional Animal Care and Use Committee. Mice were sex and age matched for all experiments, ranging in age from 6–10 wk old. Recombinant Eo red fluorescent protein (Eo RFP) containing the pEa T cell epitope was generated as previously described (20). The Y-Ae mAb is specific for a complex composed of pEa bound to the I-A\d{d} MHC class II molecule (21, 22), and was produced and used as previously described (20).

T cell activation

For studies relying on the in vitro activation of Thy1.1+ CD4+ T cells, an equal mixture of purified, CFSE-labeled TEa and wild-type PL polyclonal CD4+ T cells was incubated for 24 h at 37°C on plates precoated either with the combination of anti-CD3 (145–2C11) (23) and anti-CD28 (37.51) (24) mAbs, or with hamster IgG (BD Biosciences) as an irrelevant Ab control, as previously described (14). For in vivo adoptive transfers, the indicated number of Thy1.1+ CD4+ T cells was injected i.v. into Thy1.1+ B6 mice. In all experiments, mice were challenged with either 100 μg EoRFP plus 25 μg LPS (Sigma-Aldrich) to initiate a T cell clonal expansion, or with LPS alone as a negative control. Thy1.1+ TEa CD4+ T cells were then distinguished from the majority of transferred Thy1.1+ polyclonal CD4+ T cells using Vβ6 mAb (b292-TEa).

Magnetic bead enrichment and identification of CD4+ T cells after adoptive transfer

Enrichment and detection of low numbers of transferred CD4+ T cells were performed at the times indicated after priming as previously described (18). In brief, single cell suspensions of spleen and mesenteric, inguinal, brachial, axial, and cervical lymph nodes were suspended in 2 ml of PBS containing 2% FCS and 0.1% NaN3 (referred to as sorter buffer). Biotin-labeled anti-CD90.1 (BD Pharmingen) and anti-CD16/32 (eBioscience) Abs were added to a final concentration of 2 μg/ml and incubated on ice for 20 m. Cells were then washed and resuspended in 2 ml sort buffer containing 40 μl of streptavidin beads (Miltenyi Biotec) and incubated on ice for an additional 20 m. Cells were then washed, resuspended in 3 ml sort buffer, and passed over magnetized LS columns (Miltenyi Biotec). Finally, columns were washed 3× with 5 ml sort buffer to remove unbound labeled cells, and bound cells were eluted in 5 ml following removal from the magnet. A small aliquot of each sample was used to determine the viable total cell count using the Caltag/Invitrogen Life Technologies Counting Bead System. The remainder of the sample was then stained with anti-Vβ6-PE, anti-CD4-FcRPe Cy5.5, SA-allophycocyanin (BD Pharmingen), anti-CD6-Pacific blue, anti-B220-Pacific blue, and anti-CD11b-Pacific blue (Caltag/Invitrogen Life Technologies), and subsequently analyzed for evidence of CFSE dye dilution in the CD90.1+ Vβ6+ CD4+ TEa T cells using a BD LSR II flow cytometer (BD Pharmingen) and FlowJo (Tree Star).

Measurement of cell cycle progression in vivo

T cell G0→G1 cell cycle entry was monitored by flow cytometry using the forward scatter (FSC) determination. The average change in FSC (ΔMean FSC) during blastogenesis was calculated as the ratio of the transferred Thy1.1+ CD4+ TEa T cell FSC determination to the endogenous Thy1.1+ CD4+ polyclonal T cell population FSC at each time point. The average cell division rate for a CFSE-labeled CD4+ T cell population at various times after stimulation was calculated as previously described (14, 25). In brief, based on the peaks of CFSE fluorescence intensity within the population, each T cell was assigned to a particular cell division group d (with d = 0 to n cell divisions), and the number of T cell events (Ed) observed within each cell division group (Ed) was determined. Average cell division (D) was then calculated using the following equation:

\[ D = \frac{\sum_{d=0}^{n} (2^{-d})E_d}{\sum_{d=1}^{n} (d(2^{-d})E_d)} \]

Note that this calculation of the average number of cell divisions is corrected for the 2-fold increase in cell number that is associated with each division. To allow for a comparison of D values between groups within an experiment, the SEM was determined for each sample using the following formula:

\[ \text{Sample Variance} \sigma^2 = \frac{\sum_{d=0}^{n} (2^{-d})E_d(d - D)^2}{\sum_{d=1}^{n} (d(2^{-d})E_d)} - \frac{2^{-D}E_D}{D} \]

