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Real-Time Manipulation of T Cell-Dendritic Cell Interactions In Vivo Reveals the Importance of Prolonged Contacts for CD4+ T Cell Activation

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SUMMARY

T cells interact with dendritic cells (DCs) for periods lasting from minutes to hours. However, a causal link between the duration of this interaction and the efficiency of T cell activation has not been established in vivo. Employing intravital two-photon imaging, we manipulated T cell-DC interactions in real time and found that the first T cell-DC encounter often resulted in a long-lived interaction. Moreover, the cessation of T cell receptor-major histocompatibility complex signals promoted cellular dissociation, suggesting that antigen availability on DCs regulates contact duration. Finally, at least 6 hr of in vivo T cell-DC interaction were required for naive CD4+ T cells to undergo clonal expansion. These results establish the importance of prolonged T cell-DC interactions for efficient CD4+ T cell activation in vivo.

INTRODUCTION

Adaptive responses are initiated through the cellular interaction between rare naive antigen (Ag)-specific T cells and Ag-bearing dendritic cells (DCs) in the lymph node (LN). During this process, the duration of T cell-DC contact can be highly variable in vivo, ranging from a few minutes to several hours (Breart and Bousso, 2006). Several studies observed primarily transient interactions during the first hours of Ag recognition. During this period (also referred to as phase 1), T cells were seen contacting Ag-bearing DCs for 5–10 min, a period found to be no different from (Mempel et al., 2004a) or slightly longer than (Miller et al., 2004) that corresponding to nonspecific interactions. This phase of transient contacts lasted 8 hr (Mempel et al., 2004a) and 15 hr (Hugues et al., 2004) in studies exploring CD8+ T cell-DC interactions and 3 hr in a study analyzing CD4+ T cell-DC contacts. Subsequently, long-lived contacts (phase 2) were observed, and T cells began to secrete cytokine (Mempel et al., 2004a). The transition from short-lived to long-lived interactions suggested that changes at the level of T cells and/or DCs were necessary for the formation of a stable contact. For example, it has been hypothesized that transient T cell-DC contacts could be required for T cells and/or DCs to gain the ability to interact more stably later on (Henrickson and von Andrian, 2007; Sumen et al., 2004). Somewhat distinct results were obtained by Shakhar and colleagues, who observed CD4+ T cells engaged in stable interactions with DCs a few hours (1–6 hr) after transfer into an immunized animal (Shakhar et al., 2005). Whether the phase of transient T cell-DC contacts was absent in this system or occurred in the first 1–2 hr is unclear. More generally, how T cells behave during the first minutes of Ag recognition has not been addressed so far.

Once a stable T cell-DC interaction is established, it can be maintained for several hours, although the precise duration of individual contacts has not been measured. Under which circumstances T cells disengage from DCs is still largely unknown. Changes in T cell receptor (TCR) signals, adhesion molecules, or chemokine environment could potentially participate in the termination of T cell-DC contacts. In addition to parameters that dictate the beginning and the termination of prolonged CD4+ T cell-DC contact, it is unclear how the duration of these contacts ultimately influences T cell activation in vivo. Although correlations between T cell-DC contact stability and T cell activation efficiency have been reported in some studies (Hugues et al., 2004; Tadokoro et al., 2006; Tang et al., 2006; Zinselmeyer et al., 2005), no causal link has been established so far between these two parameters. One way to address this question would require the ability to experimentally manipulate T cell-DC contact duration in vivo, and this has remained technically challenging so far.

In the present report, we establish an experimental system that allowed us to synchronize Ag recognition by CD4+ T cells in vivo. We show that CD4+ T cells could establish long-lived interactions during their first encounter with Ag-bearing DCs. We also provide evidence that the termination of TCR signals is sufficient to induce T cell detachment from DCs. By using this system, we are able to temporally control the formation and dissociation of stable T cell-DC interactions in vivo. We establish the importance of T cell-DC contact duration for efficient T cell activation in vivo.

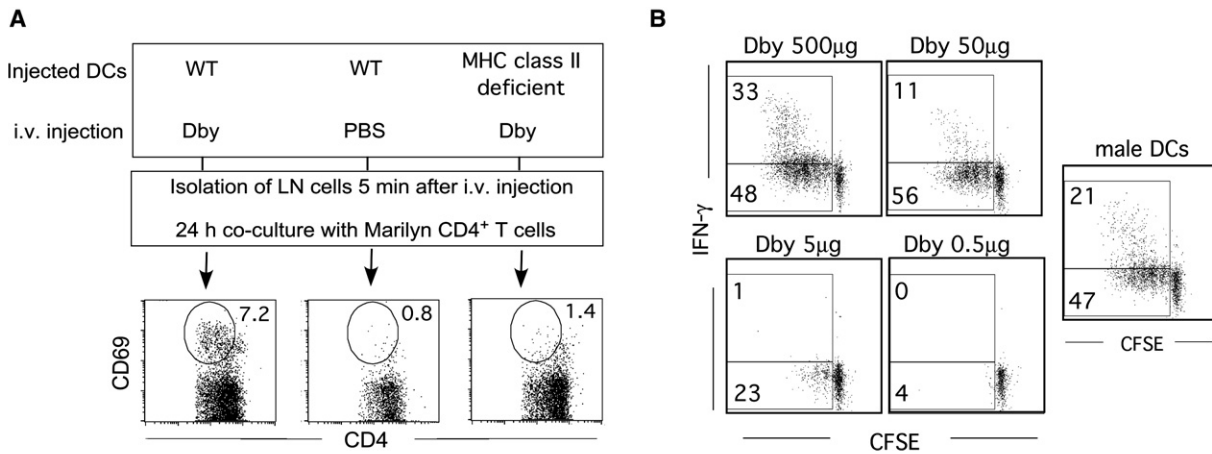

Figure 1. A Novel Approach for the Synchronization of Antigen Presentation by DCs In Vivo

(A) MHC class II-deficient recipients were injected with either WT or MHC class II-deficient DCs. After 24 hr, 50 μg of Dby peptide (or PBS as a control) was injected intravenously. Popliteal LNs were harvested 5 min later, and LN cells were cocultured with naive Marilyn $CD4^+$ T cells for 24 hr. CD69 upregulation was measured by flowcytometry. Data are gated on $CD4^+$ $CD45.1^+$ cells.

