The Proliferative Capacity of Individual Naive CD4+ T Cells Is Amplified by Prolonged T Cell Antigen Receptor Triggering

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Abstract

Strong antigenic encounter by T cells rapidly induces immunological synapse formation and surface T cell receptor (TCR) downregulation. Although surface TCR expression can remain low for several days, T cells can still sustain antigenic signaling. It has been unclear whether prolonged antigenic signaling occurs in the absence of surface TCR replenishment, being maintained by a few “nondownregulatable” surface TCRs that might reside in a synaptosomal structure. Alternatively, the low surface TCR level induced by antigen might represent a dynamic state of expression involving continual surface TCR replenishment, reengagement by antigen, and ongoing downregulation. To resolve this issue, we studied in vivo–generated, dual-specificity primary naive CD4+ T cells. On these cells, antigenic stimulus exclusively downregulated antigen-specific, but not antigen-nonspecific, TCRs. In addition to providing a means to track TCR engagement, this also allowed us to use the antigen nonspecific TCR to track TCR expression in isolation from TCR engagement by antigen. Surface TCR replenishment began within the first day of stimulation, and occurred synchronously with continuous antigen-specific TCR engagement and downregulation. Furthermore, by enhancing CD25 expression, extended signaling through surface-replenishing TCRs significantly amplified the number of daughter cells generated by naive CD4+ T cells that had already committed to proliferate. This effect required TCR engagement and could not be substituted for by interleukin 2. These data demonstrate that TCR triggering and consumption can occur over an extended period of time, with a significant impact on the effector responses evoked from naive CD4+ T cells.

Key words: TCR downregulation • TCR upregulation • TCR serial triggering • T cell commitment • T cell proliferation

Introduction

Sustained antigenic signaling is required to elicit T cell responses. To account for the mechanism by which T cells accomplish this, two models have been proposed. The “serial triggering” model asserts that a few peptide/MHC complexes can engage many TCRs in a process that involves TCR signaling and downregulation (1, 2). Downregulation is complete within 5 or fewer hours, after which low surface TCR expression appears to prevail for up to 5–8 d (3). Alternatively, in the “immunological synapse” model, it has been speculated that TCR consumption might occur as a process that functions as an “investment” (4), while a finely structured supercluster of engaged TCR, adhesion, costimulation, and signaling molecules is being constructed (5). Once the synapse is mature, MHC molecules become “locked in” at the contact site, and the intrinsically transient TCR/MHC interaction is predicted to be stabilized (4). This prediction stems from (a) the inability to observe TCR downregulation in the synapse itself (6, 7), and (b) the reasoning that by confining many TCR/MHC complexes to an attoliter volume, synapse formation amplifies the effective valency of TCR/MHC occupancy, overcoming the naturally low affinity of monomeric interactions (4). These models are not necessarily mutually exclusive, as it has been previously proposed that TCR clustering and downregulation could be envisioned to occur as complementary processes (7, 8). In both models, surface TCR expression reaches a quasistable state after the initiation of TCR engagement, which is described quantitatively by “serial triggering,” and geographically by the “immunological synapse.”
When naive T cells receive sustained antigenic signaling at a high enough dose, for a minimum period of time, they commit to proliferate (6, 9). The concentration of antigen encountered can affect two separate aspects of the proliferative response, (a) the number of T cells recruited to divide, and (b) the number of mitoses each responder will undergo (10–12). But the extent to which these parameters of T cell proliferation can be affected by the timing of antigenic stimulus is less well characterized. At a population level, it is known that T cells require a minimum duration of stimulus to commit to divide (13, 14). What is less clear is how the duration of stimulation affects the behavior of individual T cells within that population. In particular, can individual T cells that have already committed to divide increase the number of daughter cells they generate if antigenic signaling is continued beyond this minimum time?

Recent studies suggest that this commitment to proliferation is either “on” or “off.” In one set of studies, just 2–3 h of high-dose antigenic stimulus in vitro committed naive CD8+ T cells to a maximal proliferative response that was not amplified further by continued presence of antigen (15–17). In vivo, 24 h of antigenic stimulus had the same effect, and CD8+ T cells thus stimulated developed fully into cytotoxic and memory cells (17). In a separate set of studies, naive CD4+ T cells required between 6 and 40 h of antigen contact to commit to proliferate, shorter times being permissive in more costimulatory environments (14). Antigenic signaling exceeding that required to commit CD4+ T cells to proliferate had no apparent effect on proliferation, but began to induce TCR-dependent apoptosis. These and other studies (18–23) support the idea that T cells experience two phases of antigenic signaling with regard to their ultimate proliferative response: (1) initial signaling, which, in a binary fashion, both commits cells to proliferate and simultaneously dictates the potential magnitude of the proliferative response; and (2) excessive signaling, which cannot amplify proliferation, but can kill the cells.