Standard Error SEM = \sqrt{\frac{\sigma^2}{T_n}}

Results

Intense TCR/CD28 engagement fails to program CD4+ T cells for a durable cell division response in vivo

To further investigate the in vivo TCR signaling requirements for clonal expansion, Thy1.1+ CD4+ T cells from both TEa TCR-transgenic (TCR-Tg) mice and nontransgenic control B6.PL mice were mixed together and labeled with CFSE. This T cell mixture was then preactivated using a high concentration of immobilized anti-CD3 and anti-CD28 mAb for 24 h. Previous work from our laboratory has shown that this length and intensity of stimulation is insufficient for maximal in vitro proliferation, unless the T cells are allowed to remain in contact with their IL-2-containing conditioned medium after removal from the mAb-coated plates or they are supplemented with exogenous IL-2 (14). To determine whether such a defined period of T cell activation was sufficient for sustained proliferation in vivo, the CD3/CD82 mAb-stimulated T cell mixture was adoptively transferred into Thy1.1-B6 mice. Recipient mice were then injected i.v. with either LPS alone or the recombinant Ag EoRFP plus LPS. CFSE dye dilution within the Thy1.1+ CD4+ transferred T cell population was assayed 72 h later.

Using anti-Vβ6 mAb to delineate TEa TCR-Tg T cells from the majority of polyclonal CD4+ T cells (Fig. 1A), we observed that a 24 h period of CD3/CD82 mAb stimulation in vitro was insufficient to program a durable in vivo cell division after the cells were transferred into mice exposed only to LPS (Fig. 1B). On average, nearly one half of the T cells failed to divide under these stimulation conditions, perhaps consistent with an inefficient G0→G1 cell cycle entry during in vitro activation and inability to undergo the G1→S phase transition (Fig. 1, B and C). The other half of the CD4+ T cell mixture (both TEa and polyclonal) divided once or, less frequently, twice after adoptive transfer. This failure of CD4+ T cells to proliferate well in vivo following adoptive transfer was not an artifact of reduced viability or anergy induction following the in vitro cultivation, as TEa CD4+ T cells remained fully capable of undergoing multiple rounds of cell division if exposed to pEa/I-A\d{d} complexes and LPS, in vivo. Interestingly, preactivated polyclonal CD4+ T cells even failed to proliferate in vivo when exposed to LPS plus the paracrine growth factors produced during the Ag-specific proliferative response of TEa T cells. Thus, the results supported a hypothesis in which activated CD4+ T cells remain aware of the presence or absence of relevant peptide Ag/MHC ligands at late times after initial stimulation, and adjust their rate of cell division downward if Ag cannot be detected.

Kinetics of Ag-dependent CD4+ T cell activation in vivo

To further examine the role of continuous Ag recognition in the proliferation of CD4+ T cells, the kinetics of blastogenesis and cell
FIGURE 1. CD4\(^+\) T cells cannot be programmed for Ag-independent cell cycle progression in vivo. Thy.1.1\(^+\) TEa CD4\(^+\) T cells and Thy.1.1\(^+\) pl clonal CD4\(^+\) T cells were mixed, CFSE-labeled, and preincubated for 24 h either with (CD3/CD28) or without (nil) a combination of anti-CD3 and anti-CD28 mAbs, as indicated. T cells were then adoptively transferred into Thy.1.1\(^+\) B6 mice. Recipient mice immediately received LPS plus either EaRFP or PBS and CFSE dye dilution was assayed 72 h later. A, Flow cytometric gating protocol used to identify Thy.1.1\(^+\) TEa and wild-type pl clonal CD4\(^+\) T cell populations in Thy.1.1 hosts. B, CFSE fluorescence histograms for TEa (solid black tracing) and pl clonal (shaded profile) CD4\(^+\) T cells. C, Calculation of the average cell division response for TEa CD4\(^+\) T cells (open bars) and pl clonal CD4\(^+\) T cells (filled bars). Error bars indicate the SEM for the populations indicated.