(B) Efficient $CD4^+$ T cell proliferation induced by transferred DCs pulsed in vivo by i.v. injection of the Dby peptide. The experiment was conducted as described in (A) with CFSE-labeled Marilyn $CD4^+$ T cells. Three days after i.v. injection of the indicated dose of Dby peptide, popliteal LNs were harvested. Proliferation and IFN-γ production after a 4 hr in vitro stimulation were assessed by flowcytometry. The in vivo activation profile of Marilyn $CD4^+$ T cells after injection of male DCs in MHC class II-deficient recipients is shown in the right panel. Results are representative of two independent experiments.

RESULTS

A Model for Studying the First Minutes of Ag Recognition by $CD4^+$ T Cells In Vivo

For the facilitation of the study of a particular stage of T cell priming in vivo, it is beneficial to synchronize the activation of antigen-specific T cells. To this end, previous studies have analyzed T cells at various time points after transfer in preimmunized mice and/or used the injection of CD62L to block the arrival of new incoming T cells in the LN (Mempel et al., 2004a; Miller et al., 2004). Because it takes at least 1 hr for adoptively transferred T cells to enter LNs, it has not been possible to follow the first T cell-DC encounters with these approaches. To achieve this goal, we devised a new strategy to synchronize antigen presentation by DCs in the LN. Wild-type (WT) DCs (isolated from female B6 mice) were injected in the footpad of major histocompatibility complex (MHC) class II-deficient recipients. Subsequently, dye-labeled Marilyn $CD4^+$ T cells (specific for the male Dby epitope) were injected intravenously. After 24 hr, DCs migrated to the draining popliteal LN and displayed the hallmarks of mature DC as reflected by high MHC class II and high costimulation molecules (Bousso and Robey, 2003). We reasoned that an intravenous (i.v.) injection of the Dby peptide should rapidly and specifically load the transferred DCs because these cells are the only MHC class II-expressing cells in the recipient. To validate this approach, we first verified that Dby peptide injected intravenously could gain access to transferred DCs that migrated in the draining LN. WT (or MHC class II-deficient) DCs were injected in the footpad of MHC class II-deficient recipients. After 24 hr, 50 μg of Dby peptide was injected intravenously. Five minutes later, popliteal LNs were harvested, and LN cells were cultured in vitro in the presence of an excess of Marilyn $CD4^+$ T cells. The presence of Dby-loaded antigen-presenting cells in the LN cell preparation was assessed by the measurement of whether Marilyn T cells upregulated CD69 after one day of coculture. LN cells from recipients that were immunized with MHC class II-expressing DCs and that received an i.v. injection of Dby peptide were able to stimulate Marilyn $CD4^+$ T cells as detected by CD69 upregulation (Figure 1A). Antigen presentation was mediated by the transferred DCs because no CD69 upregulation was detected when we repeated this experiment with DCs lacking the expression of MHC class II molecules. Thus, Dby-presenting DCs were present in the popliteal LN as early as 5 min after peptide injection. Next, we tested whether Dby peptide injection could induce clonal expansion and differentiation of carboxyl fluorescein succinimidyl ester (CFSE)-labeled Marilyn $CD4^+$ T cells in vivo in our system. The injection of 500 μg and 50 μg of Dby peptide resulted in robust T cell clonal expansion and interferon-γ (IFN-γ) production on day 3 (Figure 1B). Poor T cell proliferation was seen with lower doses of peptide (Figure 1B). The $CD4^+$ T cell activation profile obtained with 50 μg of Dby peptide was very similar to that induced by the injection of male DCs (presenting an endogenous amount of peptide-MHC [pMHC]). For this reason, an injection of 50 μg of Dby peptide was chosen for the rest of the study. In summary, our experimental approach enabled to precisely control the timing of Ag presentation and to induce efficient T cell activation in vivo.

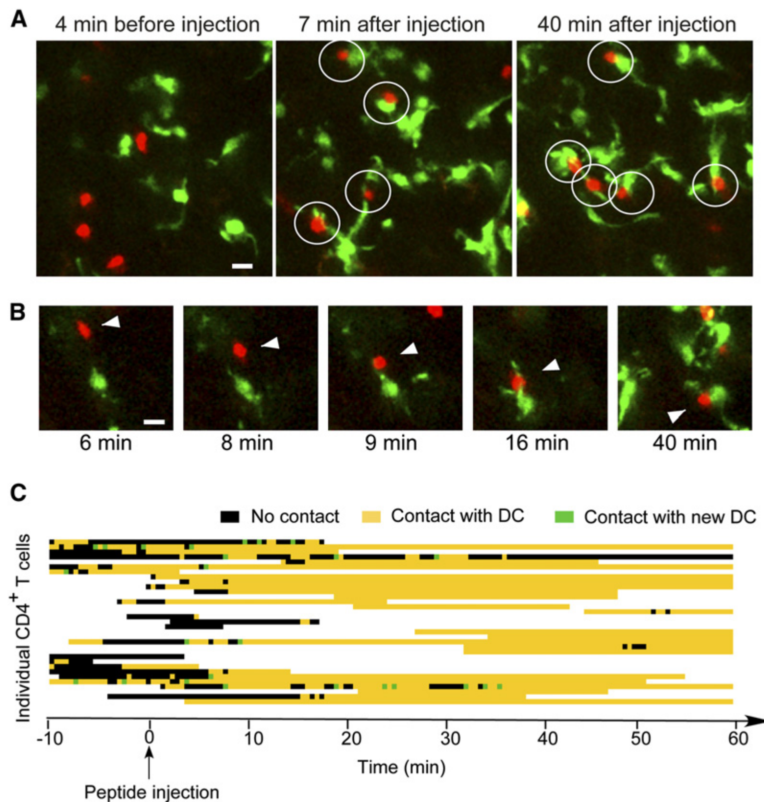

Figure 2. CD4+ T Cells Establish Long-Lived Interaction with the First Ag-Bearing DC Encountered

(A) MHC class II-deficient recipients were injected in the footpad with DCs isolated from female Ubi-GFP mice and were adoptively transferred with SNARF-labeled Marilyn CD4+ T cells. After 24 hr, mice were prepared for intravital imaging of the popliteal LN. Time-lapse images were acquired with two-photon microscopy. After approximately 10 min of imaging, 50 μg of Dby peptide was injected intravenously through the jugular vein. Imaging was then continued for 1 hr. Time-lapse images show T cells (red) and DCs (green) establishing long-lived interactions (white circles).