Since some types of APCs must effect TCR signaling well beyond 5 h in order to commit naive CD4+ T cells to proliferate (14), T cells must possess a means of maintaining antigenic signaling after the mature synapse is formed, and the major phase of TCR downregulation is complete. However, neither “serial triggering” nor “immunological synapse” in its present state sufficiently explains how sustained TCR engagement might occur longer than 5–6 h; the former, because downregulation is reported to be complete and long-lasting by this time, the latter, because single synapses have only been assayed for up to ~6 h at a maximum (6).

Therefore, the nature of TCR engagement during prolonged interactions with antigen is unclear. Bound to the immobile MHC molecules, perhaps the TCRs at the T cell/APC synapse become capable of maintaining a prolonged static engagement that can sustain antigenic signaling without requiring replenishment of the plasma membrane by new TCR, and without continued TCR downregulation. It has been suggested that TCR replenishment may be actively prevented by expression of a unique monomeric form of CD3δ (24), implying that the pool of TCRs available for sustained engagement might be restricted to a fixed group of surface TCRs that, for some reason, are not downregulated by antigen (25).

Alternatively, the plateau of surface TCR downregulation could represent a dynamic state of expression, wherein surface TCR replenishment, engagement, and internalization occur in an ongoing balance. In some systems, both in vitro and in vivo, marked surface TCR downregulation from the downregulated level has recently been observed within 1 d after the removal of antigenic stimulus (16, 26, 27). This process can be enhanced by CD28 costimulation and IL-2 signaling and cannot be accounted for as solely due to the concomitant cell enlargement induced by T cell activation (26). However, it remains unresolved whether in those systems the removal of stimulus itself causes the induction of this surface TCR replenishment, or else perhaps reveals TCR reexpression that was occurring even in the face of persistent stimulus.

Does sequential TCR engagement and consumption continue after 5–6 h of stimulation, or does a long-lasting, static pool of “nondownregulatable” surface TCRs mediate prolonged antigenic stimulus? In this paper, we used in vivo–generated naive CD4+ dual-specificity T cells to reveal the dynamic nature of surface TCR expression on T cells encountering antigen for time periods in excess of 5 h, beyond the completion of the initial phase of TCR downregulation. Using this model, we have found an unexpected functional effect that long-term TCR triggering can have on the proliferative response of naive CD4+ T cells to macrophage APCs.

Materials and Methods

**Mice.** DO11.10 mice (28) and DO11.10/RAG-2−/− mice on the BALB/c background were bred and maintained in our animal facility until their use at 6–16 wk of age.

**Igs and Peptides.** Purified antibodies used for in vitro stimulations included: anti–CD3-ε mAb (BD PharMingen); anti-CD25, anti-CD4, and anti-CD11b (hybridomas PC61, M5/114, and M1/70, respectively, purchased from American Type Culture Collection); rat γ-globulin (Jackson ImmunoResearch Laboratories). Additional mAbs purchased from BD PharMingen for use in staining for flow cytometry (FCM)* or MACS® included: anti-CD16/CD32 (2.4G2); anti-Thy1.2 (53–2.1); anti-CD25 (7D4); anti-Vα2 (B20.1); anti-CD11b (M1/70); anti-CD4 (GK1.5); anti-B220 (RA3–6B2); anti-CD44 (IM7); anti-Pan NK cell (DX5); anti-CD11c (HL3); and anti-CD8 (Ly-2). The DO11.10 clonotype-specific mAb KJ126 was purchased from Caltag Laboratories as was anti-macrophage (F4/80). The peptide derived from chicken ovalbumin amino acids 323–339 (pOVA) was synthesized by the Protein Chemistry Lab (University of Pennsylvania, Philadelphia, PA).

*Abbreviations used in this paper: FCM, flow cytometry; gMFI, geometric mean fluorescence intensity; PC, proliferative capacity; PE, R-phycocerythrin; pOVA, peptide derived from chicken ovalbumin amino acids 323-339; RF, responder frequency.
Preparation of Naive CD4⁺ T Cells. Whole splenocyte single-cell suspensions were prepared from mice with the aid of Nitex fabric (Tetko, Inc.) followed by erythrocyte lysis via hypotonic shock. To purify naive CD4⁺ T cells from DO11.10 mice by MACS® purification, splenocytes were coated with rat anti-mouse mAbs: anti–I-A<sup>+</sup>; anti-CD11b; anti-CD11c; anti-B220; anti-pan NK cell; anti-macrophage (F4/80); anti-CD8; and anti-CD44. After secondary staining with magnetic bead-conjugated polyclonal anti-rat IgG (Polysciences), cells were placed in a magnetic field. Cells not attracted to the magnet were used in experiments and were typically ≥95% CD4⁺ T cells, 70–90% CD62L-high.

