CD4 T cells integrate signals delivered during successive DC encounters in vivo

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The cellular mode of T cell priming in vivo remains to be characterized fully. We investigated the fate of T cell–dendritic cell (DC) interactions in the late phase of T cell activation in the lymph node. In general, CD4 T cells detach from DCs before undergoing cell division. Using a new approach to track the history of antigen (Ag)-recognition events, we demonstrated that activated/divided T cells reengage different DCs in an Ag-specific manner. Two-photon imaging of intact lymph nodes suggested that T cells could establish prolonged interactions with DCs at multiple stages during the activation process. Importantly, signals that are delivered during subsequent DC contacts are integrated by the T cell and promote sustained IL-2R expression and IFN-γ production. Thus, repeated encounters with Ag-bearing DCs can occur in vivo and modulate CD4 T cell differentiation programs.

Cellular encounter between antigen (Ag)-specific T lymphocytes and DCs is a central event for the initiation of CD4 and CD8 T cell responses. In lymph nodes, this encounter is favored by the high level of T cell motility and dynamic changes of DC shape. This allows an individual DC to “scan” 500–5,000 distinct T lymphocytes per hour (1, 2). Recently, dynamic imaging of T cell–DC contacts provided in vivo evidence for the existence of short- and long-lasting contacts; the latter occur preferentially between 5 and 24 h (1, 3–5). These studies suggested that T cells have the opportunity to “see” multiple Ag-bearing DCs, at least in certain experimental settings. Much less is known about the T cell–DC interaction at later stages of T cell priming (e.g., after commitment to cell division). So far, the technical difficulty in visualizing the same individual T cell for an extended period of time (>1 h) and to read-out Ag-recognition events have hampered the direct demonstration that individual T cells can engage distinct DCs successively in an Ag-dependent manner in vivo.

An additional key question is whether each encounter with Ag-bearing DCs is associated with the delivery of a signal, which is relevant for activation/differentiation, that is integrated by the T cell. This idea was developed initially by Gunzer et al. (6) who showed that T cell proliferation in a collagen matrix was the result of multiple, short T cell–DC contacts. In vitro experiments that were aimed at periodically blocking signal transduction in T cells that interacted with an APC supported the idea that IFN-γ production by T cells could be induced by intermittent signals (7). Depending on the system used, reexposure of in vitro activated CD4 T cells to Ag can sustain cell proliferation, promote IFN-γ production, or induce cell death (8, 9).

In vivo, the microenvironment of lymphoid organs dictates the choreography of T cells and DCs, and thus, influences the pattern of cellular association and dissociation between T cells and DCs (10). Whether T cells have the opportunity and the ability to accumulate signals that are delivered by different DCs in vivo, before and/or after commitment to cell division, remains to be determined.

We demonstrate that a substantial fraction of CD4 T cells interacts with several DCs in an Ag-specific manner in vivo. Importantly, by modulating the probability for a T cell to reencounter an Ag-bearing DC, we provide in vivo evidence that CD4 T cells integrate signals that are delivered during these subsequent interactions with DCs.

RESULTS
Ag–dependent CD4 T cell–DC contacts in the late stage of T cell priming
To recapitulate a CD4 T cell response, we adoptively transferred carboxyl fluorescein succinimidyl ester (CFSE)-labeled CD4 T cells bearing the anti-HY Marylin TCR into female B6 re-
recipient mice that were injected intradermally (i.d.) with 2 × 10^6 male or female splenic CD11c^+ DCs. We showed previously, using this protocol, that DCs that migrate to the draining lymph node have a mature phenotype, as detected ex vivo by high expression of MHC class II and CD86 (1). In response to Ag (injection of male DCs), Marylin T cells up-regulated CD69 within 24 h, and cell divisions were detected after 44–48 h (Fig. 1 a; not depicted). In addition, many CD4 T cells acquired the ability to produce IFN-γ as detected by intracellular cytokine staining (Fig. 1 b).