(B) Time-lapse images showing the initial interaction between a T cell and an Ag-bearing DC.

(C) Compilation of the behavior of 33 T cells over the course of the experiment, with the indicated color code. White squares indicate time points at which the T cell was not visible. Scale bars represent 20 μm. Results are representative of three independent experiments.

CD4+ T Cells Establish a Long-Lived Interaction during Their First Encounter with DCs

To determine whether early specific TCR signals were the result of transient or long-lived interactions, we took advantage of our experimental approach to visualize in real time the first encounter between CD4+ T cells and Ag-presenting DCs. MHC class II-deficient recipients were injected in the footpad with green fluorescent protein (GFP)-expressing DCs and intravenously with seminaphthorhodafluor (SNARF)-labeled Marilyn CD4+ T cells. After 24 hr, recipients were anesthetized and prepared for intravital two-photon imaging of the popliteal LN. Image acquisition was started, and fluorescent CD4+ T cells and DCs were imaged for at least 10 min in the absence of antigen. As image acquisition was proceeding, 50 μg of Dby peptide was injected intravenously through the jugular vein, and imaging was continued for an additional 60 min. This strategy allowed us to track the same cells before and after Ag presentation by DCs. Strikingly, although T cells and DCs only transiently interacted in the absence of antigen, long-lived T cell-DC interactions formed within minutes of peptide injection (Figure 2A, Movies S1–S3 in the Supplemental Data available online) and, in most cases, CD4+ T cells maintained a stable contact with the first DC encountered (Figures 2B and 2C, Movies S1–S3). The duration of these interactions was in the range of hours because virtually no T cells detached during the first 6 hr after peptide injection (Movies S4 and S5). In a few instances, T cells engaged in an interaction with one DC were grabbed by a new DC (Figure 2C). The rapid formation of long-lived T cell-DC interactions were not restricted to a particular TCR because similar results were observed when the same experiment was performed with CD4+ T cells bearing the OT-II TCR and an i.v. injection of the OVA$^{323\text{-}339}$ peptide (Movie S6). To determine whether the formation of stable interactions was Ag specific, we repeated the experiment with a mixture of specific (Marilyn) and nonspecific (OT-II) CD4+ T cells. Upon Dby injection, Marilyn T cells rapidly formed prolonged interactions with DCs, whereas OT-II T cells maintained a high level of motility (Movies S7 and S8), demonstrating that only Ag-specific T cells were stably recruited by DCs. In summary, these experiments provided direct evidence that T cell activation can be initiated though long-lived contacts with DCs in the absence of any preceding transient interactions.

To evaluate whether most Marilyn CD4+ T cells could receive TCR signals early during these contacts, we took advantage of the possibility to detect by flowcytometry c-Jun phosphorylation (Zell et al., 2001), a very early marker of Ag recognition in T cells. Thirty minutes after Dby peptide injection, c-Jun phosphorylation could be detected in 55% of Marilyn CD4+ T cells in the draining LN (Figure 3A). Less than 3% of CD4+ T cells were stained with this antibody in nondraining LNs or in phosphate-buffered saline (PBS)-injected animals (Figure 3A). Similarly, ERK (extracellular signal-regulated kinase) phosphorylation could be detected within Marilyn CD4+ T cells after Dby peptide injection (Figure 3B). These results provided additional evidence that transferred DCs displayed

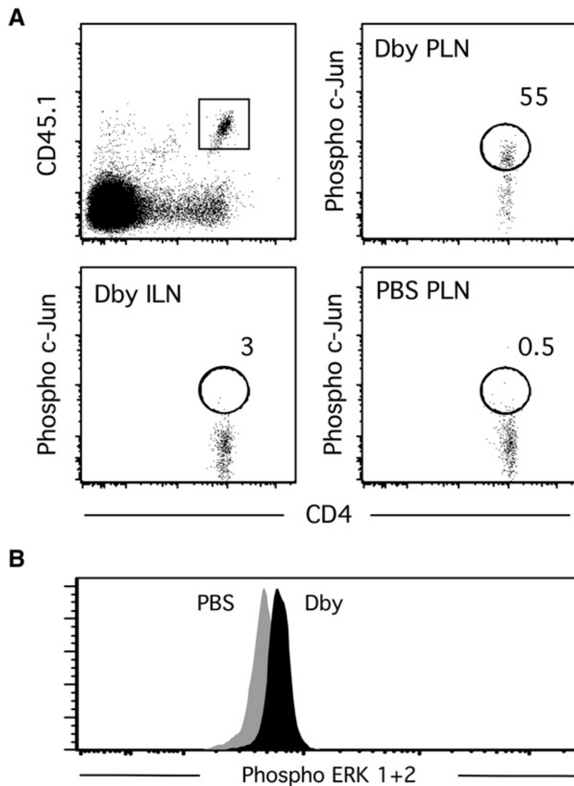

Figure 3. CD4$^+$ T Cells Show Signs of Activation Early after the Establishment of Stable T Cell-DC Interactions
MHC class II-deficient recipients were injected with 3 × 10^6 WT DCs and adoptively transferred with 10 × 10^6 Marilyn CD4$^+$ T cells. After 24 hr, 50 μg of Dby peptide was injected intravenously. Thirty minutes after Dby or PBS injection, draining popliteal (PLN) or nondraining inguinal (ILN) LNs were harvested. Cells were subjected to intracellular staining to detect c-Jun (A) or ERK1+2 (B) phosphorylation. Data are gated on CD4$^+$CD45.1$^+$ cells. Numbers represent the percentage of positive cells among the gated population. Results are representative of three independent experiments.

pMHC complexes shortly after peptide injection and showed that the stable T cell-DC contacts formed in this system were able to initiate the activation of the majority of resident Ag-specific CD4$^+$ T cells within less than 30 min.

