

# Continuous T cell receptor signaling required for synapse maintenance and full effector potential

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Although signals through the T cell receptor (TCR) are essential for the initiation of T helper cell activation, it is unclear what function such signals have during the prolonged T cell–antigen-presenting cell contact. Here we simultaneously tracked TCR-CD3 complex and phosphoinositide 3-kinase activity in single T cells using three-dimensional video microscopy. Despite rapid internalization of most of the TCR-CD3, TCR-dependant signaling was still evident up to 10 h after conjugate formation. Blocking this interaction caused dissolution of the synapse and proportional reductions in interleukin 2 production and cellular proliferation. Thus TCR signaling persists for hours, has a cumulative effect and is necessary for the maintenance of the immunological synapse.

T cell activation involves considerable reordering of cell surface and cytoplasmic components into an ‘immunological synapse’<sup>1,2</sup>. Synapse formation is dependent on TCR-mediated signals that, in concert with costimulatory signals, cause the cellular polarization of the T cell cytoskeleton, membrane receptors and selected cytoplasmic signaling effectors towards the T cell–antigen-presenting cell (APC) interface. Synapse-associated signaling also results in the spatial segregation of the TCR, CD28, lymphocyte-function-associated antigen 1 (LFA-1) and other surface molecules into supramolecular activation clusters<sup>3</sup>. The exact function of the immunological synapse in T cell activation is not well understood and probably depends on the physiological context in which it forms. Although not required for TCR signal initiation<sup>4</sup>, synapse formation has been associated with the induction of T cell proliferation, cytokine production, negative thymocyte selection and lytic granule release<sup>5–8</sup>.

Although the mechanisms that couple TCR-ligand-binding events to T cell activation are only poorly understood in the context of the immunological synapse, signaling pathways have been defined in considerable detail through biochemical and genetic experimentation. One of the earliest signaling events in T cell activation is the phosphorylation of CD3 molecules, followed by the assembly of intracellular signaling complexes<sup>9</sup>. These complexes constitute branching points for numerous signaling pathways that ultimately lead to activation-dependent changes in gene expression and cell cycle progression.

Despite the longevity of T cell–APC interactions, a recent report suggests that TCR signals may be required only in the early stages of naive T cell activation<sup>4</sup>. This is also indicated by the fact that T cells internalize up to 90% of their surface TCR pool within the first 30–60 min of cell contact<sup>10,11</sup>. Additionally, one study indicated that mature T cell blasts are maximally stimulated by only 60 min of

exposure to peptide–major histocompatibility complex (MHC) and antibodies to CD28 on a solid substrate<sup>12</sup>.

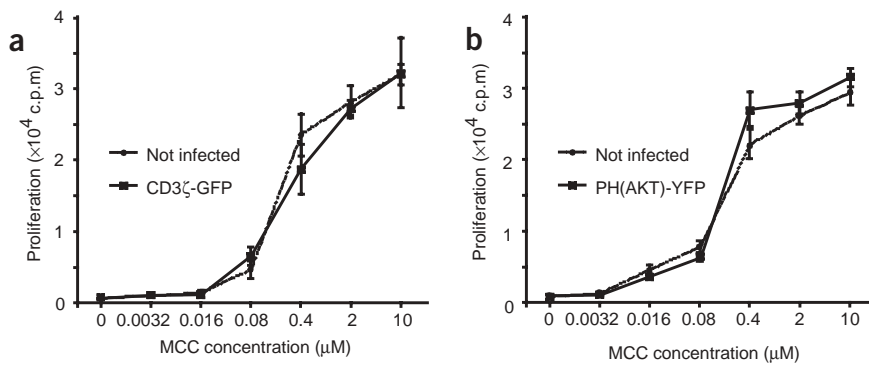
In this study we examined the involvement and function of TCR-driven signaling in the context of the immunological synapse between helper T cells and B cells from its formation to its dissolution 10–24 h later. Using dual-color, three-dimensional video microscopy, we monitored simultaneously the rapid redistribution of TCR-associated CD3 $\zeta$  and the local generation of 3'-phosphoinositides, one of the earliest second messengers downstream of TCR engagement. Despite the rapid internalization of CD3 $\zeta$  into synapse-proximal vesicles in the first few minutes, we still detected TCR-dependent signaling activity 10 h after conjugate formation. We also show that blocking the TCR ligand even many hours after conjugate formation resulted in the rapid dissolution of the synapse structure, cell detachment and a lessening of the T cell's effector potential. Continuous antigenic stimulation is thus necessary for both the integrity of the immunological synapse as well as full T cell activation.

## RESULTS

### CD3 $\zeta$ -CFP AND PH(AKT)-YFP expression in T cell blasts

A key stumbling block at present in efforts to understand the spatial relationships between different molecules involved in T cell recognition has been the simultaneous expression of different green fluorescence protein (GFP) colors in the same T cell. Although this has been achieved in T cell hybridomas and Jurkat T cells by electroporation<sup>13,14</sup>, this has proven extremely difficult in freshly isolated T cells, which require alternative gene expression methods such as retroviral infection. In our experience, simultaneous infection of cells with retroviruses encoding separate fusion proteins invariably gives rise to cells that express only one of the reporter constructs, despite the correlation between the retroviral titer during infection and the cellular

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**Figure 1** Retroviral expression of CD3 $\zeta$ -CFP and PH(AKT)-YFP did not affect the degree of T cell proliferation in response to antigen. (a,b) 5c.c7 TCR $\alpha\beta$  T cell blasts expressing CD3 $\zeta$ -CFP (a) and PH(AKT)-YFP (b) on day 9 were pooled with irradiated CH27 B cells that had been loaded with MCC peptide (concentrations, horizontal axis) and pulsed for 16 h with [ $^3$ H]thymidine 48 h after cell pooling. Error bars represent standard deviations from triplicate data sets. Data are representative of two independent experiments.

amount of reporter expression (J.B.H. & M.M.D, unpublished data). We hypothesized that successful infection could be due to viral aggregates, and thus we cultured different virus packaging lines together to promote the formation of 'mixed' aggregates. This procedure permitted expression of many combinations of fusion proteins in CD4 $^+$  and CD8 $^+$  T cells (this manuscript and M. Purbhoo, unpublished results).

Here we sought to examine the relationship between the TCR-CD3 complex and early signaling activity at the synapse. To monitor TCR-CD3 molecules, we used a CD3 $\zeta$  fused to cyan fluorescent protein (CFP; fusion, CD3 $\zeta$ -CFP), a GFP variant that is a good marker for synapse formation<sup>15</sup>. To detect TCR-derived signals, we used the pleckstrin homology (PH) domain of the serine/threonine kinase Akt, also known as protein kinase B (PKB), fused to yellow fluorescence protein (PH(AKT)-YFP). This and a similar approach using a GFP fusion of the PKB PH domain were shown to be well suited for detection of PI3 kinase activity in a macrophage cell line<sup>16</sup> and, more recently, in T cells<sup>17,18</sup>.

