Competition for antigen determines the stability of T cell–dendritic cell interactions during clonal expansion

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The regulation of T cell–dendritic cell (DC) contacts during clonal expansion is poorly defined. Although optimal CD4 T cell responses require prolonged exposure to antigen (Ag), it is believed that stable T cell–DC interactions occur only during the first day of the activation process. Here we show that recently activated CD4 T cells are in fact fully competent for establishing contact with Ag-bearing DC. Using two-photon imaging, we found that whereas prolonged interactions between activated T cells and Ag-bearing DCs were infrequent at high T cell precursor frequency, they were readily observed for a period of at least 2 days when lower numbers of T cells were used. We provide evidence that, when present in high numbers, Ag-specific T cells still gained access to the DC surface but were competing for the limited number of sites on DCs with sufficient peptide–MHC complexes for the establishment of a long-lived interaction. Consistent with these findings, we showed that restoration of peptide–MHC level on DCs at late time points was sufficient to recover interactions between activated T cells and DCs. Thus, the period during which CD4 T cells continue to establish stable interactions with DCs is longer than previously thought, and its duration is dictated by both Ag levels and T cell numbers, providing a feedback mechanism for the termination of CD4 T cell responses.

imaging | T cell activation | precursor frequency | two-photon imaging

CD4 T cell activation and differentiation are critically dependent on establishment of cell–cell interactions with antigen (Ag)-bearing dendritic cell (DCs) in lymph nodes. Taking advantage of the possibility to adoptively transfer a large number of T cells of known specificity and to track their behavior by using two-photon imaging, recent studies have indicated that T cell–DC contacts vary in stability over the course of T cell activation (reviewed in ref. 1). These results have led to the proposal that T cell activation involved distinct phases *in vivo*. A few hours after the initiation of the T cell response, T cells and DCs form long-lived conjugates. This phase of stable contacts can be preceded by a period during which only short-lived interactions are observed. Importantly, after 24 h, the formation of stable T cell–DC contacts is lost (2–4), with recently activated T cells only transiently contacting DCs. Whether changes occurring at the T cell or DC level are responsible for the modification of T cell–DC interactions during the activation process is not known.

Understanding the parameters regulating the establishment and stability of T cell–DC contacts over the course of T cell priming is essential for at least three reasons. First, the characteristics of T cell–DC contacts can encode important functional information, and, in most cases, stable T cell–DC contacts are associated with more efficient T cell priming (4–7). Second, T cell receptor (TCR) signals are not limited to a single cell–cell interaction because T cells sum up signals triggered by intermittent Ag recognition events (8, 9). In this respect, CD4 T cells can interact with DCs multiple times during the activation process, integrating signals delivered during these successive cellular encounters (10, 11). Finally, optimal CD4 T cell responses require Ag presentation to persist over several days, emphasizing the importance of late T cell–DC encounters (12).

Many factors could potentially influence the way T cells and DCs interact over the course of the response. These include modification of T cell and DC phenotype, as well as changes in T cell or DC numbers and in Ag load. In the present study we investigated the parameters regulating the dynamics of cell interactions between expanding CD4 T cells and DCs. We found that activated T cells were fully competent to establish interactions with DCs. However, when the number of responding T cells reached a certain threshold, competition for Ag sharply decreased the probability that T cells and DCs formed long-lived interactions. Our results identify T cell precursor frequency as a major parameter shaping the history of T cell–DC contacts and suggest that T cell clonal expansion exerts a negative feedback on the formation of T cell–DC contacts, helping to terminate the response.