Early T Cell Stopping Is Observed with DCs Expressing Physiological Amounts of pMHC

It was important to determine whether the early T cell stopping observed in our experimental system was the result of an abnormally high density of pMHC complexes. We addressed this point by asking whether stable interactions were formed rapidly between Marilyn CD4$^+$ T cells and male DCs (expressing the endogenous amount of I-A^b-Dby complexes). Although the level of synchronization of the activation process in this system is lower than that achieved by peptide injection, it was sufficient to test whether a phase of transient T cell-DC contacts occurred during the first hours of the response, as seen in other systems (Hugues et al., 2004; Mempel et al., 2004a; Miller et al., 2004). GFP-expressing male DCs were injected in the footpad of female B6 recipients, and one day later, Marilyn CD4$^+$ T cells were transferred intravenously. Recipients were then prepared for intravital two-photon imaging of the popliteal LNs, and T cell-DC interactions were imaged 1–3 hr after T cell transfer. As shown in Figure 4 and Movie S9, we found that CD4$^+$ T cells and male DCs establish long-lived interactions in the early phases of the activation process. In several instances, we imaged T cells being recruited in a prolonged interaction upon encounter with male DCs (Figure 4B, Movie S10). Similar results were obtained when MHC class II-deficient mice were used as recipients (Movie S11). Overall, these results indicate that early T cell stopping can be observed with DCs displaying physiological amounts of pMHC. Because these observations were made with either B6 or MHC class II-deficient recipients, they also ruled out the possibility that early T cell stopping only occurs in a MHC class II-deficient environment.

Disrupting TCR-MHC Interactions Promotes T Cell Detachment from DCs In Vitro and In Vivo

Next, we asked how long-lived T cell-DC interactions are terminated. In particular, we assessed whether the maintenance of established T cell-DC interactions relied on TCR-dependent or -independent interactions. To this end, we formed T cell-DC conjugates in vitro and subsequently incubated them for 30 min in the presence or absence of a blocking MHC class II antibody (Ab) (referred to as Y3P Ab). As shown in Figure 5, most T cell-DC conjugates had dissociated after incubation with the MHC class II Ab but not after incubation with a control Ab. In addition, most conjugates between Marilyn CD4$^+$ T cells and male DCs could also be dissociated after incubation with the Y3P Ab (data not shown). This result indicates that the cessation of TCR-MHC interactions in preformed T cell-DC conjugates is sufficient to promote T cell detachment from DCs in vitro.

To determine whether this effect was also occurring in vivo, we used intravital two-photon imaging and visualized the effect of MHC class II Ab injection on T cell-DC interactions. Recipient MHC class II-deficient mice were injected with GFP-expressing DCs and SNARF-labeled Marilyn CD4$^+$ T cells. One day later, Dby peptide was injected intravenously so that the formation of long-lived T cell-DC interactions could be initiated. Intravital imaging was started 2 hr after peptide addition, and an MHC class II Ab or a control Ab was injected intravenously. Virtually all T cell-DC contacts were highly stable before Ab injection and continued to be stable 2 hr after injection of the control Ab (Figure 6 and Movie S12). In contrast, the injection of Y3P Ab favored the termination of prolonged interactions because T cells were seen predominantly forming transient interactions (<10 min) with one or multiple DCs at 2 hr after Ab injection (Figure 6 and Movie S12). Thus, the cessation of TCR-MHC interactions appeared to be sufficient to promote T cell detachment from DCs both in vitro and in vivo.

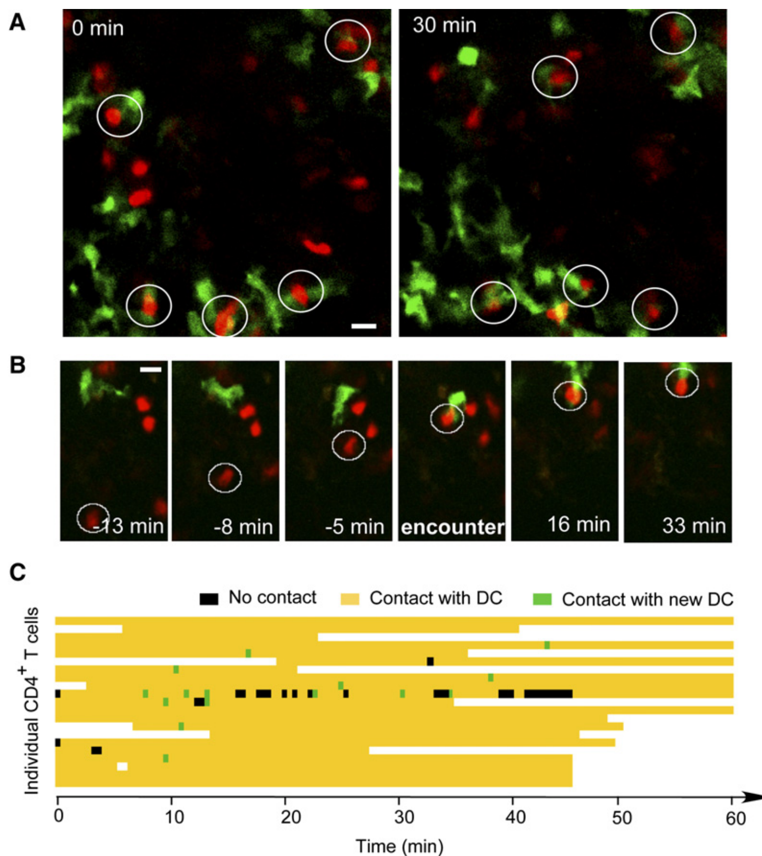

Figure 4. CD4⁺ T Cells Establish Long-Lived Interactions with Male DCs in the Initial Stages of the Activation Process

(A) B6 recipients were injected with DCs isolated from male Ubi-GFP mice. After 24 hr, 20×10^6 SNARF-labeled Marilyn CD4⁺ T cells were adoptively transferred. Mice were immediately prepared for intravital two-photon imaging, and time-lapse imaging was started 1–2 hr after T cell transfer. Time-lapse images show Marilyn CD4⁺ T cell (red) interactions (indicated by white circles) with male DCs (green) in the initial stages of the activation process.