Stimulation of Naive CD4⁺ T Cells by pOVA-pulsed Macrophages. Macrophages were matured in vitro by culturing BALB/c bone marrow in supplemented DMEM (10% FBS) (HyClone Laboratories), 100 IU/ml penicillin, 100 µg/ml streptomycin, 2 mM l-glutamine, 5 µM β-mercaptoethanol plus 30% L-929 supernatant in nonmixture culture-treated sterile Petri dishes for 1 wk (29). Macrophages were then replated in 96-well tissue culture-treated plates with 50 µg/ml pOVA and 10–20 ng/ml IFN-γ (R&D Systems) and cultured at 37°C for 48 h. Thus treated, the pOVA-pulsed macrophages were strongly adherent, and were typically ≥95% CD11b⁺ CD11c⁻. They were washed twice before addition of T cells. T cells were briefly centrifuged onto the pOVA-pulsed macrophages to synchronize conjugation, and coculture was performed at a T cell/macrophage ratio of 1:1 or 1:2. Coculture was performed in the same specialized medium required to develop the macrophages. For some cultures, stimuli was interrupted at various time points by pipetting the T cells out of the wells, leaving behind the macrophages adhered to the bottom surface, similar to a method described recently for removing T cells from an adherent APC line (16). Fluorescence microscopy showed that 85–95% of CFSE-labeled T cells could be removed from the T cell/macrophage cocultures via this method. This efficiency of removal was independent of the presence of antigen (unpublished data). We believe this may be due to the deliberate forceful separation of conjugates via pipetting that may overcome active T cell/APC adhesion processes. Furthermore, residual adhesion of 5–15% in the absence of antigen is consistent with previous results (30).

Stimulated T cells were then replated in new wells with 20 µg/ml anti–I-A<sup>+</sup> blocking mAb to inhibit any possible continued stimulus contributed by the few macrophages and membrane fragments that could potentially transit with the T cells to the new wells. The stimulated T cells thus resumed incubation in the same medium that was conditioned during coculture, but without further antigenic interaction. After various periods of time, the T cells were harvested for analysis. When stated, T cell stimulation was inhibited without removing T cells from pOVA-pulsed macrophages, but only by adding 20 µg/ml anti–I-A<sup>+</sup> blocking mAb to cultures. Some experiments involved the addition of culture of exogenous IL-2 (R&D Systems), IL-4, and IL-15 (Peprotech).

CFSE Labeling and Analysis. For some experiments, T cells were labeled with 5 µM CFSE, and the subsequent dilution of this fluorescent dye was detected by FCM and used to calculate the responder frequency (RF; number of original T cells that divided due to stimulus) and the proliferative capacity (PC; average number of daughter cells generated per responder) as described in detail in previous work from this laboratory (12, 31).

Semiquantitative FCM. Cells were washed in cold buffer containing PBS, 2% FBS, and 0.01% NaN₃, and stained with antibodies by standard means. After washing, 2–5 color FCM was performed on Becton Dickinson Immunocytometry Systems FACScan™, FACScalibur™, or FACSVantage™ cytometers calibrated with Calibrite® beads. CELLQuest™ software was used for data acquisition and analysis. Geometric mean fluorescence intensities (gMFIs) were obtained corresponding to the level of TCR or CD25 fluorescence on live T cells as calculated by CELLQuest™ software. Within each experiment, all samples were performed in duplicate, and error bars in the figure graphs represent SEM. The level of surface TCR of live cells was expressed as a percentage of that of unstimulated live T cells, while surface CD25 was expressed as a percentage of the maximum value measured in each experiment.

Quantitative FCM Analysis. Some experiments used quantitative FCM analysis via the use of microbead standards (RCP–30–5 from Spherotech) conjugated to known numbers of fluorochromes. After FCM data was acquired, the gMFI was compared with the standard fluorescence curve generated by the microbeads, and the number of mean equivalent soluble fluorochromes bound per cell was estimated. We only used this system in conjunction with several specific R-phycocerythrin (PE)—directly conjugated antibodies, having verified that the fluorochrome–protein (F/P) ratio was 1:1 (as per BD PharMingen and Caltag technical support). This allowed us to consider the number of cell-bound fluorochromes to be equal to the number of bound antibodies. Having stained the cells under conditions of saturated antibody binding, we next assumed that staining antibodies were bound to the cells monovalently, and thus that the mean equivalent soluble fluorochrome was roughly equal to the number of antigens stained on the cells.