Previous work showed that interactions with injected DCs and with endogenous DCs (that have taken up the Ag from injected DCs) are relevant to the activation process (11). As shown in Fig. S1 (available at http://www.jem.org/cgi/content/full/jem.20051018/DC1), Marylin T cells proliferated vigorously, and acquired the ability to produce IFN-γ when MHC class II–deficient recipients were immunized with wild-type male DCs (to favor direct presentation). These observations indicate that interactions between CD4 T cells and injected DCs play an important role in driving CD4 T activation and differentiation. CD4 T cell proliferation also occurred when wild-type recipients were immunized with MHC class II–deficient male DCs (indirect presentation), which indicated that endogenous APCs also can induce T cell activation.

To gain some insight into CD4 T cell–DC contacts that occur in the late phase of priming, we imaged lymph node frozen sections of mice 48 h after injection of dye-labeled T cells and DCs. Three-dimensional stacks of images were used to quantitate the CFSE content and the cell size of individual Marylin T cells. In the presence of female DCs, Marylin T cells remained small and displayed a homogenous amount of CFSE fluorescence (Fig. 2 a). In contrast, when male DCs were injected, subsets of undivided blasts (large cells with high CFSE content) and divided blasts (large cells with low CFSE content) were detected (Fig. 2, b–d). Thus, the use of high-resolution three-dimensional confocal images made it possible to visualize the hallmark of blastogenesis and cell division in situ. Most importantly, this approach enabled us to determine...
whether activated/divided T cells were interacting with an Ag-bearing DC at the time point analyzed (Fig. 2 e). 22% of undivided T cell blasts and a roughly similar fraction of divided T cell blasts (18%) were interacting with a male DC at 48 h. This might be an underestimation because some of the DC dendrites may not have been visible in our settings.

To determine if these cellular interactions were Ag-driven, male and female DCs were labeled differently and injected simultaneously at day 0. At 48 h, we compared the ability of male and female DCs to interact with Marylin T cell blasts (undivided or divided). The average number of T cell blasts per DC was 0.58 for male DCs (n = 18 DCs) but only 0.08 for female DCs (n = 39). We conclude that some Ag-driven contacts between DC and T cell blasts (undivided or divided) take place in the late phase of the priming process.

**CD4 T cells can engage different DCs in an Ag-dependent manner during activation**

We next assessed the history of Ag recognition for activated/divided T cells that were contacting a male DC at 48 h. Specifically, we asked whether these cells were engaged in a single or multiple Ag-dependent interactions during the activation process. One possibility is that T cell blasts usually interacted with a single DC and maintained this initial contact after blastogenesis and/or cell division. Alternatively, these cells could have received an initial signal from a different DC than the one with which they interacted at 48 h. To discriminate between these two possibilities, we used the following strategy (Fig. 3 a). On day zero, recipient B6 mice received an injection of CFSE-labeled Marylin T cells (i.v.) and SNARF-labeled male DCs (i.d.). At 24 h, a second injection of male DCs (labeled with a mixture of CFSE and SNARF dyes so that they appeared yellow on confocal images) was performed. CD4 T cell–DC interactions were analyzed at 48 h in the draining lymph node. A critical point of this experiment is that no proliferation was detected at 48 h when the first DC injection was missing (Fig. 3), or if female DCs were used for the first injection (depicted in Fig. 6). In these cases, proliferation was observed only at 72 h (unpublished data), which reflects a minimum delay of ~42–44 h between DC injection and the onset of cell division. As shown in Fig. 3, b and d, large T cell blasts corresponding to undivided cells also were virtually absent at 48 h in recipients that only received the second DC injection. Therefore, in recipient mice that received two DC injections, all divided T cells and the vast majority of undivided T cell blasts that were observed at 48 h received signals from DCs of the first injection. We reasoned that if these CD4 T cells made contact with only one DC, they still should have been interacting with a DC from the first injection. Conversely, if the T cell had detached from the DC that delivered the initial Ag-dependent signal and reencountered a different DC, it would have an equal probability of interacting with DCs from each injection. As shown in Fig. 4 and Fig. S2 (available at http://www.jem.org/cgi/content/full/jem.20051018/DC1), DCs from each wave had an approximately equal ability to interact with recently activated Marylin T cells (undivided blasts or divided T cells). The average number of blast T cells per DC was 0.20 (n = 178 DCs) for wave 1 and 0.18 (n = 258 DCs) for wave 2. A repetition of this experiment yielded similar results. The same conclusion held true when the analysis was restricted to undivided blasts or divided T cells (Fig. 4; not depicted). We conclude from this experiment that Marylin T cells can engage DCs multiple times (at least twice) during the activation process.