(B) Time-lapse images showing the establishment of a prolonged contact between a T cell and a male DC.

(C) Behavior of 21 individual CD4⁺ T cells in the presence of male DCs. All data were acquired between 1–3 hr after T cell transfer.

Scale bars represent 20 μm. Results are representative of three independent experiments.

Manipulating the Duration of T Cell-DC Interactions In Vivo

Because in our system injection of Dby peptide and Y3P MHC class II Ab initiate and arrest stable T cell-DC interactions, respectively, it became possible to manipulate the length of T cell-DC interactions in vivo. Recipient MHC class II-deficient mice, injected with DCs and CFSE-labeled Marilyn CD4⁺ T cells, received an injection of Dby peptide. Recipient mice were treated with Y3P Ab (or a control Ab) 2, 6, or 24 hr later. Three days after peptide injection, T cell proliferation in the draining LN was assessed by flowcytometry. No T cell proliferation could be detected in mice treated with Y3P 2 hr after Dby peptide injection (Figures 7A and 7B). This result demonstrated that a 2 hr long T cell-DC contact was insufficient to drive T cell division in this system. In mice treated with Y3P 6 hr after Dby injection, Marilyn T cells typically divided no more than once, whereas several cell divisions were observed when the interval between Dby and Y3P injection reached 24 hr. Therefore, at least 6 hr of stable T cell-DC contact in vivo were required to initiate CD4⁺ T cell clonal expansion. T cell proliferation was maximal in mice that were not Ab injected and in mice that received a control Ab, indicating that signals delivered after 24 hr also contributed to T cell activation. As shown in Figure 7C, divided CD4⁺ T cells were found to express CD44, independently of the duration of the stimulus. In contrast, the CD4⁺ T cells' ability to produce IFN-γ increased with the duration of the stimulation (Figure 7B). In addition, we measured the expression of granzyme B, which has been previously detected in activated CD4⁺ T cells (Casazza et al., 2006; Hombach et al., 2006). As shown in Figure 7C, intracellular granzyme B content within divided CD4⁺ T cells increased with the duration of T cell-DC interactions. Altogether, these data establish that the duration of T cell-DC contacts in vivo strongly influences T cell activation efficiency and emphasize the importance of long-lived interactions in this process.

DISCUSSION

In the present study, we examined the formation and the role of prolonged CD4⁺ T cell-DC interactions in vivo. We report three important findings: (1) CD4⁺ T cells can establish long-lived interactions during their first encounter with Ag-bearing DCs, (2) the termination of TCR-MHC interactions promotes T cell detachment in vivo, and (3) the continuous signals delivered during many hours of prolonged T cell-DC contacts are essential for efficient T cell activation.

Recent studies have examined how T cells and Ag-bearing DCs interact in the early phases of the immune response. Conflicting results have been reported, with several studies documenting the lack of long-lived T cell-DC interactions before 3–12 hr (Hugues et al., 2004; Mempel et al., 2004a; Miller et al., 2004) and one report visualizing

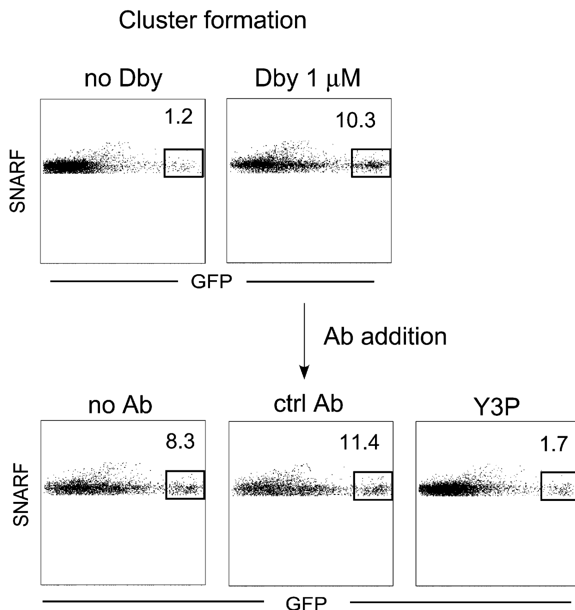

Figure 5. Blocking TCR-MHC Interactions Promotes T Cell Detachment from DCs In Vitro

GFP-expressing DCs were pulsed with 1 μM Dby peptide and incubated with naive SNARF-labeled Marilyn $CD4^+$ T cells for 60 min (upper panels). Subsequently, MHC class II Y3P or isotype control Ab was added for 30 min (lower panels). Conjugates were identified by flowcytometry as $SNARF^+GFP^+$ events. Profiles were gated on the $SNARF^+$ population. Numbers represent the percentage of T cells forming conjugates with DCs. One representative experiment out of three is shown.

stable T cell-DC contacts after 1–6 hr (Shakhar et al., 2005). We have described a new experimental system that allows the visualization of the first minutes of Ag recognition. By using this approach, we found that $CD4^+$ T cells can establish a long-lived interaction with the first Ag-bearing DC encountered. This early T cell stopping could be detected with DCs presenting physiological amounts of pMHC complexes. What dictates the T cell's ability to establish a long-lived interaction as it encounters an Ag-bearing DC for the first time? On the basis of our results and these previous reports, we favor the explanation that the integration of several parameters such as TCR affinity, Ag dose (Bousso and Robey, 2003; Miller et al., 2004), DC maturation stage (Hugues et al., 2004), and surface-bound chemokines (Friedman et al., 2006) influence the probability of triggering an early T cell stop.