Results and Discussion

Regulation of Surface TCR Expression Level in Dual-Specificity DO11.10 T Cells. When analyzed by FCM for KJ126 TCR expression, CD4⁺ T cells from DO11.10 TCR transgenic mice display a signature histogram characterized by a unimodal peak to the extreme right, and a tapering “shoulder” to the left (Fig. 1 B, left histogram). The T cells appearing in this “shoulder” are clonotype⁺, but express less surface clonotype TCR than the cells under the mode. The proportion of T cells appearing in this “shoulder” typically ranges from 15–40% of CD4⁺ T cells per mouse (unpublished data). When DO11.10 mice are bred to a RAG−2⁻⁻ background, which does not permit generation of alternative TCR-α products, all T cells fall under the modal peak of KJ126 TCR expression and no “shoulder” appears (Fig. 1 A). We speculated that the cells with decreased clonotype TCR expression in DO11.10, RAG-2 intact mice represented dual-specificity T cells, expressing the clonotype TCR-αβ plus an additional TCR-αβ, most likely generated by endogenous α-chain rearrangement (32, 33). A previous study had shown that coexpression of some αβ pairs can lead to overexpression of the total number of surface TCR by up to twofold on dual-specificity T cells (34). For our purposes, we wished to identify an alternative Vα-chain that was coexpressed on some cells with the KJ126 TCR, but did not alter the total surface TCR expression level. Therefore, we analyzed CD4⁺ DO11.10 T cells by FCM for surface KJ126 TCR (Vα13, Vβ8.2) and Vα2 TCR surface expression, the latter representing an endogenously rearranged TCR-α,
chain usually expressed on ~10% of both wild-type and DO11.10 BALB/c T cells (unpublished data). We performed quantitative FCM analysis using microbead fluorescence standards to estimate the number of KJ126 and Vα2 TCRs coexpressed on the surface of CD4⁺ DO11.10 T cells (Fig. 1 B). Since PE is considered the best fluorochrome for use in quantitative FCM (35), we examined each TCR via mAbs directly conjugated to PE, and considered jointly the data from parallel cell preparations. After converting all PE fluorescence data to receptor numbers, we calculated Vα2 TCRs plus KJ126 TCRs for each gate. The resulting data is summarized in Fig. 1 C. We observed that all KJ126⁺ Vα2⁺ dual-specificity T cells (gates 2–9, Fig. 1 C) expressed roughly the same number of total TCRs at their surface, ~30,000 TCRs per T cell, regardless of the proportion expressed of either specific TCR. 3% of T cells were KJ126⁻ Vα2⁺ and also expressed a similar total number of surface TCRs (gate 10, Fig. 1 C). In some mice tested, KJ126⁺ Vα2⁻ cells displayed a slightly increased level of TCR expression, but only by ~10–15% at a maximum (an example is shown in Fig. 1 C, gate 1). We have previously shown that wild-type BALB/c T cells also express ~30,000 TCRs per T cell (31). We conclude that quiescent CD4⁺ DO11.10 T cells which express combinations of KJ126 and Vα2 TCRs, express a constant number of total surface TCRs, a number similar to that of clonotype-negative (KJ126⁻ Vα2⁺) and wild-type cells (31), independent of the category or quantity of either TCR specificity. Thus, the T cells which express both KJ126 and Vα2 TCRs do not appear predisposed to anomalous regulation of surface TCR expression level. Therefore, we measured the expression patterns of these two receptors in the experiments described below, and consider their responses to likely represent that of surface TCRs that are controlled by the “normal” regulatory mechanisms of naive CD4⁺ T cells.

Specific Downregulation of Engaged, but Not Nonengaged, TCRs Upon Stimulation of Naive CD4⁺ Dual-Specificity T Cells with Antigen-pulsed APCs. Strong antigenic engagement of TCR leads to surface TCR downregulation in a

**Figure 1.** Assessment of KJ126 (clonotype) and Vα2 (endogenous) surface TCR expression on dual-specificity DO11.10 T cells. (A) CD4⁺ splenic T cells from DO11.10 mice bred onto a RAG-2⁻/⁻ background homogenously express the transgenic clonotype TCR, KJ126 (solid line), while non-T cell splenocytes do not express KJ126 (dashed line). (B) CD4⁺ T cells from DO11.10 whole splenocytes were analyzed by FCM after 5 × 10⁵ events were collected. This large number of events facilitated defining the inflection point where the modal peak of KJ126 fluorescence intersects with the characteristic left “shoulder” of the peak (see text). The modal KJ126 fluorescence peak was assigned as gate 1, and gate 2 began at the inflection point (left histogram), judged subjectively but defined before any analysis was performed. Consecutive gates were identical in size and placed, descending the KJ126 fluorescence histogram (unpublished data). The PE gMFI within these narrow gates was converted to an average number of KJ126 TCRs per cell through the use of microbead fluorescence standards (Materials and Methods). To continue to quantify TCR numbers using PE, parallel tubes were used to estimate surface Vα2 using anti–Vα2-PE and KJ126-FITC. For this analysis, the inflection point on the KJ126-FITC histogram (center histogram) was identified and a gate identical in size and placement to that for the KJ126-PE sample was made and labeled as an equivalent gate 2. The PE fluorescence of Vα2⁺ cells was then measured and quantified (right histogram). Consecutive KJ126-FITC gates descended the histogram as was done with KJ126-PE, and Vα2 surface TCR expression was quantified for each gate. (C) The average number of KJ126 and Vα2 TCRs expressed per cell in each gate (1–10), as determined in B, were summed. Data from 1 of 4 experiments are shown.
dose- and time-dependent fashion; however, there is conflicting data as to whether this downregulation is specific for engaged TCRs, or general for all surface TCRs of a cell (1, 36–40), since opposing results were obtained using various cell types and TCR expression systems. The resolution of this discrepancy is important both for understanding how T cells regulate their antigenic sensitivity during cellular activation, and also for evaluating the accuracy with which TCR triggering can be measured. Since the DO11.10 mouse provided a source of in vivo–generated naive CD4+ dual-specificity T cells with confirmed wild-type regulation of surface TCR expression, we could address this issue in a more physiologic system than those previously reported. Therefore, naive CD4+ DO11.10 T cells were stimulated by pOVA-pulsed macrophages and the consequent expression of both KJ126 and Vα2 TCRs was charted. While KJ126 TCRs were readily downregulated from the T cell surface, Vα2 TCRs were not downregulated (Fig. 2 A). This remained the case when dendritic cells were used as APCs, and at all time points examined after stimulus from 1 to 144 h (unpublished data). Limiting the analysis gate exclusively to Vα2+ cells showed that Vα2+ cells had specifically downregulated KJ126, confirming that the dual-specificity cells were stimulated and that TCR downregulation was detectable on them (unpublished data). Significantly, 9% of CD4+ T cells were Vα2+ regardless of stimulation, indicating that cells were not downregulating Vα2 and exiting the analysis gate. Conversely, stimulation of DO11.10 T cells by anti-Vα2 mAb resulted in downmodulation of Vα2 TCRs, but not KJ126 TCRs; anti-CD3 mAb downmodulated both TCR species (Fig. 2 B). We conclude that when naive CD4+ T cells encounter strong antigenic contact, engaged TCRs become downregulated, while nonengaged TCRs do not.