To confirm that the interactions between T cell blasts and DCs from the second wave were Ag dependent, we gave a first DC injection using unlabeled male DCs and a second injection using a mixture of differently labeled male and female DCs. The ability of male and female DCs from the second injection to interact with T cell blasts was compared on confocal images of lymph node sections. On average, male DCs engaged 3.2-fold more T cell blasts than did female DCs (n = 99 male DCs; n = 20 female DCs). We conclude from this result that DC reengagement by activated T cells is Ag dependent. Overall, these experiments show that T cells often reengage a DC that is distinct from the one that promoted the initial activation event. These subsequent interactions were Ag dependent.

**Figure 3. Kinetics of CD4 T cell blastogenesis and division.** (a) Experimental design. Recipient mice were adoptively transferred with 10⁶ CFSE-labeled Marylin T cells and received one or two of the following i.d. injections: injection 1 performed on day 0 with 2 x 10⁶ SNARF-labeled male DCs, and injection 2 performed at 24 h with 2 x 10⁶ male DCs labeled with a mixture of CFSE and SNARF dyes. Draining inguinal lymph nodes were harvested at 48 h. (b) Lymph node cells from recipients that received the first or second DC injection only were analyzed by flow cytometry. Data are gated on CD4⁺CD45R1⁺ lymphocytes. c and d) Lymph node cells from recipients that received the first (c) or the second (d) DC injection only were sectioned and processed for confocal imaging. Marylin T cells (green); DCs from wave 1 (red); DCs from wave 2 (orange). The presence of large undivided T cell blasts and divided T cells was not seen at the time of analysis in recipient mice that received the second DC injection only. Bars, 20 μm.
interactions occur at various stages of T cell activation, including after commitment to cell division.

Dynamics of T cell–DC contacts in the late phase of T cell activation

To extend our findings, we analyzed the T cell–DC contacts using two-photon imaging of intact lymph nodes. First, we examined the dynamics of individual T cell–DC contacts at 24 h (Fig. 5 a). As a result of the rapid shape changes of DCs, sometimes it was difficult to follow T cell–DC contacts for long periods of time (>20 min), because conjugates often left the area imaged. To minimize this problem, we followed the fate of individual T cell–DC contacts for a period of 10 min, which is the typical duration of a short-lived interaction. As shown in Fig. 5 a and Videos 1 and 2 (available at http://www.jem.org/cgi/content/full/jem.20051018/DC1), the vast majority (90%, 18/20) of T cell–DC interactions lasted >10 min. This observation suggests that the contact duration between Marylin T cells and male DCs that were observed at 24 h is in the range of hours, and confirms earlier results that were obtained with other transgenic TCRs. In contrast, when female DCs were used as stimulators, virtually all contacts (95%, 52/55) were terminated within 10 min (Fig. 5 b; Videos 3 and 4, available at http://www.jem.org/cgi/content/full/jem.20051018/DC1).