Here, we have shown that, at least in some cases, the probability can be very high, with $CD4^+$ T cells establishing long-lived interactions with the first Ag-bearing DC encountered. This result implies that the phase of transient interactions is not required for the establishment of prolonged T cell-DC interactions. In other cases, possibly if the stimulus is too low, T cells initially only make short-lived contacts with DCs before transiting to a phase of stable interactions. As proposed previously (Hugues et al., 2004; Mempel et al., 2004a; Miller et al., 2004), such transitions could be the result of changes at the T cell or the DC level, occurring over the course of the activation process. Alternatively, the transition from short-lived to long-lived contacts seen in some systems could simply reflect the gradual accumulation of stable contacts as more and more T cells succeed in stably binding DCs after a series of unsuccessful tries.

After cell interaction, T cells need to detach from the DC. However, the basis for this event is poorly understood. Previous studies have shown that interrupting TCR-MHC interactions with mAb results in the cessation of calcium signals and the disruption of the immunological synapse but only induces the dissociation of 20% of T cell blast-B cell conjugates in vitro (Huppa et al., 2003). With respect to T cell-DC interactions, we demonstrate that blocking TCR ligands induces the dissociation of virtually all established T cell-DC conjugates in vitro within less than 30 min. Similarly, the intravenous injection of MHC class II-blocking Ab in vivo promoted the termination of stable T cell-DC contacts in LNs, although the effect was somewhat slower than that observed in vitro, possibly because of the time required for the Ab to reach a high enough concentration in the LN. Thus, TCR-MHC interactions and associated signaling appear to be a driving force in maintaining T cell-DC conjugates. These observations suggest that T cells could detach from DCs once pMHC amounts fall below a certain threshold. Hence, the number of specific pMHC at the DC surface might regulate the period of time during which the T cell remains attached to the DC. As shown previously, other parameters such as DC maturation state (Hugues et al., 2004), T cell precursor frequency (Garcia et al., 2007), CTLA-4 expression on T cells (Schneider et al., 2006), and CYTIP on DCs (Hofer et al., 2006) could also influence contact length.

As mentioned above, the formation of T cell-DC interactions lasting several hours has been firmly established in vivo with the dynamic imaging of intact LNs (Bousso and Robey, 2003; Hugues et al., 2004; Mempel et al., 2004a; Miller et al., 2002; Shakhar et al., 2005; Stoll et al., 2002; Tadokoro et al., 2006; Tang et al., 2006). Noteworthily, because typical imaging periods last 1 hr or less, the actual duration of these so-called ''long-lived'' contacts is not precisely known, falling somewhere between 1 and 24 hr. The occurrence of long-lived contacts raises two important questions. First, it is not known whether signals are delivered only at the beginning of the cell-cell contact or continuously as long as the cellular conjugate is maintained. Second, it is unclear how long these interactions need to be in order to trigger efficient T cell activation. Elegant imaging studies performed on T cell–antigen-presenting cell conjugates in vitro showed evidence of sustained phosphoinositide 3-kinase activity during cell conjugation (Costello et al., 2002; Harriague and Bismuth, 2002; Huppa et al., 2003). Studies that used in vitro stimulation have also provided evidence that the duration of TCR stimulation influences T cell activation, differentiation, and fitness (Gett et al., 2003; van Stipdonk et al., 2003). In different systems using CD3 Ab, recombinant pMHC, Ag-loaded B cells, or macrophages, the duration of the stimulation needed to observe T cell proliferation

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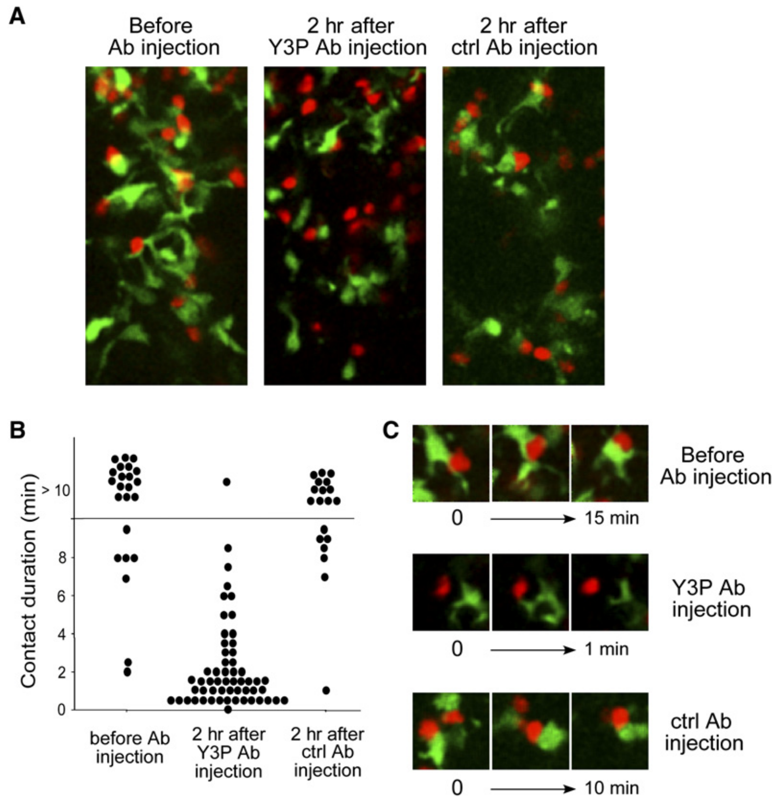

Figure 6. In Vivo Disruption of Long-Lived T Cell-DC Interactions upon Injection of an MHC Class II Ab

MHC class II-deficient recipients were injected with DCs isolated from female Ubi-GFP mice and were adoptively transferred with SNARF-labeled Marilyn $CD4^+$ T cells. After 24 hr, mice were injected intravenously with 50 μg of Dby peptide. Mice were prepared for intravital two-photon imaging of the popliteal LN. Time-lapse images were acquired 2 hr after peptide addition. After approximately 30 min of imaging, 500 μg MHC class II Y3P Ab or a control Ab was injected intravenously through the jugular vein. Imaging was then continued for 3 hr. Results are representative of two independent experiments.