For our studies, we used primary lymph node T cell blasts from 5c.c7  $\alpha\beta$  TCR transgenic mice retrovirally transduced to express PH(AKT)-YFP as well as CD3 $\zeta$ -CFP. Infected T cells expressed moderate amounts of the reporter constructs and were indistinguishable from uninfected T cells in their response to moth cytochrome C 88-103 (MCC) peptide bound to the mouse class II molecule I-E $^k$  (Fig. 1). We were able to image the dynamics of both fluorophores nearly simultaneously, separated by less than 100 ms within each image plane.

We were able to see antigen-dependent generation of 3'-phosphoinositides by the rapid recruitment of PH(AKT)-YFP from cytoplasmic and nuclear locations to the interface after B cell contact (Fig. 2). As shown before<sup>17-19</sup>, this relocation was strictly dependent on the presence of antigen. At the time of B cell contact, CD3 $\zeta$ -CFP was already present in detectable clusters at the interface, then accumulated rapidly at sites of increased PI3K activity (Fig. 2d,e and Supplementary video online). This colocalization indicated a causal relationship between ligand recognition through the TCR and PI3K activation and was indicative of an early burst in TCR-mediated signaling activity. Subsequently, most of the TCR-associated CD3 $\zeta$ -CFP at the interface was internalized into a dense central cluster of vesicles minutes after APC contact. Measurement of TCR surface expression by flow cytometry showed that 5c.c7 T cells had internalized 70% of their TCRs 30 min after B cell contact (Supplementary Fig. 1 online). Despite this loss, PH(AKT)-YFP recruitment to the interface remained high. The localization of the PH domain and, by inference, PI3K activity continued throughout the duration of the assay (Fig. 2g) and for many hours (discussed below). PH(AKT)-YFP colocalized with the membrane-proximal but not the membrane-distal subset of internal CD3 $\zeta$ -CFP, indicating a gradual dissociation of PI3K

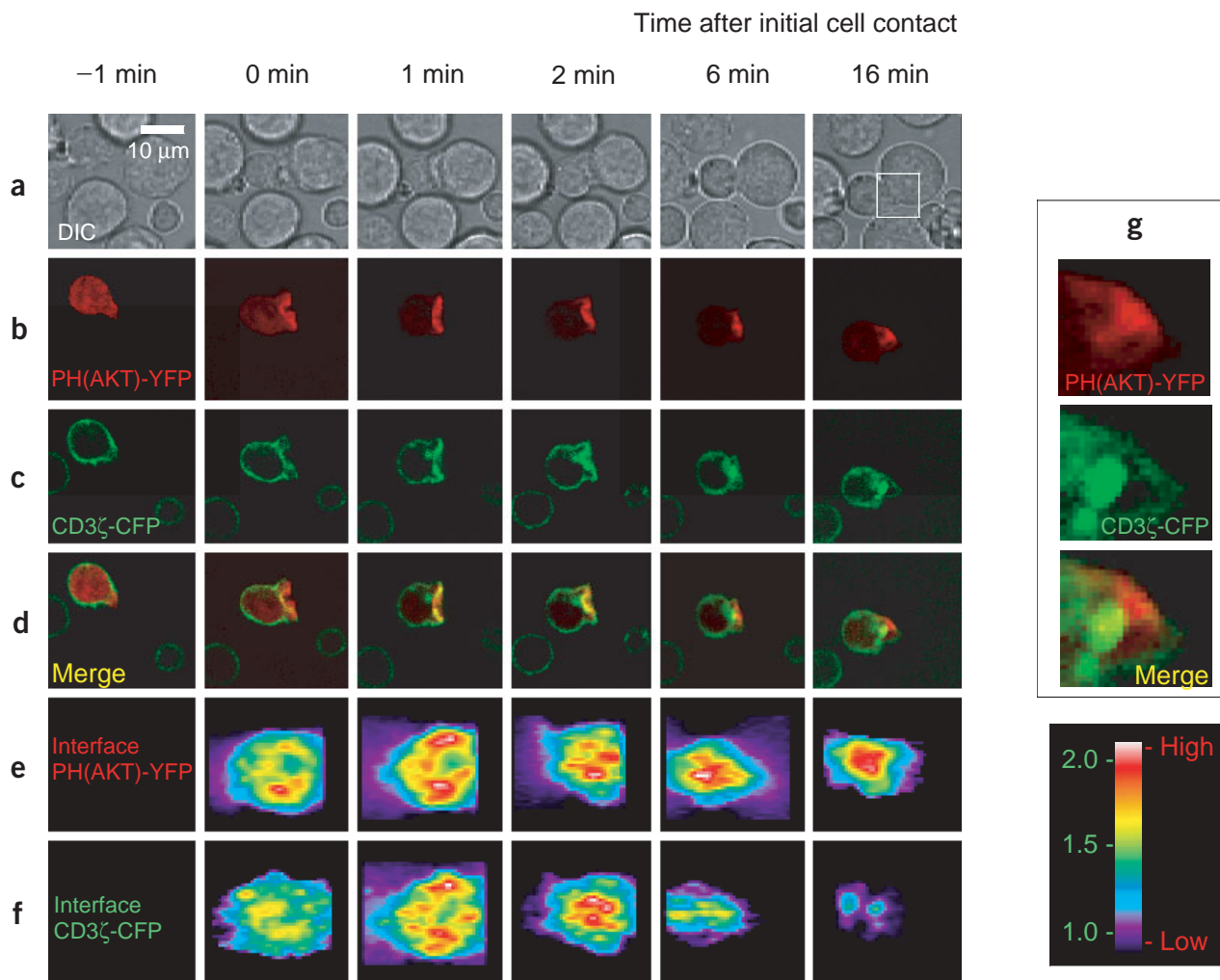
activity from internalized TCR destined for endosomal destruction or recycling<sup>11,20</sup>. In summary, removal of activated TCRs from contact interface had no appreciable effect on the amount of synapse-associated PI3K activity.

### TCR signals propelled sustained PI3K activity

Although the rapid onset of PI3K activity is antigen dependent and is at least in part directly linked to TCR-mediated activation of the transmembrane adaptors LAT (linker for activation of T cells) and TRIM (TCR-interacting molecule), costimulation through CD28 and activation of chemokine receptors also causes PI3K activation<sup>21</sup>. In addition, PI3K remains active for several hours after initial B cell contact<sup>17</sup>. To determine just how long PI3K activity persists and to what extent it depends on TCR-ligand engagement, we used monoclonal antibodies specific for I-E $^k$  (mAb 14.4.4) or I-E $^k$ /MCC (mAb D4), or isotype-matched control antibodies, to block TCR-mediated signaling in T cell-APC conjugates at 1, 3, 6 and 10 h after the experiment began. Typically, more than 90% of the T cells had made B cell contact within 15 min of cell pooling (data not shown). The ratio of cytoplasmic and membrane associated YFP fluorescence intensity served as a measure of 3'-phosphoinositide production.