Next, we examined subsequent interactions between activated T cells and Ag-bearing DCs (Fig. 5, c and d). Naive female mice were adoptively transferred with CFSE-labeled Marylin CD4 T cells; they received injections of unlabeled male DCs on day 0 and SNARF-labeled male DCs on day 1. Intact lymph nodes were subjected to two-photon imaging on day 2. We focused our analysis on T cell blasts that were engaged with a labeled DC, because they had interacted with at least two DCs. T cell blasts that were not in contact with a labeled DC at the beginning of the experiment usually were highly motile, and made only brief (<5 min) contacts with the labeled DCs that happened to be in their trajectories (Fig. 5 c; Video 5, available at http://www.jem.org/cgi/content/full/jem.20051018/DC1). Conversely, of the contacts that were not terminated during the first 5 min (15/31), all were maintained at 10 min (Fig. 5 c). Typically, these conjugates were maintained during the entire imaging period (30 min) or...
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moved out of the imaged area, which precluded further analysis (Fig. 5d; Videos 5–7, available at http://www.jem.org/cgi/content/full/jem.20051018/DC1). These observations indicate that (a) most T cell blasts are motile, (b) migrating T cell blasts do not necessarily stop upon DC encounter, and may receive signals during transient contact with DCs, and (c) a subset of activated T cells that make subsequent contact with a DC forms prolonged interactions (>30 min). Overall, our results suggest that T cells can establish prolonged interactions with DCs at multiple stages during the activation process.

CD4 T cells integrate signals that are delivered by successive encounters with Ag-bearing DCs

Next, we assessed whether subsequent encounters between recently activated T cells and DCs influenced T cell differentiation. We compared the activation of T cells that had a low probability to encounter several Ag-bearing DCs during the activation process. Injection of different numbers of DCs is one way to vary the probability of T cell–DC encounters in the lymph node. The caveat of this approach is

Figure 5. CD4 T cells can establish prolonged interactions with DCs at multiple stages during the activation process. (a and b) T cell–DC contacts at 24 h. Recipient mice were injected in the footpad with 2 × 10^6 SNARF-labeled male (a) or female (b) DCs, and were adoptively transferred with 10^7 CFSE-labeled Marylin T cells. Popliteal lymph nodes were subjected to two-photon imaging at 24 h. The fate of individual T cell–DC contacts was followed over time (n = 20 for male DCs; n = 55 for female DCs). Plots show the percentage of interactions that had not terminated at the indicated time points. (c) Imaging subsequent interactions between activated T cells and DCs. On day 0, mice were injected in the footpad with unlabeled male DCs and at 24 h with SNARF-labeled male DCs. Two-photon imaging was performed at 48 h. The fate of individual contacts between T cell blasts and labeled DCs was followed over time (n = 31). Plot shows the percentage of interactions that had not terminated at the indicated time points. (d) Time-frame images showing that upon reencounter with Ag-bearing DCs, activated T cells also can establish prolonged contact. One example that is representative of 15/31 contacts between a T cell blast and a labeled DC is shown. Bar, 10 μm.

Figure 6. Successive encounters with Ag-bearing DCs in vivo promote CD25 expression on T cells and IFN-γ production. (a) Experimental design. At day 0, recipient mice were adoptively transferred with CFSE-labeled Marylin T cells. Injections of 10^6 DCs were performed at days 0 and 1. The first wave of DCs contained a limited amount of Ag-bearing DCs (10^5) to reduce the opportunity for T cells to interact with multiple DCs. The second wave of DCs (10^6 male or female DCs) was used to modulate the probability for a recently activated T cell to reengage another Ag-bearing DC. Cells from draining lymph nodes from the indicated recipient were analyzed by flow cytometry 38 h after the second DC injection. (b) Reencounter with Ag-bearing DCs had little effect on T cell proliferation. Data are gated on CD4^+CD45.1^ cells. (c) Additional encounters with Ag-bearing DCs promote CD25 expression. Data are gated on CD4^+CD45.1^ cells. (d) The percentage of CD25-positive Marylin T cells is graphed as a function of the number of cell divisions undergone. Results are means ± SD (n = 4). Results are representative of four independent experiments. (e) Additional encounters with Ag-bearing DCs promote IFN-γ production. Lymph node cells were restimulated in vitro for 4 h in the presence of Dby peptide, and subjected to intracellular staining. (f) The percentage of IFN-γ–producing Marylin T cells is graphed as a function of the number of cell division undergone. Results are means ± SD (n = 4). One representative experiment out of three is shown.
that it also affects the extent of competition between T cells by altering the T cell/DC ratio. For this reason, we devised an alternative strategy that allowed us to modulate the probability that a T cell will reencounter additional Ag-bearing DCs, without influencing T cell competition (Fig. 6 a).