(A) Representative images of T cells (red) and DCs (green) before Ab injection (left) and 2 hr after Y3P (middle) or control (right) Ab injection.

(B) Y3P injection results in the termination of prolonged T cell-DC interactions. The duration of individual T cell-DC contacts is shown before and 2 hr after Ab injection.

(C) Time-lapse images showing representative T cell-DC contacts before (upper panel) or 2 hr after Y3P (middle panel) or control Ab (lower panel) injection.

was quite variable, ranging from 2 to 20 hr (Curtsinger et al., 2003; Huppa et al., 2003; Iezzi et al., 1998; Lee et al., 2002; Schrum and Turka, 2002). One caveat of these approaches is that they do not take into consideration the critical influence of the LN microenvironment on T cell motility and their interactions with DCs. In vivo imaging studies have visualized distinct T cell-DC contact durations in immunological contexts leading to different functional outcome, i.e., immunity versus tolerance (Hugues et al., 2004; Tadokoro et al., 2006; Tang et al., 2006; Zinselmeyer et al., 2005). Nevertheless, these important observations remain correlative, and it was not clear whether contact dynamics or other unmeasured parameters were primarily responsible for the divergent T cell fates observed. Others have examined the role of antigen persistence on T cell responses but have not related their observations to cell-cell contacts (Obst et al., 2005; Prlic et al., 2006). To our knowledge, the present report is the first study establishing a causal link between T cell-DC contact length and T cell activation efficiency in vivo. Our ability to control the duration of T cell-DC contacts in situ showed that a 2, 6, or 24 hr long interaction triggers strikingly different activation profiles. This indicates that signals continue to be delivered to the T cell for many hours (>6 hr) during its conjugation with a DC. Although c-Jun phosphorylation was detected within minutes, $CD4^+$ T cell proliferation required at least 6 hr of T cell-DC interaction. Although our data do not exclude a role for shorter interactions in the activation process, it is likely that the formation of a stable contact with a DC is the most efficient way for $CD4^+$ T cells to receive the prolonged stimulus required for their commitment to cell division.

It is interesting to note that contacts occurring after 24 hr continue to be integrated in the $CD4^+$ T cell activation program, favoring in particular the production of IFN-γ. This observation is fully consistent with a recent report showing that antigen persistence can increase clonal expansion (Obst et al., 2005) and with our previous work demonstrating that late signals could be delivered through additional interactions with DCs (Celli et al., 2005). Thus, although a long-lived T cell-DC contact is required to trigger T cell division, additional stimulations through DC re-encounters are important in order to sustain $CD4^+$ T cell proliferation.

In summary, we have provided in vivo evidence that $CD4^+$ T cells can rapidly form long-lived interactions with Ag-bearing DCs, integrate signals during many hours of contact, and detach upon termination of TCR signals. Our study establishes a central role for long-lived T cell-DC interactions in driving efficient clonal expansion in vivo. Finally, the possibility of synchronizing and manipulating T cell-DC interactions in vivo will offer new opportunities to dissect the temporal regulation of T cell differentiation.

EXPERIMENTAL PROCEDURES

Mice

Six-week-old C57BL/6 (B6) mice were purchased from Charles River Laboratories. Female Marilyn (anti-H-Y) TCR transgenic *RAG-2$^{-/-}$CD45.1$^{+/+}$* mice and female MHC class II-deficient mice were

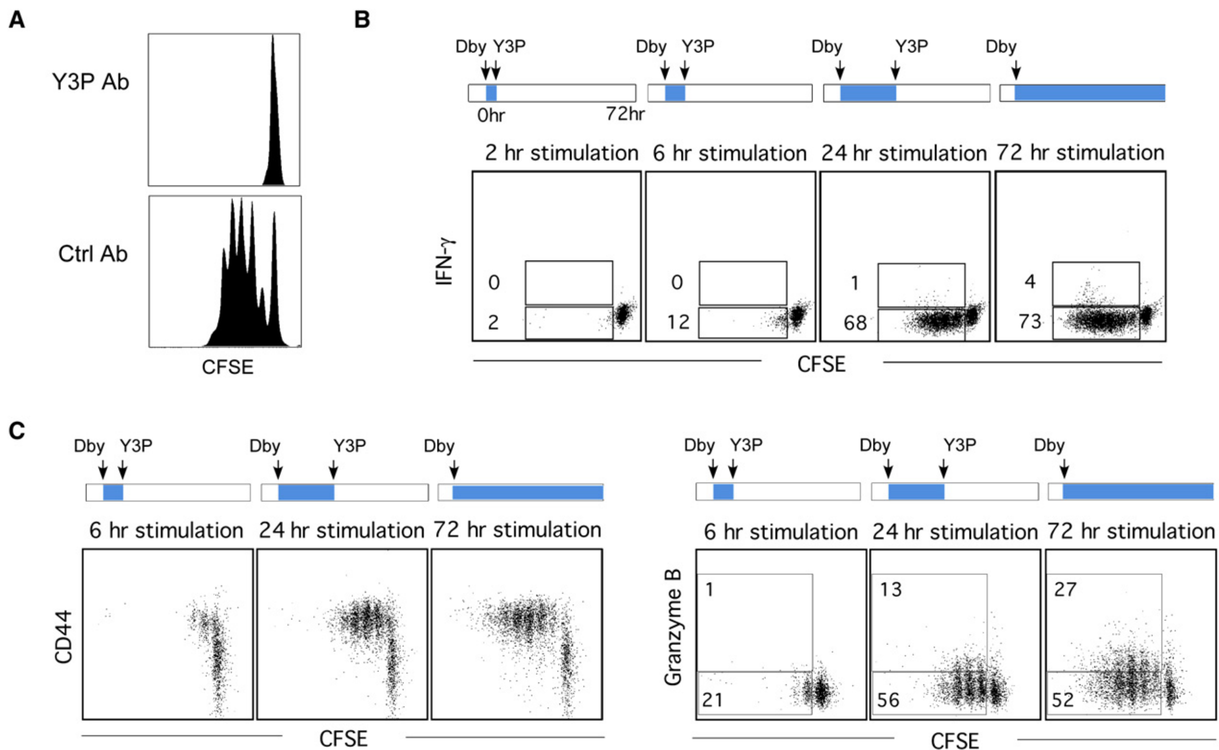

Figure 7. Manipulating the Duration of T Cell-DC Contacts In Vivo

MHC class II-deficient recipients were injected with DCs and were adoptively transferred with CFSE-labeled Marilyn CD4$^+$ T cells. The following day, the recipients received 50 μg of Dby peptide intravenously, and 500 μg of MHC class II (Y3P) or control Ab was injected at various time points. Proliferation and IFN-γ production was assessed 72 hr after Dby peptide injection by flowcytometry. Results are representative of three independent experiments.