Results

Activated T Cells Are Fully Competent for Establishing Cognate Interactions with DCs *in Vitro* and *in Vivo*. Previous reports have documented an absence of stable contacts between activated T cells and peptide-pulsed DCs at 48 h, raising the possibility that activated T cells have an inherent defect in forming conjugates with Ag-bearing DCs (2–4). To test this hypothesis, we measured by flow cytometry the ability of naïve and *in vitro* activated CD4 T cells bearing the Marilyn TCR to form conjugates with Dby-pulsed DCs. As shown in Fig. 1 *a* and *b*, naïve as well as activated CD4 T cells formed Ag-specific conjugates with DCs *in vitro*. In fact, cell conjugation was more efficient with activated T cells than with their naïve counterparts, being more rapid and requiring less Ag (Fig. 1*c*). To extend these results and assess the ability of divided T cells to interact with DCs, we labeled naïve T cells with carboxyl fluorescein succinimidyl ester (CFSE) and activated them *in vitro* for 2 days before incubating them with an excess of seminaphtharhodafluor (SNARF)-labeled Dby-pulsed DCs. Using this approach, it was possible to analyze the efficiency of DC conjugation as a function of the round of cell division undergone by T cells. Interestingly, the most divided T

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Abbreviations: Ag, antigen; pMHC, peptide–MHC; CFSE, carboxyl fluorescein succinimidyl ester; TCR, T cell receptor; APC, Ag-presenting cell; SNARF, seminaphtharhodafluor.

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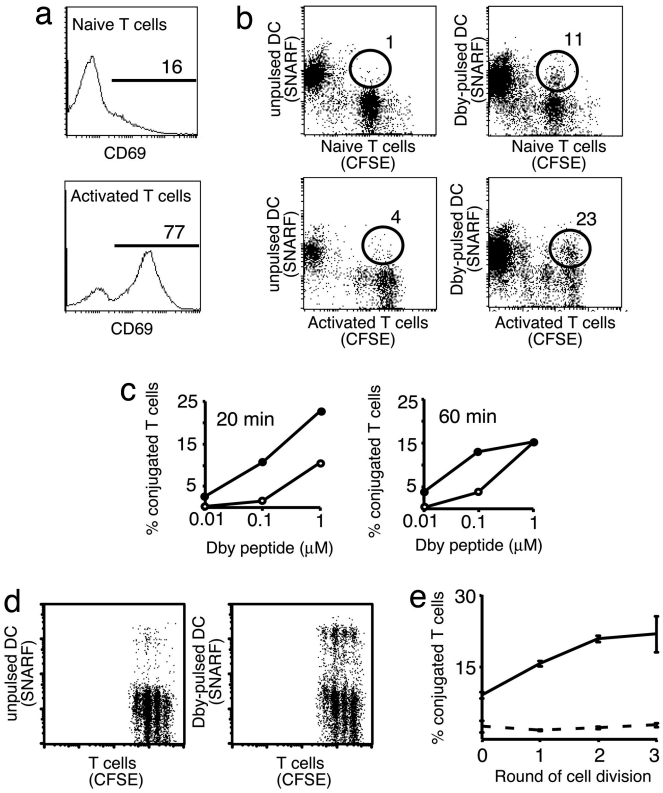

Fig. 1. Activated T cells are fully competent for establishing conjugates with Ag-bearing DCs *in vitro*. (*a*) Naïve Marilyn CD4 T cells were activated *in vitro* by using anti-CD3/CD28 beads. After 48 h, T cells were stained for CD69. (*b* and *c*) Naïve or activated Marilyn CD4 T cells were labeled with 1 μM CFSE and incubated with DCs pulsed with the Dby peptide or left unpulsed and labeled with 1 μM SNARF. (*b*) The percentage of naïve or activated T cells conjugated to a DC was quantified by flow cytometry at 20 min. DCs were pulsed with 1 μM Dby peptide. (*c*) The percentage of conjugated naïve (open circles) or activated (filled circles) T cells was measured at 20 and 60 min for different peptide concentrations used to pulse DCs. (*d*) Naïve Marilyn T cells were labeled with CFSE and stimulated *in vitro* with anti-CD3/CD28 beads. After 48 h, activated T cells were incubated with an excess of SNARF-labeled DCs that were either left unpulsed (*Left*) or pulsed with the Dby peptide (*Right*), and cell conjugation was assessed after 60 min. (*e*) The graph shows the percentage of conjugated T cells among those T cells that have undergone the indicated number of cell division. Cell conjugation was performed by using Dby-pulsed DCs (solid line) or unpulsed DCs (dashed line). Values are means ± SD. Results are representative of three independent experiments.

cells were the most likely to be recruited by DCs (Fig. 1 *d* and *e*). Altogether, these *in vitro* experiments argued against an intrinsic defect of activated T cells to engage Ag-bearing DCs.