T cells continued to generate 3'-phosphoinositides throughout the entire time course of the experiment, though in somewhat smaller amounts in the older conjugates (Fig. 3). This activity was strictly dependent on TCR-mediated signaling at all times, as antibody blockade of TCR ligands led to a complete dissipation of PH(AKT)-YFP localization at the interface.

We showed in real time the collapse of PI3K activity as a result of antigen neutralization in a PH(AKT)-YFP-expressing 5c.c7 TCR $\alpha\beta$  transgenic T cell that had been in contact with an MCC-loaded CH27 B cell for 5 h. Before addition of the blocking antibody, YFP fluorescence was confined mainly to the T cell plasma membrane contacting the B cell, but was also detectable to a minor extent outside the contact area. Withdrawal of stimulatory peptide-MHC ligands resulted in loss of surface-associated PI3K activity only 2 min after antibody addition. We thus infer that neither the synaptic architecture nor the binding status of stimulatory ligands provided appreciable protection against the blocking antibody. This result was consistent with a dynamic and permeable membrane apposition, allowing fast access of the antibody to the TCR ligands. It is also likely that fewer TCRs were bound to ligand at this stage. In a separate control experiment, we also included two monoclonal antibodies binding to I-A $^k$ , which are present on CH27 B cells but irrelevant for antigen recognition (Supplementary Fig. 2 online). Targeting nonantigenic MHC class II molecules by these antibodies had no effect on the PI3K signal. The blockade of antigenic stimuli led to a



**Figure 2** Antigen-induced PI3K activity colocalized with TCR-CD3 complexes within the nascent immunological synapse and remained mainly synapse associated at later stages despite substantial TCR internalization. T lymphocytes were isolated from 5c.c7  $\alpha\beta$  TCR transgenic mice and infected with two batches of retroviruses expressing PH(AKT)-YFP and CD3 $\zeta$ -CFP. Usually 15% of the T cells were positive for the expression of both constructs at the time of imaging (day 6). CH27 B cells had been pulsed with the MCC peptide (0.4  $\mu$ M) and were pooled with transduced T cells. **(a)** Differential interference contrast (DIC) acquisitions. **(b–d)** Epifluorescent midplane acquisitions of PH(AKT)-YFP **(b)**, CD3 $\zeta$ -CFP **(c)** and their corresponding overlays **(d)**. **(e,f)** Three-dimensional interface reconstructions of PH(AKT)-YFP **(e)** and CD3 $\zeta$ -CFP **(f)**. **(g)** A 'close-up' view of the area of contact at the 16-min time point (white rectangle, far right panel of **a**) of PH(AKT)-YFP (red) and CD3 $\zeta$ -CFP (green) and their corresponding overlay. To improve image quality, out-of-focus light was removed from fluorescent image stacks using a blind deconvolution algorithm. The white bar (far left panel of **a**) indicates object size; the 'false-color look-up table' (bottom right) indicates intensity values for interface reconstructions (high-low representation for PH(AKT)-YFP and fold increase (left margin) over average surface intensity for CD3 $\zeta$ -CFP).

breakup of the T cell–APC conjugate in 20% of cases. Thus, continuous stimulation through the TCR is required not only for the PI3K signal but also for cell adhesion that involves multiple signals.

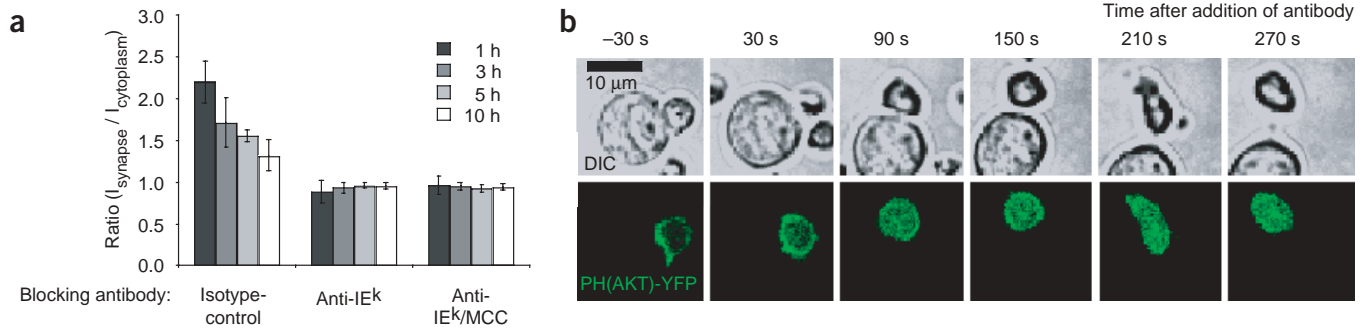
#### TCR-driven calcium mobilization

An increase in cytoplasmic intracellular calcium is essential for and is an early marker of T cell activation<sup>22</sup>. It can thus serve as another early indicator of the T cell response. Using the calcium-sensitive dye fura-2, we monitored T cell calcium and, analogous to the antibody blockade experiments described above, its dependence on continuous TCR-derived stimuli at various times after conjugate formation. A previous study successfully used this approach to provide a causal link between TCR occupancy and the calcium signal 1 min after conjugate formation<sup>23</sup>. Ratiometric analysis of fura-2-loaded T cells showed that the calcium signal persisted for more than 10 h after conjugate formation (Fig. 4). As with PI3K, the increased calcium depended on continuous

TCR signaling, as it could be suppressed at all time points by the anti-IE<sup>k</sup> blockade.

#### Sustained TCR signals maintain immunological synapse

Withdrawal of stimulatory antigenic stimuli resulted in the breakup of about 20% of the T cell–B cell couples (Fig. 3b and data not shown). The T cell integrin LFA-1 promotes T cell adhesion in an activation-dependent way by increasing its affinity for intracellular adhesion molecule 1 (ICAM-1) after stimulation<sup>24,25</sup>. One hallmark of synapse formation is the accumulation of ICAM-1–LFA-1 ligand pairs in a ring-like pattern<sup>3</sup>, with an occasional central enrichment within the T cell–B cell interface. We analyzed the extent to which maintenance of the ICAM-1 ring requires continuous TCR-mediated signaling in antibody-blockade experiments (Fig. 4b). We incubated MCC-loaded CH27 B cells expressing ICAM-1 fused to GFP (ICAM-1–GFP) with 5c.c7 T cells for up to 10 h and assessed ICAM-1–GFP distribution by fluorescence



**Figure 3** PI3K activity remained synapse-associated for more than 10 h in an antigen-dependent way. **(a)** Day-6 5c.c7 TCR $\alpha\beta$  transgenic T cells expressing PH(AKT)-YFP were pooled with CH27 B cells that had been pulsed with the MCC peptide (0.4  $\mu$ M). About 90% of the T cells were found in conjugates 15 min after cell pooling (data not shown). Monoclonal antibodies were added to conjugates (addition times, in key) and T cells were inspected for localization of PH(AKT)-YFP 15 min later. The ratio of synapse-associated fluorescence intensity to cytoplasmic fluorescence intensity ( $I_{\text{synapse}}/I_{\text{cytoplasm}}$ ) served as a relative measure for the amount of PI3K activity. Error bars represent the standard deviation of intensity ratios derived from 25 T cells that were inspected in each group. Blockade of stimulatory ligands resulted in detachment of about 20% of the T cells independently of the time after cell pooling. Anti-, antibody to. **(b)** Addition of antibody to I-E<sup>k</sup>-MCC (D4) led to a rapid cessation of synaptic PI3K activity and caused T cell detachment. Data represent the time course of the response of a 5-hour-old conjugate to antibody treatment. Times above the differential interference contrast (DIC) and PH(AKT)-YFP midplane images refer to the time of D4 addition.