(A) A 2 hr long T cell-DC interaction is not sufficient to promote T cell proliferation. In this experiment, MHC class II Y3P Ab or control Ab was injected 2 hr after Dby peptide injection. Data are gated on CD4$^+$CD45.1$^+$ cells.

(B) Proliferation and IFN-γ production are determined by the duration of T cell-DC interactions. MHC class II Ab was injected at various time after Dby peptide injection.

(C) Granzyme B expression but not CD44 upregulation by divided T cells is determined by the duration of T cell-DC interactions.

obtained from the CDTA. Mice expressing the OT-II transgenic TCR and mice expressing GFP under the human ubiquitin C (Ubi-GFP) (Schaefer et al., 2001) were bred in our animal facility. All animal experiments were performed according to institutional guidelines for animal care and use.

Cell Preparation and Transfer

Splenic DCs were purified as described (Bousso and Robey, 2003) and injected in the footpad of female recipient mice. CD4$^+$ T cells were isolated from the LNs of female Marilyn TCR Tg *RAG-2*$^{-/-}$ mice, labeled with 5 μM SNARF or CFSE (Molecular Probes) and injected intravenously.

Ex Vivo T Cell Activation Assay

MHC class II-deficient mice were injected in the footpad with 5 × 10^6 DCs. After 1 day, recipients were injected intravenously with 50 μg of Dby (NAGFNSNRANSSRSS) peptide (NeoMPS). Five minutes after peptide injection, LNs were treated with 1 mg/ml collagenase D (Sigma) for 15 min. LN cells were mixed at a 1:1 ratio with naive Marilyn CD4$^+$ T cells and cultured for 24 hr.

T Cell-DC Conjugate Assay

Splenic DCs obtained from Ubi-GFP female mice were pulsed for 30 min at room temperature with 1 μM Dby peptide and were cultured at 37°C with Marilyn T cells previously stained with 1 μM SNARF (DC:T ratio = 4:1). After 1 hr, some samples were collected and placed on ice. Others were incubated for an additional 30 min at 37°C in the presence of 10 μg/ml of an I-A^b Ab (clone Y3P) (Janeway et al., 1984) or an IgG2a isotype control Ab (eBioscience). All samples were then analyzed by flowcytometry for conjugate quantitation (FACS Canto, BD Biosciences). Y3P hybrodima was a kind gift of Dr Ana-Maria Lennon-Duménil and was propagated in VectraCell bioreactors (BioVectra). Y3P Ab was purified with HiTrap rProteinA columns on an Akta Purifier 10 (GE Healthcare).

FACS Analysis

LNs were incubated at 37°C for 15 min in RPMI-1640 containing 1 mg/ml collagenase. Cell suspensions were prepared and stained with a combination of the following antibodies: allophycocyanin (APC)- or PE-labeled anti-CD4, PE-labeled anti-CD69 (BD Biosciences), PE-Cy7-labeled anti-CD45.1, and APC-conjugated anti-CD44 (eBiosciences). For intracellular staining, LN cells were fixed in PBS containing 2% formaldehyde (Labonord) for 15 min at room temperature. Cells were incubated with APC- or PE-conjugated CD4 and PE-Cy7-conjugated CD45.1 antibodies and then subjected to intracellular staining with a PE-labeled anti phosphorylated c-Jun antibody (KM-1, Santa Cruz Biotechnology) or a Alexa 488-conjugated phospho ERK1/2 (BD Biosciences) or a APC-conjugated Granzyme B (Caltag) Ab and the Cytofix/Cytoperm kit (BD Biosciences) according to the manufacturer's instructions. Intracellular staining for IFN-γ was performed

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after a 4 hr phorbolmyristate acetate (PMA) (50ng/ml) and ionomycin (1μg/ml) stimulation and with a PE-conjugated IFN-γ Ab (BD Biosciences).

Intravital Two-Photon Imaging

The procedure for intravital two-photon imaging was adapted from previous studies with some modifications (Mempel et al., 2004b). In brief, a cannula was inserted in the jugular vein of the anesthetized mouse, and a popliteal LN was microsurgically exposed. The mouse was then placed on a custom-designed heated stage. So that the region of interest could be immobilized, plaster bandages were placed on each side of the posterior leg. A coverslip was placed on top of the popliteal LN and glued onto the plaster cast. The LN temperature was maintained at 37°C by a heated metal ring placed onto the coverslip and filled with water so that a 20×/0.95 NA dipping objective (Olympus) could be immerged. Two-photon imaging was performed with an upright microscope DM 6000B with a SP5 confocal head (Leica Microsystems). Excitation was provided by Chameleon Ultra Ti:Sapphire laser (Coherent) tuned at 900 nm. Typically five z planes spaced 8 μm apart and located at least 150 μm below the LN surface were imaged every 30 s. In some experiments, 50 μg Dby peptide or 500 μg of purified Y3P or control Ab were injected intravenously through the cannula during image acquisition. Movies were processed and analyzed with either Imaris software (Bitplane) or the Image5D plugin of the Image J software. Cell contacts were scored by the examination of individual z planes.

Supplemental Data

Twelve movies are available at http://www.immunity.com/cgi/content/full/27/4/625/DC1/.

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