Optimal Ag-induced cell cycle progression requires uninterrupted recognition of TCR ligands

To develop a model system that would give us control over the kinetics of TCR occupancy in vivo, we made use of the Y-Ae mAb that specifically recognizes pEaI-A\(^b\) complexes and competes for binding with the TCR on the TEa T cells (19, 21). One million CFSE-labeled CD4\(^+\) TEa T cells were again adoptively transferred into B6 mice and challenged with EaRFP/LPS for a period of 120 h. Y-Ae mAb was also administered to the mice at various time points following the Ag injection. As expected, CD4\(^+\) T cell proliferation detected at 120 h was nearly abolished (95 ± 2% division were defined in vivo following an adoptive transfer of 10\(^6\) resting (G\(_0\)) TEa TCR-Tg T cells and an i.v. exposure to EaRFP and LPS. Flow cytometry of TEa T cells recovered at various times during the 5-day immunization indicated a rapid period of cell enlargement (G\(_0\)→G\(_1\)) that peaked between 24 and 36 h (Fig. 2A). During this same time period, the TEa CD4\(^+\) T cell population also progressed from the G\(_1\)→S, G\(_2\) phase of the cell cycle, with an average time to first cell division of ~34 h (Fig. 2B). To estimate the rate of cell cycle progression throughout the time course, we fit these average cell division data to a curve using a least-squares method, and then calculated the first derivative of the resultant polynomial expression. The results indicated that CD4\(^+\) T cells achieved their maximal cell division rate of one division every 11.5 h (0.087 divisions/h) beginning at the time of their first detectable cell division (Fig. 2C). By 54 h of stimulation, however, the T cells had significantly reduced both their mean cell size and their average rate of division. No evidence was found for a burst of cell cycle progression programmed to occur at a high fixed rate. Instead, both the state of growth and the rate of cell division varied continuously throughout the clonal expansion.

FIGURE 2. Kinetics of TCR-dependent CD4\(^+\) T cell blastogenesis and cell division in vivo. A total of 10\(^6\) CFSE-labeled TEa CD4\(^+\) T cells were transferred to B6 mice, and 24 h later 100 \(\mu\)g EaRFP/LPS was administered i.v. A, Delta mean FSC of TEa T cells. B, Average cell division response for TEa T cells. Note that data from 24 h and beyond were fit to a polynomial expression (dashed line) using a least-squares method with result: \(y = 0.00000460t^3 - 0.00135t^2 + 0.144t - 2.51\) (\(R^2 = 0.87\)). C, Cell division rate, calculated for Ag-stimulated TEa T cells as the first derivative with respect to time for the polynomial function shown in B: \(y = 0.0000138t^2 - 0.00270t + 0.144\). D, CFSE histograms are shown for TEa T cells stimulated with EoRFP/LPS for 120 h, either in the absence of any Y-Ae mAb (shaded profile) or with an infusion of Y-Ae mAb beginning at the indicated time point (solid black tracing). E, Percent inhibition \(I\) of cell divisions by the addition of Y-Ae mAb at the time points indicated: \(I = 100\%(1 - (D_{obs}/D_{pre}))\), where observed division \(D_{obs}\) is calculated as the average number of TEa cell divisions that occurred in Y-Ae-treated animals subsequent to the mAb addition, and predicted division \(D_{pre}\) is defined as the average number of cell divisions expected during the same period in the absence of Y-Ae mAb, using the polynomial function in panel B to calculate average cell divisions before the time of the Y-Ae addition. Error bars indicate SEM and reflect a propagation of errors.
CD4+ T cell competition for Ag/MHC ligands regulates the rate of G1→SG2M phase cell cycle progression

Hataye et al. (18) previously reported that the duration of CD4+ DO11.10 TCR-Tg T cell clonal expansion and the extent of cell division following OVA Ag immunization are reduced when the starting naive population is present at high frequency. Although the mechanism for this control of clonal expansion is unknown, it is thought to depend on competition between T cells of identical TCR specificity for Ag/MHC complexes (26). This led us to the hypothesis that CD4+ T cells at high frequency respond suboptimally to immunization, not because of a failure to enter the cell cycle (G0→G1 phase), but because of slowed G1→SG2M phase cell cycle progression as a consequence of increased competition for available Ag/MHC complexes and reduced TCR signaling as the clonal expansion proceeds.

To investigate whether TEa T cells demonstrate a comparable control of cell division, we utilized a similar magnetic bead enrichment column protocol and flow cytometric gating strategy as Hataye et al. (18) to allow for the detection of low numbers of Thy1.1+ V6β6+ CD4+ TEa T cells 4 days after adoptive transfer, both with and without immunization (Fig. 3A). Four days after their adoptive transfer in the absence of Ag, ~2 to 3% of the naive TEa T cells could be recovered from the secondary lymphoid tissues (Fig. 3B). As a result of immunization, TEa T cells expanded 65.7 ± 19.7-fold when transferred at 1×105 cells per mouse, but only 2.4 ± 1.3-fold when transferred at 5×106 cells per mouse. Fitting these data to a curve allowed us to derive a mathematical expression for clonal expansion as the clonal expansion proceeds.