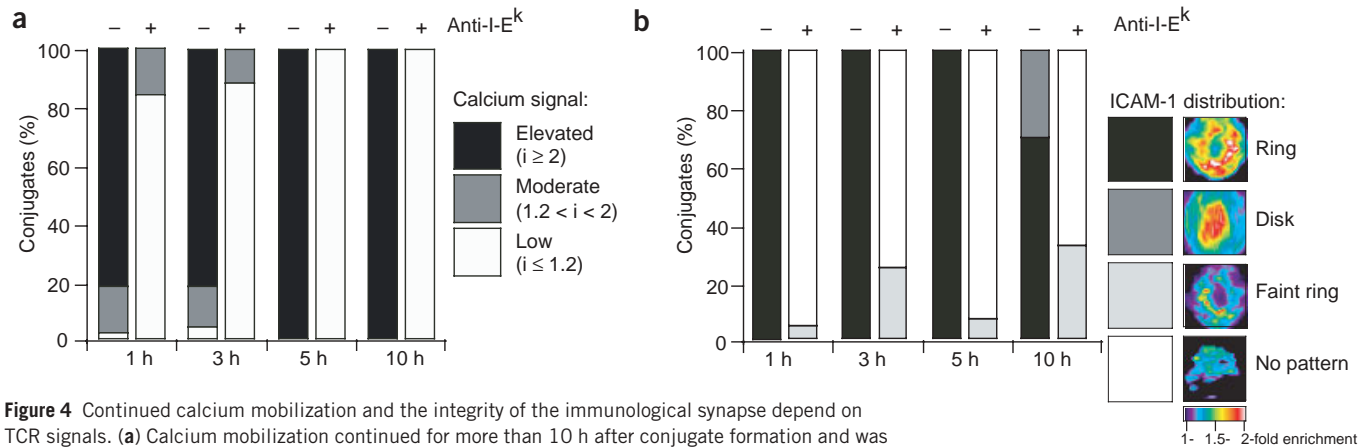
microscopy. Without antibody blockade, B cells showed the characteristic ring-like ICAM-1 accumulation pattern. After about 10 h, this changed to a disk-like pattern in about one third of the conjugates, presumably reflecting of a gradual termination of T cell–B cell communication. Addition of the antibody to I-E<sup>k</sup> (mAb 14.4.4) induced dissipation of the ICAM-1 ring at all time points after B cell encounter. Continuous TCR-mediated signals are therefore also required to maintain functional cell adhesion mediated by LFA-1 affinity up-regulation.

**Continued TCR signaling shapes effector potential**

We examined the importance of continuous T cell antigen recognition for the secretion of interleukin 2 (IL-2) and cell proliferation by T helper cells (Fig. 5). To enable rapid and synchronized conjugate

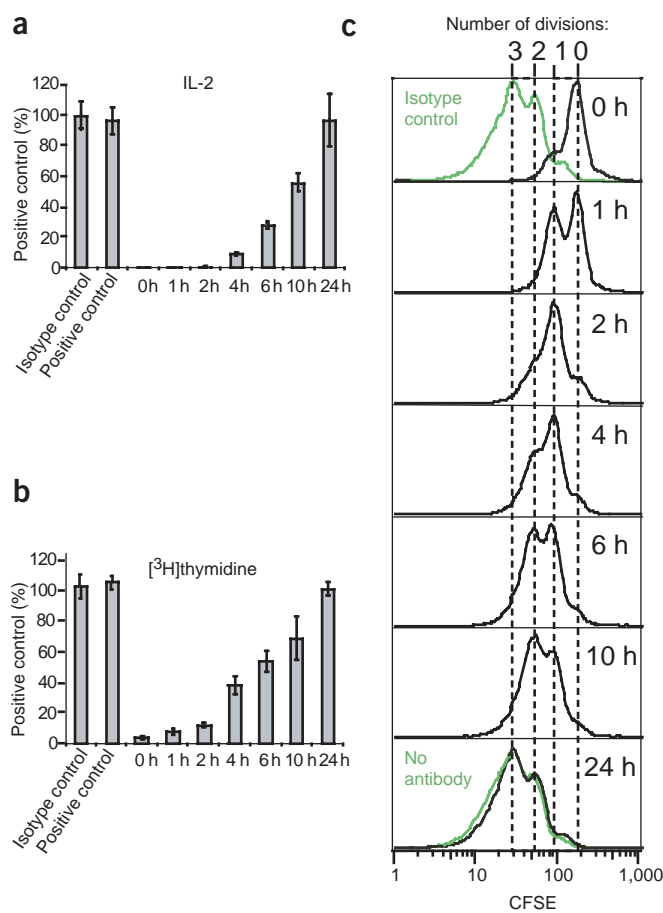
formation, we pooled T cells with irradiated and antigen-loaded B cells at a ratio of 1:2 and gently centrifuged them in 96-well round-bottomed plates. We determined the amount of IL-2 secreted into the media by enzyme-linked immunosorbent assay (ELISA) 48 h later (Fig. 5b). The detection of IL-2 amounts above background amounts required 4 h of uninterrupted T cell antigen recognition, yet more than 10 and less than 24 h were required for maximal IL-2 production. T cell proliferation, as measured by [<sup>3</sup>H]thymidine uptake 48 h after cell pooling, also required continuous antigenic stimulation, though to a lesser degree (Fig. 5c). Again, between 10 and 24 h of TCR stimulation were necessary for a full proliferative response.