To extend our results to an *in vivo* setting, activated and naïve Marilyn CD4 T cells were labeled with distinct vital dyes and adoptively transferred in recipient mice that were deficient for MHC class II molecules. Of note, we found that a large fraction of these recently activated T cells homed efficiently to lymph nodes. Recipients were injected in the footpad with unpulsed dye-labeled DCs isolated from wild-type mice. After 1 day, 50 μg of Dby peptide was injected i.v., resulting in the rapid (<5 min) and specific loading of injected DCs [the only MHC class II-expressing Ag-presenting cells (APCs) in the recipient] that had migrated to the draining lymph node (data not shown). Thirty minutes after peptide injection, draining lymph nodes were harvested and T cell–DC contacts were quantified by confocal microscopy. As shown in Fig. 2, peptide injection rapidly caused a substantial fraction of both activated and naïve T cells to be conjugated to DCs. In summary, recently activated

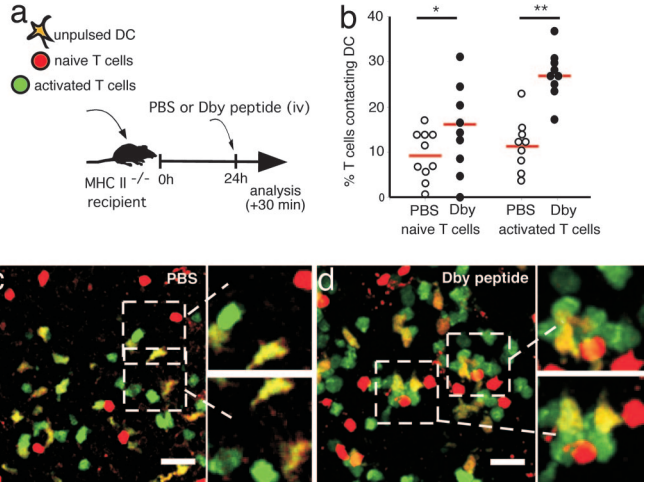

Fig. 2. Activated T cells interact with Ag-bearing DCs *in vivo*. (*a*) Experimental setup. MHC class II-deficient mice were injected in the footpad with 2×10^6 unpulsed wild-type female DCs labeled with a mixture of CFSE and SNARF dyes (0.5 μM and 1 μM, respectively). Recipients were also adoptively transferred with a 1:1 mixture of SNARF-labeled naïve Marilyn T cells and CFSE-labeled activated Marilyn T cells. At 24 h, each recipient received an i.v. injection of Dby peptide (50 μg) to specifically pulse the injected DCs present in the draining lymph node. After 30 min, popliteal lymph nodes were harvested, sectioned, and analyzed by confocal microscopy. (*b*) The percentage of activated or naïve T cells contacting a DC was measured on confocal images of lymph node sections. Each dot represents the value derived from an individual section. Mean values are indicated by a red bar. *, $P = 0.06$; **, $P < 0.001$. Results are representative of two independent experiments. (*c* and *d*) Confocal images of lymph node sections 30 min after injection of PBS (*c*) or Dby peptide (*d*). DCs are yellow, naïve T cells are red, and activated T cells are green. (Scale bar: 20 μm.)

CD4 T cells appear to be fully competent to establish Ag-specific interactions with DCs. This result provides a rationale for the analysis of T cell–DC dynamics during clonal expansion.