The studies that demonstrated downregulation of both engaged and nonengaged TCRs used T cell hybridomas (37), Jurkat cell lines (38, 40), and murine T cells expressing a CD25−CD3ζ chimera in addition to endogenous TCRs (40). To our knowledge, the present data are the first to address the specificity of TCR downregulation in naive CD4+ T cells in which wild-type regulation of surface TCR expression level has been confirmed. It seems clear that under some conditions, both engaged and nonengaged TCRs can be downregulated during antigenic stimulation. We speculate that the factors leading to this response may include surface TCR hyperexpression and/or a constitutively activated T cell phenotype. However, we conclude that under normal physiologic conditions, naive CD4+ T cells do not exhibit this behavior, and only downregulate engaged TCRs upon antigenic encounter.

**Dynamic Surface TCR Expression during Prolonged Antigenic Stimulation.** We and others have reported that after the initial antigen–induced downregulation, removal of T cell stimulation is followed by surface TCR recovery, both in vitro (26, 27) and in vivo (16). The level to which surface TCR returns depends on CD28 costimulation and IL-2 signaling, and can surpass the original by up to twofold in vitro. Here, we wished to determine (a) whether surface TCR replenishment occurred exclusively for the engaged TCR specificity, or for both TCRs of dual-specificity T cells, and (b) whether surface TCR replenishment occurred or was prevented during persistent antigenic stimulation. Naive CD4+ DO11.10 T cells were stimulated by peptide-loaded macrophages for various lengths of time. KJ126 TCRs became downregulated and remained low for as long as the stimulus lasted (Fig. 3 A, left). Removal of stimulus after 18 h (and also as late as 72 h; unpublished data) permitted recovery of surface KJ126 TCR levels over time. Interestingly, Vα2 TCR levels also increased over time, and to a greater degree with a longer period of stimulus (Fig. 3 A, right). In fact, uninterrupted stimulus by pOVA-pulsed macrophages through the clonotype TCR yielded

![Figure 2](image-url)
the highest Vα2 TCR expression, so that when KJ126 TCR levels were lowest, Vα2 TCR levels were highest (Fig. 3 A). Therefore, accepting Vα2 as a marker for non-engaged surface TCR, these data directly demonstrate that T cells undergoing antigenic stimulation export TCRs to replenish the plasma membrane (marked by Vα2) at the same time during which TCR engagement and downregulation by antigen continues to occur (marked by antigen-engaged KJ126).

This result demonstrates that persistent antigenic stimulus does not prevent export of new TCRs to the plasma membrane. Previously, it has been shown that the coreceptors CD4 and CD8 can be upregulated by T cell activation (41, 42). We now demonstrate that the same appears true for the TCR itself, although this is not apparent as long as antigen remains present. When antigen is removed, recovery of surface TCR expression does not appear to be due to newly initiated TCR export, but rather due to cessation of TCR downregulation. Furthermore, the processes of TCR engagement, downregulation, and replenishment begin to occur synchronously within the first day of T cell activation, revealing a dynamic state of TCR expression that can persist for days during prolonged antigenic stimulation.

The upregulation of surface Vα2 reached ~200% of the original Vα2 level over time; however, upon interruption of stimulus, the KJ126 TCR was observed to increase from 350% (having been downregulated) to 350% of the original KJ126 level over time (Fig. 3). Previously, activation-induced surface TCR upregulation was observed to be maximal at ~200% of the original level (26, 27), equivalent to the increase observed here for Vα2. In the present system, the increase in KJ126 expression is not only due to increased expression per T cell, but also inflated due to selective expansion of the highest KJ126 expressers; as expected, these cells proliferate better to antigenic stimulus than dual-specificity T cells, due to the quantitative difference in antigen-specific TCR expression (references 43 and 44 and unpublished data), and have begun to proliferate by 72 h after stimulus. In support of this explanation, DO11.10/RAG-2−/− T cells, which solely and uniformly express the KJ126 TCR (Fig. 1), increase their surface TCR expression upon activation to only ~200% of the original value after stimulus withdrawal (unpublished data). For these reasons, the average increase in surface TCR expression per activated T cell is best estimated to be approximately twofold.