To obviate the possibility that we had missed earlier or later proliferative events in our analysis, we determined the total number of cell



**Figure 4** Continued calcium mobilization and the integrity of the immunological synapse depend on TCR signals. **(a)** Calcium mobilization continued for more than 10 h after conjugate formation and was dependent on persistent TCR signals. Day-6 5c.c7 TCR $\alpha\beta$  transgenic T cell blasts were pooled at a ratio of 1:2 with CH27 B cells that had been pulsed with 0.4  $\mu$ M MCC peptide. Cells were loaded with the calcium-sensitive dye fura-2 15 min before and images were obtained 15 min after the addition of antibody to I-E<sup>k</sup> (Anti-I-E<sup>k</sup>; +, with; –, without) after cell pooling (antibody addition times, below graph). T cells could be easily identified by their smaller size. Based on the ratiometric analysis of fura-2 fluorescence, the increase (i) in calcium signal was classified as ‘elevated’ (more than 2-fold), ‘moderate’ (between 1.1-fold and 2-fold) or ‘low’ (between 1-fold and 1.2-fold).  $n = 50$ . **(b)** The integrity of the immunological synapse depended on persistent TCR signals. Day-6 5c.c7 TCR $\alpha\beta$  transgenic T cell blasts were pooled at a ratio of 1:2 with ICAM-1-GFP expressing CH27 B cells that had been pulsed with 0.4  $\mu$ M MCC peptide. Conjugates were inspected 15 min after the addition of antibody to I-E<sup>k</sup> (Anti-I-E<sup>k</sup>; +, with; –, without) after cell pooling (antibody addition times, below graph). Synaptic ICAM-1-GFP distributions were classified as ‘ring’ (ring-shaped enrichment of  $\geq 1.4 \times$  average surface density with an occasional central enrichment), ‘disk’ ( $\geq 1.4 \times$  average density spread over the entire contact area), ‘faint ring’ (ring-shaped ICAM-1 enrichment of  $< 1.4 \times$  average surface density) or ‘no pattern’ (random pattern with maximal enrichment of  $\leq 1.4 \times$  average density).  $n = 50$ .





**Figure 5** Sustained TCR signaling exerted a cumulative effect on T cell activation. **(a,b)** Antibody blockade interfered with the IL-2 and the proliferative response in a time-dependent way. Here, 5c.c7 TCR  $\alpha\beta$  transgenic T cell blasts were pooled with irradiated MCC-pulsed (0.4  $\mu$ M) CH27 B cells (0-h time point) and antibody to IE<sup>k</sup>/MCC was added after cell pooling (antibody addition times, below graphs). The positive control was left untreated. The isotype-matched control antibody (mAb Tü36, mouse IgG2b) was added at the 0-h time point. **(a)** Secreted IL-2 was determined from media supernatant by ELISA 48 h after cell pooling. **(b)** Cells were then pulsed for 16 h with [<sup>3</sup>H]thymidine and analyzed for thymidine uptake. Negative controls (T cells alone, B cells alone, and B cells and T cells pooled in the absence of MCC peptide) showed background amounts (data not shown). [<sup>3</sup>H]thymidine incorporation of the 0-h samples can be attributed to residual proliferation of irradiated B cells (<5% of positive control). Error bars refer to the standard deviation derived from quintuple data sets. The data shown are representative of three independent experiments. **(c)** Analysis of the number of cell divisions showed a cumulative effect of persistent TCR signals on the proliferative response. Here 5c.c7 TCR  $\alpha\beta$  transgenic T cell blasts were labeled with CFSE and pooled with irradiated MCC-pulsed CH27 B cells (0-h time point). Antibody to IE<sup>k</sup>/MCC (D4) was added after cell pooling (addition times, below graphs). The positive control was left untreated (no antibody; bottom). The isotype-matched control antibody (Tü36) was added at the 0-h time point. Cells were analyzed by flow cytometry 120 h after cell pooling. Data are representative of three independent experiments.

divisions by staining the T cells with carboxyfluorescein diacetate succinimidyl ester (CFSE)<sup>26</sup> (Fig. 5d). As is typical for effector and memory T cells, most T cells underwent three cell divisions after 5 d. In contrast, addition of the D4 antibody (to I-E<sup>k</sup>-MCC) at any time point up to 10 h resulted in a reduction in the number of cell divisions. This was most evident at the 0-h time point (no division) and was barely detectable at the 24-h time point (three divisions). Notably, 10 h of

uninterrupted TCR signaling gave rise to only two cell divisions and was thus insufficient for the T cells to proliferate to their full capacity.

## DISCUSSION

In this report we demonstrated that TCR signals were sustained throughout the lifetime of the T cell-APC interaction and that this signaling activity was required for the maintenance of the immunological synapse and for a full commitment to T cell activation. At its lowest point, the TCR density was approximately 30% of its original amount, consistent with previous reports<sup>10,11,20</sup>. Here we showed that TCR-CD3 internalization was well underway as early as 6 min after T cell-APC contact and seemed to localize to one central cluster of vesicles. Even with this loss, there were still many TCRs at the interface. Similarly, it seems likely that the failure to find TCR-proximal tyrosine phosphorylation events 30–60 min after initial cell contact<sup>4</sup> reflects the limitations of available detection methods rather than the absence of these events. Here, we accessed localized activation of PI3K as well as the mobilization of intracellular calcium to assess the contribution of TCR signaling. Although both signals could derive from signaling activities other than TCR-proximal tyrosine phosphorylation, the antibody-blockade studies confirmed their dependence on TCR signals at all times.

The requirement of persisting TCR signals for the progression of the T cell activation program sheds light on the physiology of T cell help but leaves many questions unanswered. Why are these conjugates long lived? Signal integration itself might require time and certainly concludes in time-consuming cellular processes such as gene transcription, translation, protein secretion and induction of mitosis. Although continued TCR signals seem to ‘fuel’ these processes, their requirement might exert a proofreading mechanism to avoid the activation of bystander APCs. Conversely, APCs might be given the necessary time to process T cell-derived stimuli. CD4<sup>+</sup> effector T cells become committed after only 1 h of stimulation by plate-bound MHC-peptide complexes and antibodies to CD28; prolonged antigenic stimulation (>5 h) led to activation-induced cell death rather than T cell proliferation<sup>12</sup>. The disparity between those results and ours reported here may indicate the importance of signals not provided by the plate-bound method of stimulation (such as cytotoxic T lymphocyte antigen 4 (CTLA-4)-mediated attenuation).

The activation of effector or memory T cells and the priming of naive T cells are distinct events in the immune response; they involve T cells of different developmental stages, engage different APCs, occur at different anatomical sites and fulfill different physiological functions<sup>27,28</sup>. Nonetheless, both types of activation are TCR-signal dependent. Although there is considerable controversy over the lifespan of individual APC-T cell interactions<sup>29–31</sup>, there is strong evidence that T cell priming is influenced by the duration of TCR stimulation. A recent report suggested that only 2 h of cell interaction are required for the priming of naive CD4<sup>+</sup> T cells through contact with T cell- and B cell-depleted splenocytes<sup>4</sup>. Consistent with the results reported here, those conditions gave rise to only one cell division, which probably represents a premature termination of priming. Indeed, prolonged TCR stimulation had a cumulative effect on the proliferative capacity of naive CD4<sup>+</sup> T cells that divide up to nine times<sup>32</sup>. A previous study indicated that the number of cell divisions in the T helper cell type 1 versus type 2 polarization of the effector T cell compartment might be involved<sup>33</sup>. The duration of TCR signaling might influence the development of helper T cell subsets, as suggested before<sup>34</sup>.