The Stability of T Cell–DC Contacts Is Regulated by T Cell Precursor Frequency. In the late phase of priming, T cell number increases as a result of T cell expansion while Ag levels may diminish with both phenomenon promoting T cell competition. Therefore, we asked how the behavior of expanding T cells varied in the lymph node based on their frequency in the T cell pool. We undertook the comparison of T cell–DC contacts at a late time point (48 h) using two-photon imaging of intact lymph nodes when 1×10^6 or 10×10^6 T cells were transferred. Recipients were injected in the footpad with Dby-pulsed DCs and adoptively transferred with 1×10^6 SNARF-labeled Marilyn CD4 T cells alone or in conjunction with 9×10^6 unlabeled Marilyn CD4 T cells (hereafter referred to as competitors). Using a similar strategy, we have previously shown that injected DCs that migrate to the draining lymph node displayed in mature phenotype (13). We used DCs purified from GFP expressing mice, as their brightness enabled us to visualize fine dendrite dynamics. In this set of experiments, we chose not to label the competitors to facilitate the identification of contacts made by SNARF-labeled T cells. As shown in Fig. 3 and in supporting information (SI) Movie 1, numerous stable contacts were made between CD4 T cells and DCs in the absence of competitors. Approximately half of T cell–DC contacts lasted at least 20 min (many of those lasted >1 h, but it was not systematically possible to follow the fate of these interactions as some cells left the imaged volume). Under these conditions, CD4 T cells had relatively stationary trajectories, low velocities and low confinement ratio, indicative of constrained movement (Fig. 3 and SI Fig. 7). In sharp contrast, the vast