Naive female mice were adoptively transferred with CFSE-labeled Marylin CD4 T cells, and received two DC injections. The first injection was performed on day 0, and contained a limited number (10^6) of male DCs to minimize the number of Ag-bearing DCs that a T cell could see in the initial phase of activation. These male DCs were mixed with an excess of female DCs so that a constant number of 10^6 DCs was always injected. The second injection—10^6 male DCs (condition Ag+Ag) or 10^6 female DCs (condition Ag+no Ag)—was performed at 24 h and modulated the probability that recently activated T cells would encounter additional Ag-bearing DCs. Analyses were performed 38 h after the second injection to ensure that signals that were delivered by the first wave of Ag-bearing DCs were mandatory to observe proliferation. As expected, in control animals that received only female DCs during the first injection and male DCs during the second injection (condition no Ag or no Ag versus 23.3 ± 5.2% for condition Ag+no Ag, mean ± SD; n = 4). Interestingly, this was also true for T cells that had undergone few (0–2) rounds of cell division. Thus, signals that are delivered during subsequent T cell–DC encounters in vivo are integrated by CD4 T cells and rapidly modulate their phenotype and effector functions.

DISCUSSION

We followed the occurrence and consequences of T cell–DC interactions subsequent to the initial activation events. In general, T cells detached from DCs before cell division but often reengaged a different DC in an Ag-dependent manner. This seemed to happen at various stages of the activation process, including after commitment to division. Importantly, our results provide in vivo evidence that signals that are delivered through different DC encounters can be integrated by T cells and reflected in their differentiation program. Our approach took extensive advantage of the 2-d delay that is required to observe T cell proliferation after DC injection. By performing two DC injections that were spaced so interactions with DCs from the first injection were necessary to observe proliferation, we could determine the history of Ag recognition of some activated T cells, and assess the functional consequences of DC reencounter after the initial activation events.

Most undivided and divided T cell blasts did not seem to interact with male DCs at 48 h, which supported the idea that T cell–APC contact falls apart before T cell division. This observation differs from a previous study that concluded that most T cells proliferated while clustering with DCs (14). However, it is in agreement with a two-photon imaging study that visualized T cells undergoing cell division without contacting any Ag-bearing APCs (5). Differences in the overall avidity of T cell–APC interactions might explain these discrepancies. The remaining 20% of undivided blasts and divided T cells contacted male DCs in an Ag-dependent fashion. Experiments that aimed at tracking the history of Ag recognition events for these T cells revealed that the DC that contacted activated/divided T cells usually was different from the one that delivered the initial signal that was required for proliferation. The frequency of recently activated T cells reencountering DCs is likely to be >20%, because our analyses provided a snapshot image of contacts at a fixed time point. Whereas high DC numbers likely favors DC reengagement by T lymphocytes, the high motility of T cells may suffice to promote multiple T cell–DC contacts, even when a relatively low number of Ag-bearing DCs is present in the lymph node (1, 2, 15). This view is supported by an in silico model of T cell activation in the lymph node, which predicted that divided T cells have the opportunity to reencounter rare Ag-bearing DCs (16). Because the lifespan of activated DCs is ~3 d (17), T cells have the opportunity to contact multiple DCs at the early stages of activation, and at later time points, including after commitment to cell division.