**Effect of Continued Antigenic Signaling on Naive CD4+ T Cell Proliferative Responses to Macrophage APCs.** After removal of stimulus at 18 h, significant recovery of KJ126 expression was detected even as soon as 6 h later (Fig. 3), which, together with the Vα2 trend, indicated that surface TCR replenishment was well underway during the first day of stimulation. Since new TCR expression was being masked by continual TCR engagement and consumption during uninterrupted stimulation, we hypothesized that this prolonged antigenic signaling might affect the magnitude of the T cell proliferative response. To examine this possibility, we stimulated naive CD4+ DO11.10 T cells with high-dose peptide-loaded macrophages for 18 h, enough time to commit T cells to proliferate, or alternatively, for 72 h. When T cells were harvested and counted after 120 h of culture, we found that T cells stimulated for 72 h expanded to a greater degree than did those stimulated for 18 h, without any remarkable change in the percentage of viable T cells harvested (Fig. 4). To more closely analyze the contribution of cell division to T cell expansion in this system, we stimulated CFSE-labeled naive CD4+ DO11.10 T cells with high-dose peptide-loaded macrophages for 18 or 72 h. All cells were harvested and analyzed by FCM after 120 h of culture. The proliferative response of naive CD4+ cells responding to 18 h of stimulus resulted in daughter

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**Figure 3.** During prolonged antigenic signaling, surface TCR replenishment occurs at the same time that TCR engagement and downregulation are ongoing. (A) MACS®-purity naive CD4+ DO11.10 T cells were stimulated by pOVA (50 μg/ml)pulsed macrophages for 18 h, after which the T cells and macrophages were separated, and T cell culture immediately resumed in the presence of anti-I-A<sup>4</sup> in new tissue culture wells (white squares, dashed line). Alternatively, T cells remained in the presence of macrophages throughout the entire culture period (white diamonds, bold line). T cells were harvested for analysis at different times, as indicated on the x-axes of the graphs. The graphs depict the kinetics of KJ126 (left) and Vα2 surface (right) TCR expression under the conditions specified. Timeline key; bold line, the hours of coculture of pOVA-pulsed macrophages with naive CD4+ T cells; dashed line, continued T cell culture of T cells after their separation from APCs; arrow, the hour of reagent addition to culture. The total hours of T cell culture before harvest is indicated at the right end of the timeline. (B) These overlain histograms provide examples of the changes in surface TCR expression displayed in some of the data in A. Removal of stimulus after 18 h allowed T cells to upregulate surface expression of KJ126 TCRs by 96 h after stimulus (left, bold line) beyond the level expressed by unstimulated cells (left, thin line). With 96 h of continuous stimulation by pOVA-pulsed macrophages, surface KJ126 expression was downregulated (left, dashed line), while surface Vα2 expression was upregulated (right, bold line) when compared with unstimulated levels (right, thin line). The cytometer acquisition settings were identical at all time points as revealed by a broad range of microbead fluorescence standards (unpublished data). Data from 1 of 3 experiments are shown.
cells having divided up to 3 and 4 times (Fig. 5 B); however, the response to 72 h of stimulus yielded the appearance of cells having divided up to 7 times (Fig. 5, D and E). Antigenic signaling between 18 and 72 h could not be replaced by exogenous IL-2 (Fig. 5 C), IL-4 or IL-15 (unpublished data) to produce this effect. If antigenic signaling was permitted beyond 72 h, induction of T cell death prevailed (unpublished data) via TCR hyperstimulation (18, 45, 46).

To quantitatively analyze the proliferative responses, we used the CFSE cell division profiles to calculate the number of original T cells that had divided (RF) and the average number of daughter cells produced per original responder (PC) as we have reported previously (12, 31). The marked increase in RF between 6 and 18 h of stimulus indicates that during this time new T cells were being recruited into the pool of cells which would eventually proliferate (Fig. 5 F, left). However, the number of daughter cells that each recruited cell committed to generate (PC) remained constant between these times (Fig. 5 F, right). Therefore, differences in proliferation between T cells stimulated 6 versus 18 h can be attributed almost entirely to differences in the number of original cells that commit to proliferate (RF) during this time, and not to the number

![Graph](image)

**Figure 4.** Extended antigenic signaling increases accumulation of naive CD4+ T cells. The timelines displayed are structured as outlined in Fig. 3. MACS®-pure naive CD4+ DO11.10 T cells were stimulated by pOVA (50 μg/ml)-pulsed macrophages for 18 or 72 h. At the serial time points indicated on the x-axes, live and dead T cells from duplicate wells were counted. The bar graphs display average T cell numbers. SEM bars are too small for visualization on this scale. Data from 1 of 4 experiments are shown.