At low frequency demonstrated a relatively short 5.5 h cell cycle period (0.18 divisions/h), as compared with the 15.5 h period (0.06 divisions/h) measured for T cells at the higher adoptive transfer frequency and at the same time point (Figs. 2C and 4C). Furthermore, low frequency CD4+ T cells continued to divide at a relatively high rate (>0.1 divisions/h) through 60 h of stimulation, whereas the T cells immunized at the higher frequency never divided that quickly and seldom divided beyond 60 h of stimulation. Therefore, the increased clonal expansion observed during the priming of very low frequency CD4+ T cells resulted from a G1→SG2M cell cycle progression rate that was faster right from the start, and remained higher at all subsequent time points. This result suggested that competition between Ag-specific CD4+ T
cells for a particular peptide Ag/MHC ligand has little effect on the initial selection of naive CD4+ T cell clones for expansion, but quickly affects their rate of cell division.

CD4+ T cells scan for TCR ligands throughout the primary Ag response to avert cell cycle arrest

Taken together, these data were most consistent with a model in which CD4+ T cells regulate their rate of G1—S transition according to peptide Ag/MHC availability. A total of 10^4 TEa T cells were transferred to B6 mice and challenged with 100 μg EoRFP/LPS, with some mice receiving Y-Ae mAb 36 h later. A, FSC (top) or CFSE (bottom) histograms for TEa T cells recovered between 44–96 h after immunization, as indicated, either with (solid black tracing) or without (shaded profile) the Y-Ae mAb addition at 36 h of stimulation. Numbers within FSC panels indicate percent-enlarged cells. B, Average cell division responses for Ag-stimulated TEa T cells in mice treated with Y-Ae mAb (circles) or PBS (squares) at the 36 h time point. Data from 36 h and beyond were fit to polynomial expressions (dashed lines) using a least-squares method: y = 0.000000184x^3 - 0.00179x^2 + 0.302x - 7.89 (No mAb; R^2 = 0.99) and y = 0.0000243x^3 - 0.00618x^2 + 0.529x - 11.4 (Y-Ae mAb addition; R^2 = 0.99). C, Cell division rates, calculated for Ag-stimulated TEa T cells as the first derivative with respect to time for the polynomial functions shown in panel B; y' = 0.00000552x^2 - 0.003558x + 0.302 (No mAb; fine dashed line) and y' = 0.0000730x^2 - 0.0124x + 0.529 (Y-Ae mAb addition; thick dashed line). D, Total TEa T cell counts as in panel B. E, Delta mean FSC of TEa T cells as in B. Error bars represent the SEM.

FIGURE 4. CD4+ T cells dynamically regulate their size and cell division rate according to peptide Ag/MHC availability. A total of 10^4 TEa T cells were transferred to B6 mice and challenged with 100 μg EoRFP/LPS, with some mice receiving Y-Ae mAb 36 h later. A, FSC (top) or CFSE (bottom) histograms for TEa T cells recovered between 44–96 h after immunization, as indicated, either with (solid black tracing) or without (shaded profile) the Y-Ae mAb addition at 36 h of stimulation. Numbers within FSC panels indicate percent-enlarged cells. B, Average cell division responses for Ag-stimulated TEa T cells in mice treated with Y-Ae mAb (circles) or PBS (squares) at the 36 h time point. Data from 36 h and beyond were fit to polynomial expressions (dashed lines) using a least-squares method: y = 0.000000184x^3 - 0.00179x^2 + 0.302x - 7.89 (No mAb; R^2 = 0.99) and y = 0.0000243x^3 - 0.00618x^2 + 0.529x - 11.4 (Y-Ae mAb addition; R^2 = 0.99). C, Cell division rates, calculated for Ag-stimulated TEa T cells as the first derivative with respect to time for the polynomial functions shown in panel B; y' = 0.00000552x^2 - 0.003558x + 0.302 (No mAb; fine dashed line) and y' = 0.0000730x^2 - 0.0124x + 0.529 (Y-Ae mAb addition; thick dashed line). D, Total TEa T cell counts as in panel B. E, Delta mean FSC of TEa T cells as in B. Error bars represent the SEM.