Antigen-dependent initiation of integrin-mediated cell adhesion is well characterized; however, much less is known about the mechanisms that lead to synapse termination. Representing a key feature of

the mature immunological synapse, the ring-shaped localization of the ICAM-1 adhesion molecule requires continuous TCR signaling, a finding with broad implications for cell-contact-dependent communication in adaptive immunity. A decrease in TCR signals may also be central to the physiological dissolution of T cell–B cell conjugates. Downregulation of surface TCR might constitute one but probably not the only mechanism contributing to signal attenuation. After an initial decrease in TCR surface density, equilibrium is probably reached through ongoing ligand-induced TCR internalization and replenishment of the cell surface pool with newly synthesized TCRs<sup>32</sup>. However, as shown here, TCR-mediated signaling continues for many hours even at reduced receptor surface expression. Signal attenuation might also result from the inhibitory function of CTLA-4, which competes with the costimulatory receptor CD28 for binding to B7-1 and B7-2, and disrupts TCR-proximal tyrosine phosphorylation events through recruitment of the intracellular SHP-2 phosphatase<sup>35</sup>. Undetectable in naive CD4<sup>+</sup> T cells, CTLA-4 protein expression is up-regulated in effector T cells, yet is confined mainly to endosomal compartments in the absence of antigenic stimuli<sup>36</sup>. CTLA-4 requires transport to the immunological synapse to exert its inhibitory function, and its accumulation there seems proportional to the strength of the antigenic signal<sup>37</sup>. Further studies might show a relationship between CTLA-4 recruitment and synapse termination.

Probably reflecting their distinct function in acquired immunity, a very different requirement for TCR signals applies to the activation of naive and effector or memory CD8<sup>+</sup> cytolytic T cells (CTLs). Without the need for costimulation, effector CTLs can kill any antigenic MHC class I-expressing nucleated cell during a comparatively short interaction through Fas-induced apoptosis or by delivery of lytic granules<sup>38</sup>. In the absence of a clearly identifiable regulatory function of CTLs, the main *raison d'être* of the CTL–target cell synapse might be to help verify antigenicity and provide spatial orientation for swift delivery of a lethal hit. A brief period of antigenic stimulation is sufficient to trigger clonal expansion and differentiation during CTL priming<sup>39,40</sup>. This notably different behavior might underlie the distinct regulation of the CTL response to pathogenic challenge, but should also be considered in the physiological context of cross-priming through CD4 helper T cells. APCs must up-regulate costimulatory ligands as a precondition for CTL priming, in a process that often involves much longer antigen-dependent interactions with naive CD4 helper T cells<sup>41–43</sup>.

In summary, our findings provide insight on the interdependence between signal reception through the immunological synapse and cellular signal integration. Antigenic ligands not only drive synapse formation but are also equally important for its maintenance. The need for some TCR signaling at all times demonstrates the rapid reversibility of the cellular mechanisms required to preserve an ordered cell–cell interface. Such a requirement also seems to represent a regulatory feedback mechanism, which would help to determine the response to an antigenic challenge.

## METHODS

**Plasmids and cell lines.** DNA encoding PH(AKT) as cloned into pEYFP-C1 (ref. 16) was provided by T. Meyer (Stanford University). PCR using the primers JH3/01 (5'-CCATCGATGGAGTGAACCGTCAGATCCGCTAGCGCTACCGGTC-3') and JH4/01 (5'-GGATCGATCCTCTACAATGTGGTATGGCTGATTA TGATCA-3') gave rise to a 1.2-kb fragment that was inserted through *Clal* (underlined sequence) into the retroviral expression vector pMG-1B (a gift from G. Nolan, Stanford University). DNA encoding full-length mouse CD3ζ-CFP (provided by M. Krummel, University of California at San Francisco) was cloned into the retroviral expression vector pIB II using the 5' *XhoI* and 3' *NotI* restriction sites. Both retroviral expression vectors are derived from the MFG

series of retroviral vectors and have full-length Moloney long-terminal repeats, an extended Ψ sequence and an internal ribosomal entry site upstream of the blasticidin-S deaminase gene. For virus production, the ecotropic virus packaging line Phoenix E (a gift from G. Nolan, Stanford University) was stably transfected with these expression vectors.

**Antibodies and mice.** Antibodies 14.4.4 (to IE<sup>k</sup>)<sup>44</sup> and D4 (to IE<sup>k</sup>-MCC)<sup>45</sup> were purified from hybridoma culture supernatant. RM4-5 (antibody to mouse CD4) conjugated to Cy5-phycoerythrin or fluorescein isothiocyanate<sup>46</sup>, Tü36 (mouse IgG2b, κ antibody to HLA DR), Tü39 (mouse IgG2a, κ antibody to HLA DP, DQ, DR)<sup>47</sup> and 2.4G2 (antibody to mouse CD16/CD32, FcγIII/II)<sup>48</sup> were purchased from BD PharMingen. The 5c.c7 TCRαβ transgenic mice (on a B10A background) were purchased from Jackson laboratories.

**Retroviral transduction.** Virus-producing lines were grown at 32 °C for 2 d in 15-cm culture dishes. For dual-color experiments, packaging lines (producing CD3ζ-CFP and PH(AKT)-YFP expressing viruses) were pooled and cultured together. Collected media supernatant was filtered through syringe filters with a pore size of 0.45 μm (Schleicher and Schuell) and centrifuged overnight at 10,000 r.p.m. (Sorvall, RC5B centrifuge, SA600 rotor). At 24 h before infection (day 0), lymphocytes were isolated from lymph nodes of 5c.c7 transgenic mice and stimulated in 24-well plates in the presence of 2 μM HPLC-purified MCC peptide (ANERADLIAYLQADK) at a density of 7.5 × 10<sup>6</sup> cells per well and per milliliter of full medium (RPMI containing 10% FCS (Gemini), 2 mM L-glutamine (Irvine Scientific), 100 U/ml penicillin/streptomycin, 50 μM β-mercaptoethanol (Sigma-Aldrich) and 1 mM sodium pyruvate (Irvine Scientific)). For infection, the virus-containing pellets from the overnight centrifugation and 5 μg/ml polybrene (Sigma-Aldrich) were added to cells (one pellet per milliliter of cell culture), which were then subjected to a 1-h centrifugation in 24-well plates at 2,500 r.p.m. (Allegra 6KR; Beckman Coulter); this was considered day 1. Cells were cultured at 37 °C. On day 2, 100 U/ml IL-2 was added, and on day 3, 40 μg/ml blasticidin-S (Invitrogen) was added. On day 5, dead cells were removed by centrifugation through a Histopaque-1119 (Sigma-Aldrich) cushion (7 min at 2,000 r.p.m. in a Beckman Allegra 6KR tabletop centrifuge). Cells were used for experiments on day 6 and day 9.