Previous studies analyzed the dynamics of T cell–DC contacts in intact lymph nodes at various time points (1, 3–5,
18). During the first hours, transient contacts (<10 min) dominate; these are followed by long-lived interactions and then by the reacquisition of a motile behavior as T cells are blasting. In good agreement with these reports, we found that at 20 h, long-lasting interactions were dominating, and that most T cell blasts were crawling freely at 48 h. However, our experimental strategy enabled us to identify a subset of T cell blasts that makes prolonged interactions (>30 min) with DCs that are encountered in the late phase of priming (DCs from the second injection). These results suggest that opportunities for T cell blast to receive signals at late time points are not limited to brief contacts with DCs, but include the establishment of relatively long-lived interactions. Based on our observations and the work of others, we propose that Ag recognition for an individual T cell could involve a series of short-lived contacts with DCs, as well as several relatively prolonged (>30 min) interactions.

The strength of the signal that is received by CD4 or CD8 T cells dictates their differentiation programs (19). In particular, short in vitro stimulations induce an abortive clonal expansion that is associated with low CD25 expression, whereas longer stimulations promote sustained CD25 expression and optimal T cell expansion (20, 21). Prolonged TCR stimulation also is required for CD4 T cells to acquire the ability to produce IFN-γ (22). We found that increasing the probability of DC reencounter in vivo promoted expression of CD25 and IFN-γ production. Our findings provide in vivo evidence that the overall signal that is received by a T cell integrates stimuli that are delivered by multiple DCs before and after commitment to cell division. They also indicate that, under certain circumstances, signals that promote cell cycle and CD25 expression can be delivered by different Ag-bearing DCs. A recent study by Jenkins et al. demonstrated that distinct populations of DCs affect T cell activation programs differently after immunization with a soluble Ag (23). Together with our results that showed that T cells can accumulate sequential signals in vivo, this suggests that the T cell activation program integrates the type and the number of DCs that are encountered. Conversely, there may be an upper limit to the number of DC encounters that promote optimal CD4 T cell activation. A recent report found that CD4 T cells that were stimulated by a high number of DCs for 5 d in a row displayed reduced protective function (24).

Although our results do not exclude that a single T cell–DC interaction may be sufficient to trigger an optimal activation program under optimal conditions of Ag presentation, they do suggest that T cells that received suboptimal activation signals (e.g., because of low Ag amount or low TCR affinity) can be rescued by additional DC encounters. Such an additional effect might explain why some CD4 T cell responses are dependent on the presence of Ag for several days (25, 26), and why the life span of DCs can influence the extent of T cell activation (27–29). In this respect, it is tempting to speculate that the 3–4 d period during which activated T cells are sequestered in the lymph node (30, 31) may increase the number of subsequent T cell–DC contacts. Although imaging experiments that were performed in the present study were focused on interactions between CD4 T cells and injected DCs, interactions with endogenous DCs that have engulfed dead DCs can be an additional source of Ag reencounter for activated T cells. In an infectious context, the capacity for T cells to integrate signals from multiple APC encounters may adapt the strength of the adaptive response to the extent of Ag dissemination.

In summary, we documented that recently activated CD4 T cells can reinteract with Ag-bearing DCs in vivo, and subsequently, integrate these late signals in their differentiation program. Thus, the number of Ag-bearing DCs that reaches the draining lymph node may act as an important parameter by dictating the number of Ag-specific T cells that is recruited into the immune response, and by qualitatively modulating the activation program of T cells through APC reencounter.

MATERIALS AND METHODS

Mice. C57BL/6 and C3H/HeJ mice were purchased from Charles River Laboratories. 6–9-wk-old female Marylin (anti-HY) TCR transgenic RAG-2−/− CD45.1+ mice (32) and MHC class II−/− deficient mice were obtained from the Centre de Distribution, Typage et Archivage animal. Animals were housed in specific pathogen-free conditions in our animal facility. All animal experiments were performed according to institutional guidelines for animal care and use.

Cell preparation and transfer. Splenic DCs from male or female C57BL/6 mice were purified using anti-CD11c-conjugated microbeads (Miltenyi Biotec) and an AutoMacS system as described previously (1). Cell purity was measured by flow cytometry and was ~90%. DCs were labeled with 5 μM SNARF (Invitrogen) alone or in conjunction with 5 μM CFSE (Invitrogen) for 10 min at 37°C, washed, and injected i.d. in the inguinal region or footpad as specified. CD4 T cells were isolated from the spleen and lymph nodes of female Marylin TCR Tg RAG-2−/− mice, labeled with 5 μM CFSE, and injected i.v.