**Figure 5.** Antigenic signaling beyond 18 h amplifies CD4+ T cell division in response to pOVA-pulsed macrophages. The timelines displayed are structured as outlined in Fig. 3. CFSE-labeled MACS®-pure naive CD4+ DO11.10 T cells were stimulated by pOVA (50 μg/ml)-pulsed macrophages for 3–72 h. Then stimulus was interrupted by removing the T cells from the presence of the macrophages and reculturing the T cells with anti–I-Ad until their harvest at 120 h, as described in Fig. 3. The peak to the far right in each histogram is labeled “0” because it contains cells that have not divided. Consecutive peaks to the left contain cells having divided once, twice, three times, etc. (A) Stimulus was interrupted at 3 h. (B) Stimulus was interrupted at 18 h. (C) Same as B except that 100 U/ml exogenous IL-2 was added to culture when antigenic stimulus was removed. (D) Stimulus was interrupted at 72 h. (E) An isotype control for anti–I-Ad was added to culture at 18 h, followed by stimulus interruption at 72 h. (F) RF (percent of original T cells that divide) and PC (average number of daughter cells per responder) were calculated for the experimental groups indicated. Data from 1 of 4 experiments are shown.
of daughter cells each precursor will generate (PC). Surprisingly, the situation was reversed for T cells stimulated 18 versus 72 h. By 18 h most all cells that would ever divide had already committed to do so, and thus RF rose only minimally between 18 and 72 h of stimulus (Fig. 5 F, left). But PC displayed a marked increase during this time, rising from ~3.5 to 7.5 daughter cells per responder on average (Fig. 5 F, right). So differences in proliferation between T cells stimulated 18 versus 72 h is not appreciably due to recruitment of new T cells to the response (RF), but is due instead to an increase in the number of daughter cells that each committed cell will generate (PC). As noted previously, dual-specificity T cells have a decreased proliferative response to antigen than single--specificity cells (43, 44). However, αβ2+ T cells which responded to pOVA (which therefore by definition are also KJ126+) displayed a similar enhancement of PC when antigenic stimulus was continued between 18 and 72 h (unpublished data). Thus, in our experiments, although dual specificity T cells proliferated somewhat less, they were still governed by the same rules and constraints as single specificity cells. We conclude that the continued triggering of surface-replenishing TCRs has the effect of amplifying the proliferative response of individual naive CD4+ T cells to macrophage APCs, by increasing the number of mitoses that responding T cells undergo.

**CD25 Expression and Signaling Operate Downstream of Prolonged Antigenic Signaling To Amplify PC.** We wished to determine whether continued antigenic signaling amplified T cell PC by a mechanism dependent on IL-2, the main T cell growth factor in vitro (47). Since endogenous IL-2 was clearly not limiting for the T cell proliferative response with macrophages as APCs (Fig. 5, C and F), we examined the IL-2R. To determine the relationship between continued antigenic signaling and inducible CD25 (IL-2Rα) expression, we stimulated naive CD4+ DO11.10 T cells with pOVA-pulsed macrophages for 18 or 72 h, but cultured the cells for a total time of 72 h. T cells stimulated for 18 h expressed CD25 by 72 h, but at half the magnitude compared with T cells stimulated for the full 72 h (Fig. 6 A). Significantly, ~95% of stimulated T cells expressed CD25 regardless of the length of stimulus (unpublished data), indicating that the increase in expression noted here occurs on a per-cell basis, and is not due to mobilization of T cells into or out of the CD25+ fraction. We conclude that in addition to inducing CD25 expression initially, antigenic signaling, when continued, enhances CD25 expression on naive CD4+ T cells.

To test whether IL-2R signaling was required in order for sustained antigenic signaling to amplify T cell PC, we stimulated CFSE-labeled naive CD4+ DO11.10 T cells with pOVA-pulsed macrophages. After 18, 48, or 72 h, reagents were added to culture to block I-Aδ, CD25, or both. After 144 h of culture, cells were harvested and analyzed by FCM to determine PC as before. We observed that T cell PC was not amplified whenever CD25 was blocked, regardless of the timing of reagent addition to culture (Fig. 6 B). Therefore, IL-2R signaling is required in