**Microscopy.** Experiments were done using a Zeiss Axiovert S100TV microscope (Carl Zeiss) and a 40× Fluor (NA 1.3) objective. The microscope was fitted with a high-speed piezo-driven Z-dimension motor (Physik Instrumente). A Sutter DG-4 high speed optical changer (Sutter Instruments) equipped with a 300-W Xenon light bulb served as a bright excitation source and supports changes in excitation wavelengths within 1 ms. For dual-color experiments, emission was optically split using a Dual-View Micro-Imager (Optical Insights) and analyzed synchronously using CFP and YFP emission filters (Chroma Technology). Images were acquired with a cooled interline Coolsnap hq camera (Roper Scientific). Z-stacks usually contained 21 image planes that were 1 μm apart that were acquired in the camera streaming mode to minimize exposure time. Imaging experiments were done at 37 °C and 7% CO<sub>2</sub> in imaging buffer (full RPMI medium without phenol red) using an objective heater (Bioprotech) and a Zeiss environmental control system.

For calcium imaging, T cells were pulsed for 30 min at room temperature with 5 μg/ml fura-2-am (Molecular Probes) and washed once in imaging buffer. The ratio of the fura-2 emission intensity at 510 nm resulting from 340-nm and 380-nm excitation was determined to quantify the relative intracellular calcium concentration. Data acquisition and analysis were done in Metamorph 5.0 (Universal Imaging). To remove out-of-focus light, three-dimensional fluorescence image stacks were subjected to 40 iterations of a blind deconvolution algorithm (Deblur 8.0; AutoQuant Imaging).

**IL-2 secretion ELISA and [<sup>3</sup>H]thymidine incorporation.** T cell blasts (1 × 10<sup>5</sup>; day 9) were pooled with irradiated (12,000 rad) CH27 B cells (2 × 10<sup>5</sup>) that had been pulsed overnight with 0.4 μM MCC peptide, in 96-well round-bottomed plates in a volume of 200 μl and spun at 500 r.p.m. in a Beckman Allegra 6KR tabletop centrifuge for 2 min to enforce cell contact formation. After 48 h, 50 μl culture supernatant were removed and assayed for IL-2 by ELISA as described before<sup>49</sup>. Each well was then pulsed for 16 h with 1 μCi [<sup>3</sup>H]thymidine/200 μl. Incorporated [<sup>3</sup>H]thymidine was quantified as described before<sup>49</sup>.

**CFSE assay.** T cells (day 9) were washed twice in Hank's balanced salt solution plus 2 mM calcium and adjusted to a concentration of  $1 \times 10^6$  cells per ml, then labeled for 10 min at 37 °C with 1  $\mu$ g CFSE (Molecular Probes). Cells were washed twice in full media. T cells ( $1 \times 10^5$ ) were pooled with irradiated ( $1.2 \times 10^4$  rad) and MCC-pulsed (0.4  $\mu$ M) CH27 B cells ( $2 \times 10^6$ ) in 96-well round-bottomed plates in a volume of 200  $\mu$ l and spun at 500 r.p.m. in a Beckman Allegra 6KR tabletop centrifuge for 2 min to enforce cell contact formation. After 3 d, cells were transferred to 48-well plates and 300  $\mu$ l fresh media was added to each sample. At 120 h after cell pooling, cells were stained with monoclonal antibody to mouse CD4 conjugated to Cy5-phycoerythrin and analyzed by flow cytometry (Epics Elite ELP; Beckman-Coulter). Samples were analyzed using Flow-Jo software (Treestar).