FACS analysis. Lymph nodes were incubated at 37°C for 15 min in RPMI 1640 containing 1 μg/ml collagenase. Cell suspensions were prepared and stained with a combination of the following antibodies: APC-labeled anti-CD8, PE-labeled anti-CD69 or PE-labeled anti-CD25 (all purchased from BD Biosciences), and PE-Cy7–labeled anti-CD45.1 (eBioscience). Samples were analyzed on a FACSCalibur (BD Biosciences). For intracellular IFN-γ staining, lymph node cells were cultured for 4 h in the presence of 1 μM Doby peptide NAGFNSNRRANSSRSS (NeoMPS) and 1 μg/ml Brefeldin A. Alternatively, in experiments that included MHC class II−/− deficient recipients, lymph node cells were restimulated with 12.5 ng/ml PMA and 1 μg/ml ionomycin (Sigma-Aldrich) in the presence of 1 μg/ml Brefeldin A. Cells were incubated with APC-conjugated anti-CD4 and PE-Cy7–conjugated anti-CD45.1 antibodies, and were subjected to intracellular staining using a PE-labeled anti-IFN-γ antibody (BD Biosciences) and the Cytofix/Cytoperm kit (BD Biosciences) following the manufacturer’s instructions.

Confocal and two-photon imaging. Recipient mice were killed, and the inguinal lymph nodes that drain the area of the DC injection were removed carefully and fixed in 4% PFA for 15 min at 4°C. After two washes in PBS, lymph nodes were incubated in PBS 20% sucrose for 1 h at 4°C and washed again. Samples were dried off, put in OCT compound (Tissue-Tek Sakura Finetek Europe), and frozen in liquid nitrogen. Cryosections were mounted using Vectashield mounting medium (Vector Laboratories). Slides were analyzed with a confocal microscope (Leica); Z-stacks of images spaced 1 μm apart were collected, and images were processed using ImageJ software. The size and the total CFSE fluorescence amount of T cells that were con-

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tained entirely within the Z-stacks of images were calculated using ImageJ software. The total CFSE amount for an individual T cell was obtained by summing the CFSE fluorescence that was contained in each plane (spaced by 1 μm), whereas cell size corresponds to the area delimiting the T cells after a projection of the stack of images in the Z direction. Two-photon imaging was performed using an upright Axioscope 2 FS microscope (Carl Zeiss MicroImaging, Inc.). Excitation (780 nm) was provided by a Ti:sapphire laser (Coherent), and was focused onto the specimen using an achroplan IR 40X/0.8 NA dipping objective (Carl Zeiss MicroImaging, Inc.). Popliteal lymph nodes were maintained at 37°C, and were superfused with RPMI 1640, without phenol red, bubbled with a gas mixture containing 95% O2 and 5% CO2 as described previously (1). Typically, three to five planes located at least 100 μm below the lymph node capsule and spaced 5–10 μm apart were imaged every 15–30 s. Time-lapse videos were obtained after performing a maximum intensity projection, and were processed further using ImageJ software.

Online supplemental material. Videos 1–7 show T cell–DC interactions visualized in intact lymph nodes using two-photon imaging. Videos 1 and 2 illustrate that Marylin TCR CD4 T cells establish long-lasting contacts with Ag-bearing DCs at 24 h. Videos 3 and 4 show that, in the absence of Ag, T cell–DC contacts are short lived. Video 5 shows examples of short- and long-lived interactions between activated T cells and DCs. Videos 6 and 7 illustrate that a subset of T cell blasts establish prolonged interactions upon reencounter with Ag-bearing DCs. Fig. S1, demonstrates that direct and indirect modes of Ag presentation promote CD4 T cell activation. Fig S2 shows additional examples of activated CD4 T cells interacting different waves of DCs (similar to Fig. 4). Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20051018/DC1.

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