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**Figure 6.** CD25 expression and signaling operate downstream of prolonged antigenic signaling to amplify the PC of naive CD4+ T cells. The timelines displayed are structured as outlined in Fig. 3. (A) MACS®-pure naive CD4+ DO11.10 T cells were stimulated by pOVA (50 μg/ml)-pulsed macrophages for 18 or 72 h and cultured until harvested for FCM analysis at 72 h. For some cultures, T cell stimulation was interrupted by removing the T cells from the presence of the macrophages and adding anti-I-Aδ after 18 h (white squares). In other cultures, the T cells remained in the presence of the macrophages throughout, and anti-I-Aδ (gray diamonds), rat IgG (white circles), or nothing (black triangles) was added to the culture medium at 18 h. Data are expressed as a percentage of the maximum CD25 gMFI observed in the experiment. Data from 1 of 2 experiments are shown. (B) CFSE-labeled MACS®-pure naive CD4+ DO11.10 T cells were stimulated by pOVA (50 μg/ml)-pulsed macrophages for 18–72 h and cultured until harvested for FCM analysis at 144 h. At the time points indicated on the x-axes, combinations of anti-CD25 blocking mAb, anti-I-Aδ blocking mAb, and/or rat IgG (a control for either blocking mAb) were added to culture. In this experiment, addition of anti-I-Aδ mAb was always accompanied by transfer of T cells to new wells to continue their culture, as indicated in the timelines. A second set of Ig additions were administered at 72 h, as shown in the timelines, after which T cells from all experimental groups had received equal quantities of total rat IgG, and had been transferred to new wells to continue their culture. The 72 h treatments were done to avoid TCR-induced T cell death that can be caused by prolonged antigen exposure (see text). Data from 1 of 3 experiments are shown.
order for sustained antigenic signaling to amplify T cell PC. We conclude that CD25, once induced, can be expressed in a graded fashion, and is a limiting factor which determines the magnitude of the proliferative response of naive CD4+ T cells. CD25 expression, and thus T cell proliferation, is tunable by the duration of antigenic stimulus.

Concluding Remarks. The persistent presence of antigen can mask new levels of surface TCR expression that result from cellular activation, so that what appears to be a steady, low surface TCR expression level, represents concurrent export of new TCRs replacing constantly downregulated, engaged TCRs. Therefore, although qualitative changes in membrane compartmentation are probably important to TCR engagement and signaling, the process of TCR triggering, involving occupancy and downregulation, appears unaltered by T cell activation. Our data support a role for the export of new TCRs to the T cell membrane in sustaining antigenic signaling for prolonged periods of time, and expand upon the idea that the separately characterized processes of TCR clustering and TCR triggering are best understood in a unified model of TCR engagement (8). In 1999, Bernard Malissen suggested the importance of determining whether GFP-tagged TCRs are dynamically expressed in immunological synapses (48), despite the fact that MHC molecules were stably and statically expressed there (4). The results of such an experiment have not yet been published. However, two research groups have recently generated GFP-tagged TCR chains that can be functionally expressed at the cell surface in a T cell hybridoma (49) and Jurkat cells (50). Our results evoke the prediction that microscopic studies will eventually show both antigen-dependent TCR internalization and export in T cells undergoing prolonged stimulation. Whether such membrane dynamics can occur at the site of the immunological synapse itself will be interesting to learn.

Despite our findings regarding a very long period of TCR consumption, a major role for prolonged antigenic signaling during naive T cell stimulation was unexpected. Previous work had demonstrated that once T cells had committed to proliferate, further sustenance of antigenic signal did not amplify the proliferative response. Iezzi et al. (14) showed that 15–20 h of TCR engagement in the presence of CD28 costimulation was sufficient to commit naive CD4+ T cells to later proliferate to full capacity; with highly costimulatory APCs, the signaling time of commitment was decreased to 6 h. In those experiments, commitment to and magnitude of the naive CD4+ proliferative response appeared to be simultaneously decided, as seems to be the case with naive CD8+ cells (15–17). With macrophages as APCs, however, we found that each responding naive CD4+ T cell generated a greater quantity of daughter cells, on average, in response to 72 than to 18 h of antigenic stimulus, without significantly altering the number of original precursors that divided. Very recently, Lee et al. (51) observed that naive CD4+ T cells committed to undergo a single mitosis if stimulated by splenocyte APCs for 2 h, but divided more times if stimulated up to 6 h. Together with this study, these data demonstrate a temporal sequence that segregates commitment to and maximization of the proliferative response of individual T cells based on the duration of antigenic stimulus. These data clearly demonstrate that when individual naive CD4+ T cells commit to proliferate, they do not necessarily commit to the potential magnitude of their proliferative response. The number of daughter cells the “committed” T cells will generate is determined, at least in part, by the extent of continued TCR-dependent signaling after commitment.

Iezzi et al. (14) also showed that the induction of CD25 expression correlated with the time of antigenic stimulus required to commit T cells to proliferate. We concur with Iezzi et al. (14), in that IL-2R signaling likely determines both the induction and magnitude of the proliferative response, but add that IL-2R expression can occur in a graded fashion, dependent on the duration of antigenic stimulus. We think the most likely explanation for the discrepancy between our results and those of Iezzi et al. (14) is that CFSE dilution and FCM analysis may be a more sensitive measure of differences in proliferation than the standard tritiated thymidine-incorporation assays used in the cited work, as this has proven the case for others (52). We conclude with the proposal that naive CD4+ T cells can experience three phases of antigenic signaling with respect to their ultimate proliferative response: (1) induction, initial signaling during which time all cells with the potential to proliferate commit to do so; (2) amplification, continued signaling which enhances CD25 expression, ultimately causing committed cells to divide more times; and (3) management, excessive signaling which can kill the cells. Surface TCR replenishment, which succeeds the initial TCR downregulation, supplies the plasma membrane with new TCRs that can be triggered to contribute to antigenic signaling during each of these phases.
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