*Note: Supplementary information is available on the Nature Immunology website.*

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#### COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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- Grakoui, A. *et al.* The immunological synapse: a molecular machine controlling T cell activation. *Science* **285**, 221–227 (1999).
- Dustin, M.L., Bromley, S.K., Davis, M.M. & Zhu, C. Identification of self through two-dimensional chemistry and synapses. *Annu. Rev. Cell. Dev. Biol.* **17**, 133–157 (2001).
- Monks, C.R., Freiberg, B.A., Kupfer, H., Sciaky, N. & Kupfer, A. Three-dimensional segregation of supramolecular activation clusters in T cells. *Nature* **395**, 82–86 (1998).
- Lee, K.H. *et al.* T cell receptor signaling precedes immunological synapse formation. *Science* **295**, 1539–1542 (2002).
- Kupfer, H., Monks, C.R. & Kupfer, A. Small splenic B cells that bind to antigen-specific T helper (Th) cells and face the site of cytokine production in the Th cells selectively proliferate: immunofluorescence microscopic studies of Th-B antigen-presenting cell interactions. *J. Exp. Med.* **179**, 1507–1515 (1994).
- Delon, J., Stoll, S. & Germain, R.N. Imaging of T-cell interactions with antigen presenting cells in culture and in intact lymphoid tissue. *Immunol. Rev.* **189**, 51–63 (2002).
- Richie, L.I. *et al.* Imaging synapse formation during thymocyte selection: inability of CD3z to form a stable central accumulation during negative selection. *Immunity* **16**, 595–606 (2002).
- Stinchcombe, J.C., Bossi, G., Booth, S. & Griffiths, G.M. The immunological synapse of CTL contains a secretory domain and membrane bridges. *Immunity* **15**, 751–761 (2001).
- Kane, L.P., Lin, J. & Weiss, A. Signal transduction by the TCR for antigen. *Curr. Opin. Immunol.* **12**, 242–249 (2000).
- Valitutti, S., Muller, S., Cella, M., Padovan, E. & Lanzavecchia, A. Serial triggering of many T-cell receptors by a few peptide-MHC complexes. *Nature* **375**, 148–151 (1995).
- Liu, H., Rhodes, M., Wiest, D.L. & Vignali, D.A. On the dynamics of TCR:CD3 complex cell surface expression and downmodulation. *Immunity* **13**, 665–675 (2000).
- Iezzi, G., Karjalainen, K. & Lanzavecchia, A. The duration of antigenic stimulation determines the fate of naive and effector T cells. *Immunity* **8**, 89–95 (1998).
- Zal, T., Zal, M.A. & Gascoigne, N.R. Inhibition of T cell receptor-coreceptor interactions by antagonist ligands visualized by live FRET imaging of the T-hybridoma immunological synapse. *Immunity* **16**, 521–534 (2002).
- Bunnell, S.C. *et al.* T cell receptor ligation induces the formation of dynamically regulated signaling assemblies. *J. Cell. Biol.* **158**, 1263–1275 (2002).
- Krummel, M.F., Sjaastad, M.D., Wulfig, C. & Davis, M.M. Differential clustering of CD4 and CD3zeta during T cell recognition. *Science* **289**, 1349–1352 (2000).
- Botelho, R.J. *et al.* Localized biphasic changes in phosphatidylinositol-4,5-bisphosphate at sites of phagocytosis. *J. Cell. Biol.* **151**, 1353–1368 (2000).
- Costello, P.S., Gallagher, M. & Cantrell, D.A. Sustained and dynamic inositol lipid metabolism inside and outside the immunological synapse. *Nat. Immunol.* **3**, 1082–1089 (2002).
- Harriague, J. & Bismuth, G. Imaging antigen-induced PI3K activation in T cells. *Nat. Immunol.* **3**, 1090–1096 (2002).
- Marshall, J.G. *et al.* Restricted accumulation of phosphatidylinositol 3-kinase products in a plasmalemmal subdomain during Fc  $\gamma$  receptor-mediated phagocytosis. *J. Cell Biol.* **153**, 1369–1380 (2001).
- Valitutti, S., Muller, S., Saiio, M. & Lanzavecchia, A. Degradation of T cell receptor (TCR)-CD3-z complexes after antigenic stimulation. *J. Exp. Med.* **185**, 1859–1864 (1997).
- Ward, S.G. & Cantrell, D.A. Phosphoinositide 3-kinases in T lymphocyte activation. *Curr. Opin. Immunol.* **13**, 332–338 (2001).
- Feske, S., Giltman, J., Dolmetsch, R., Staudt, L.M. & Rao, A. Gene regulation mediated by calcium signals in T lymphocytes. *Nat. Immunol.* **2**, 316–324 (2001).
- Valitutti, S., Dessing, M., Aktories, K., Gallati, H. & Lanzavecchia, A. Sustained signaling leading to T cell activation results from prolonged T cell receptor occupancy. Role of T cell actin cytoskeleton. *J. Exp. Med.* **181**, 577–584 (1995).
- Lollo, B.A., Chan, K.W., Hanson, E.M., Moy, V.T. & Brian, A.A. Direct evidence for two affinity states for lymphocyte function-associated antigen 1 on activated T cells. *J. Biol. Chem.* **268**, 21693–21700 (1993).
- Labadia, M.E., Jeanfavre, D.D., Caviness, G.O. & Morelock, M.M. Molecular regulation of the interaction between leukocyte function-associated antigen-1 and soluble ICAM-1 by divalent metal cations. *J. Immunol.* **161**, 836–842 (1998).
- Lyons, A.B. & Parish, C.R. Determination of lymphocyte division by flow cytometry. *J. Immunol. Methods* **171**, 131–137 (1994).
- Banchereau, J. & Steinman, R.M. Dendritic cells and the control of immunity. *Nature* **392**, 245–252 (1998).
- Mellman, I., Turley, S.J. & Steinman, R.M. Antigen processing for amateurs and professionals. *Trends Cell Biol.* **8**, 231–237 (1998).
- Gunzer, M. *et al.* Antigen presentation in extracellular matrix: interactions of T cells with dendritic cells are dynamic, short lived, and sequential. *Immunity* **13**, 323–332 (2000).
- Miller, M.J., Wei, S.H., Parker, I. & Cahalan, M.D. Two-photon imaging of lymphocyte motility and antigen response in intact lymph node. *Science* **296**, 1869–1873 (2002).
- Stoll, S., Delon, J., Brotz, T.M. & Germain, R.N. Dynamic imaging of T cell-dendritic cell interactions in lymph nodes. *Science* **296**, 1873–1876 (2002).
- Schrump, A.G. & Turka, L.A. The proliferative capacity of individual naive CD4<sup>+</sup> T cells is amplified by prolonged T cell antigen receptor triggering. *J. Exp. Med.* **196**, 793–803 (2002).
- Bird, J.J. *et al.* Helper T cell differentiation is controlled by the cell cycle. *Immunity* **9**, 229–237 (1998).
- Iezzi, G., Scotet, E., Scheidegger, D. & Lanzavecchia, A. The interplay between the duration of TCR and cytokine signaling determines T cell polarization. *Eur. J. Immunol.* **29**, 4092–4101 (1999).
- Lee, K.M. *et al.* Molecular basis of T cell inactivation by CTLA-4. *Science* **282**, 2263–2266 (1998).
- Egen, J.G., Kuhns, M.S. & Allison, J.P. CTLA-4: new insights into its biological function and use in tumor immunotherapy. *Nat. Immunol.* **3**, 611–618 (2002).
- Egen, J.G. & Allison, J.P. Cytotoxic T lymphocyte antigen-4 accumulation in the immunological synapse is regulated by TCR signal strength. *Immunity* **16**, 23–35 (2002).
- Harty, J.T., Tinnereim, A.R. & White, D.W. CD8<sup>+</sup> T cell effector mechanisms in resistance to infection. *Annu. Rev. Immunol.* **18**, 275–308 (2000).
- Kaech, S.M. & Ahmed, R. Memory CD8<sup>+</sup> T cell differentiation: initial antigen encounter triggers a developmental program in naive cells. *Nat. Immunol.* **2**, 415–422 (2001).
- van Stipdonk, M.J., Lemmens, E.E. & Schoenberger, S.P. Naive CTLs require a single brief period of antigenic stimulation for clonal expansion and differentiation. *Nat. Immunol.* **2**, 423–429 (2001).
- Carbone, F.R., Kurts, C., Bennett, S.R., Miller, J.F. & Heath, W.R. Cross-presentation: a general mechanism for CTL immunity and tolerance. *Immunol. Today* **19**, 368–373 (1998).
- Schoenberger, S.P., Toes, R.E., van der Voort, E.I., Offringa, R. & Melief, C.J. T-cell help for cytotoxic T lymphocytes is mediated by CD40-CD40L interactions. *Nature* **393**, 480–483 (1998).
- Bennett, S.R. *et al.* Help for cytotoxic-T-cell responses is mediated by CD40 signalling. *Nature* **393**, 478–480 (1998).
- Ozato, K., Mayer, N. & Sachs, D.H. Hybridoma cell lines secreting monoclonal antibodies to mouse H-2 and Ia antigens. *J. Immunol.* **124**, 533–540 (1980).
- Reay, P.A. *et al.* Determination of the relationship between T cell responsiveness and the number of MHC-peptide complexes using specific monoclonal antibodies. *J. Immunol.* **164**, 5626–5634 (2000).
- Fredrickson, G.G. & Basch, R.S. Early thymic regeneration after irradiation. *Dev. Comp. Immunol.* **18**, 251–263 (1994).
- Pawelec, G., Ziegler, A. & Wernet, P. Dissection of human allostimulatory determinants with cloned T cells: stimulation inhibition by monoclonal antibodies TU22, 34, 35, 36, 37, 39, 43, and 58 against distinct human MHC class II molecules. *Hum. Immunol.* **12**, 165–176 (1985).
- Unkeless, J.C. Characterization of a monoclonal antibody directed against mouse macrophage and lymphocyte Fc receptors. *J. Exp. Med.* **150**, 580–596 (1979).
- Irvine, D.J., Purbhoo, M.A., Krosggaard, M. & Davis, M.M. Direct observation of ligand recognition by T cells. *Nature* **419**, 845–849 (2002).