Differentiation of this average cell division response as a function of time further revealed that when Y-Ae Ab was administered at the 36 h time point, the rate of cell division immediately began to decline at a much more rapid pace than control (Fig. 4C). Consistent with these cell division results, the administration of Y-Ae mAb at 36 h also inhibited T cell clonal expansion (Fig. 4D).

Growth inhibition accompanies the deceleration of cell division, when CD4+ T cells lose contact with Ag/MHC complexes

In vitro, it has been shown that 24 h of CD3 and CD28 ligation alone cannot maintain CD4+ T cells in a blasted state over the remainder of a 5 day incubation period (14). Cell growth is a prerequisite for G1—S transition, and the 100 μg EoRFP/LPS administered at 36 h also inhibited T cell clonal expansion (Fig. 4D).

This observation that a reduction in mean cell size accompanied the decrease in the average rate of cell division following Y-Ae mAb administration prompted us to compare forward scatter profiles to rates of cell division under both high and low adoptive transfer conditions. The comparison revealed that cell size was an excellent predictor of the average rate of cell division at any point.
FIGURE 5. Cell size predicts the rate of cell division throughout clonal expansion. The average TTel CD4^+ T cell δ mean FSC and cell division rate at each time point were plotted for the EoRFF/LPS-challenged animals examined in the time courses shown in Figs. 2, A and C and 3, C and E. Groups included: No mAb treatment at high (10^6) TTea adoptive transfer number ( ), no mAb treatment at low (10^4) adoptive transfer number ( ), and Y-Ae mAb treatment (from 36 h) at low (10^4) adoptive transfer number ( ). Delta mean FSC directly correlated with the cell division rate with an R^2 = 0.88.

in time, independent of the number of T cells transferred or the addition of the Y-Ae mAb (R^2 = 0.88) (Fig. 5). Therefore, the data suggested that a failure to maintain cell growth after cessation of TCR signaling causes a premature cell cycle arrest that limits CD4^+ T cell clonal expansion.

Discussion

The experiments shown in this study were designed to optimally stimulate an entry of quiescent naive CD4^+ T cells into G_1 phase of the cell cycle, but then simulate the effects of a later clearance of Ag, either by the removal of T cells from a CD3 and CD28 mAb-coated plate in vitro or through the neutralization of the relevant peptide Ag/MHC complexes in vivo. We achieved G_1–G_2 cell cycle progression in both cases, based on the development of blastogenesis in the responder populations. Although in vitro stimulation with immobilized mAb cannot mimic all aspects of Ag encounter within a secondary lymphoid organ, it did allow for a specific and uniformly intense activation of CD4^+ T cells through molecules known to be important to the commitment to cell cycle progression. In contrast, use of the Y-Ae mAb to neutralize available peptide Ag/MHC complexes at late time points during T cell activation in vivo ensured that all normal early activation biology would occur, with the caveat that the rate at which this mAb completely quenches ongoing TCR ligation is uncertain. Using these two complementary approaches to investigate the control of cell cycle progression by late TCR signaling, we confirmed the observation of Obst et al. (17) that cell division eventually ceases in the absence of persistent Ag recognition.

How does the TCR regulate the proliferative outcome, once a CD4^+ T cell has entered the cell cycle? To begin to address this question, we determined whether cessation of TCR signaling in activated CD4^+ T cells that had achieved an optimal proliferative state would lead to an immediate deceleration of cell cycle progression and fewer cell divisions per unit time, or whether the T cells would continue to divide at a high rate for the remainder of a defined, albeit shortened, proliferative program. A careful kinetic analysis of the rate of cell division supported the former hypothesis: Cell cycle times were always shortest immediately following the onset of cell division, and then became progressively longer over time, in parallel with waning peptide Ag/MHC availability after the i.v. Ag infusion and rising responder T cell frequencies. Furthermore, the initial rate of cell division of CD4^+ T cells responding to Ag from very low numbers (approaching frequencies expected for the naive repertoire) was at least twice that of T cells competing for peptide Ag/MHC complexes at 500–fold higher adoptive transfer frequency, and this rate remained higher throughout their time course. Therefore, the rate of cell cycle progression was never programmed, and instead appeared related to the availability of peptide Ag/MHC complexes at any particular time as well as the number of Ag-specific responder CD4^+ T cells.

Decreases in precursor cell recruitment and proliferative burst size have previously been reported for CD4^+ T cells responding to Ag at very high precursor frequency (18, 26). High competition for TCR ligands also inhibits the development of stable T cell/APC conjugates 48 h into a clonal expansion response, perhaps suggesting that limiting peptide Ag/MHC complexes can be sequestered on a dendritic cell by individual Ag-specific T cells (27). Consistent with this notion, a reduction in the available peptide Ag/MHC concentrations achieved following i.v. immunization at low Ag dose leads to essentially no change in the time to first cell division, but ultimately stimulates only a reduced number of cell divisions by CD4^+ T cells (Ref. 26, and data not shown). The cell division rate model presented in this study now suggests that, independent of any effect on the recruitment of naive T cells to undergo a clonal expansion, high competition for peptide Ag/MHC complexes puts a continuous brake on cell cycle progression that leads to an earlier cessation of cell division. Thus, it appears reasonable to conclude that throughout a clonal expansion response, responder T cells “share” access to relevant peptide Ag/MHC complexes, and adjust their cell cycle rate according a running average of the amount of time spent in a conjugate with an Ag-bearing dendritic cell.

The hypothesis of continuous TCR control over the rate of cell cycle progression was investigated more definitively in this study using the adoptive transfer of a very low number of Ag responders and the infusion of Y-Ae mAb at various times during the clonal expansion. Neutralization of peptide Ag/MHC at the height of blastogenesis and the peak rate of cell division (36 h) put an immediate brake on proliferation such that cell cycle arrest occurred 24 h prematurely, with the average number of cell divisions reduced by 2-fold. Consistent with this, the number of daughter cells recovered at the end of the clonal expansion was reduced by 75% when Ag recognition beyond 36 h was prevented. Although the mechanism of this cell cycle arrest cannot be inferred from these experiments, the measurement of forward scatter changes by flow cytometry provided one clue. The average increase in forward scatter profile (and by extension, the cell size) observed for Ag-stimulated T cells under any condition predicted the rate of cell division within the population at that moment (Fig. 5). Thus, the premature loss of cell size that occurred when peptide Ag/MHC was neutralized 36 h into the clonal expansion indicates that TCR signals may ensure a high proliferative rate by directly promoting continued blastogenesis after every round of cell division.

Proliferation is thought to be triggered within naive T cells by a finite number of TCR and peptide Ag/MHC serial engagements, in conjunction with the ligation of CD28 receptors by B7 molecules expressed on the surface of the APC (28, 29). Although the attainment of this Ag recognition threshold appears to be initiated during relatively dynamic interactions between Ag-specific lymph node T cell and Ag-bearing APC, within several hours a period of stable T cell/APC conjugation facilitates the formation of a functional immunological synapse designed to optimize TCR and CD28 downstream signaling events (30–32). Efficient TCR and CD28 signaling from within the immunological synapse augments and sustains the activation of PI3K, either directly through its association with tyrosine-phosphorylated CD28 molecules, or indirectly through an up-regulation of OX40 (33–36). After 18–24 h of tight clustering with the Ag-bearing APC, the T cell disengages in
anticipation of its first and subsequent cell divisions. Nevertheless, such disengagement from tight APC clusters is not indicative of disinterest on the part of the rapidly cycling T cell in further Ag presentation. Previously activated T cell blasts continue to seek out Ag-bearing dendritic cells more capably than naive T cells, with resultant sustained expression of CD25 and increased production of IFN-γ (27, 37). Our experiments now demonstrate that such late interaction of CD4+ T cells with Ag-bearing APC also sustains their blastogenesis and facilitates a continued optimal rate of cell division.

PI3K is understood to promote blastogenesis via an induction of Akt/protein kinase B, with resultant inactivation of the proliferative counterregulators tuberous sclerosis complex-2 and forkhead box subgroup-O, and the activation of the downstream effectors mammalian target of rapamycin and NF-kB (38–43). Several in vitro studies of T cell proliferation have previously implicated the persistent activation of CD28, PI3K, Akt/protein kinase B, and mammalian target of rapamycin, in addition to the TCR, in the maintenance of an optimal rate of cell growth and division at late times during the proliferative response (14, 15, 44). Furthermore, Akt/protein kinase B transgenic T cells demonstrate a more durable proliferative response (45). It will now be important to establish whether PI3K mediates this effect of continuous TCR signaling on growth and cell division in vivo.

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Disclosures

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