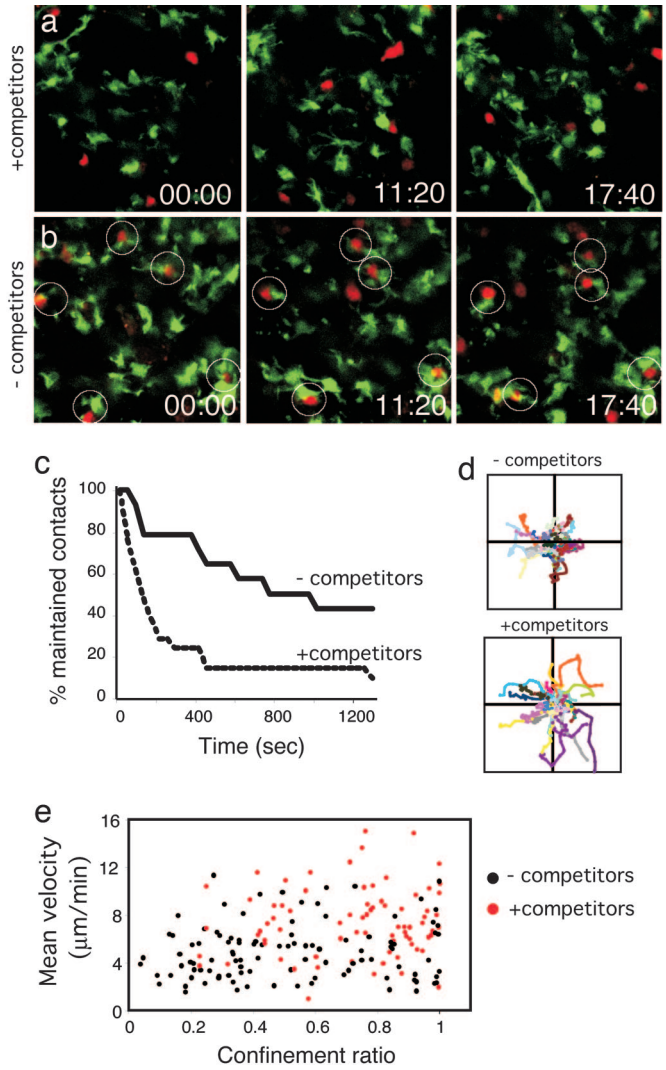

Fig. 3. T cell precursor frequency dictates the efficiency of stable T cell–DC formation. B6 recipient mice were immunized by footpad injection of 1.5×10^6 DCs derived from Ubi-GFP mice and pulsed with 100 nM Dby peptide. After 2–4 h, recipients were adoptively transferred with 1×10^6 SNARF-labeled naïve Marilyn CD4 T cells alone or together with 9×10^6 unlabeled Marilyn CD4 T cells (competitors). At 48 h after DC injection, intact popliteal lymph nodes were subjected to two-photon real-time imaging. (*a* and *b*) Time-frame images showing that long-lived T cell–DC interactions (marked by white circles) at 48 h occur in the absence (*b*) but not in the presence (*a*) of competitors. (*c*) Individual T cell–DC contacts ($n = 35$) were monitored over time, and the percentage of T cell–DC contacts maintained was graphed as a function of time. Long-lived interactions were observed only in the absence of competitors. (*d*) Individual T cell trajectories were analyzed in the absence ($n = 50$) or in the presence ($n = 48$) of competitors and graphed from a common origin. (*e*) T cells displayed higher velocities and confinement ratios in the presence of competitors. Individual CD4 T cells were tracked over time, and their mean velocity was graphed against their confinement ratio. Low confinement ratios reflect constrained movement. A total of 77 and 104 T cells tracks were analyzed in the presence and in the absence of competitors, respectively. Results are representative of at least four time-lapse movies obtained in two independent experiments.

majority of contacts made between labeled CD4 T cells and DCs in the presence of competitors were transient, lasting <10 min. Accordingly, the majority of CD4 T cells displayed high velocities and confinement ratio as well as long trajectories (Fig. 3 and SI Fig. 7).

The use of two-photon microscopy enabled us to track cells located up to 300 μm below the lymph node surface. To confirm that our findings also apply within deeper regions of the lymph node, we quantified CD4 T cell–DC conjugates at 48 h on lymph node frozen sections. We found that the frequency of CD4 T cells contacting DCs was only 12.6 ± 5.6% (mean ± SD) when 10×10^6 T cells were transferred but reached 33.8 ± 8.5% when 1×10^6 T cells were transferred (SI Fig. 8). Next, we assessed whether loss of T cell–DC interactions observed in conditions of high T cell frequency was Ag-specific. To this end, we analyzed, in the same recipient, conjugates between DCs pulsed with both Dby and $OVA^{323–339}$ peptides and dye-labeled OT-II and Marilyn T cells in the presence or in the absence of unlabeled Marilyn T cells competitors. As shown in SI Fig. 9, the presence of Marilyn T cell competitors inhibited interactions between DCs and labeled Marilyn T cells but did not significantly affect interactions between DCs and OT-II T cells. This finding indicates that the impact of T cell precursor frequency on T cell–DC interactions is largely Ag-specific.

Altogether, these results demonstrate that the probability for an individual Ag-specific T cell to establish a stable interaction with a DC at 48 h decreases sharply in condition of high T cell precursor frequency.

Lack of Stable T Cell–DC Contacts During T Cell Expansion Is Not Due to Steric Inhibition. One possibility to explain the lack of stable contacts at high T cell precursor frequency would be that T cell–DC encounters are prevented as a result of steric hindrance. According to this model, DCs would be surrounded by Ag-specific T cells and would not be accessible to other T cells. This could not be evaluated in the previous experiments as not all CD4 T cells were labeled. Therefore, we repeated the experiment, but this time, all Marilyn T cells were dye-labeled. As expected, when 1×10^6 T cells were transferred, CD4 T cells established frequently stable interactions at 48 h (SI Movie 2 *Right*). This was not the case, however, when 10×10^6 T cells were transferred, with most CD4 T cells establishing only transient contacts with DCs (SI Movie 2 *Left*). Yet, in this condition, a large part of the DCs surface appeared to be accessible to T cells (Fig. 4*a* and SI Movie 2). To further evaluate the possibility of steric inhibition, we counted, for each individual DCs, the number of transient encounters with Ag-specific T cells (over a fixed period of 25 min) as well as the number of stable interactions with T cells. We find that the number of transient encounters varied accordingly to the T cell number, with on average 0.6 and 4.5 encounters when 1×10^6 and 10×10^6 T cells were transferred, respectively (Fig. 4). We conclude that the failure to establish stable interactions at high T cell precursor frequency is not due to steric inhibition as T cells had no problem accessing the DC surface. However, we find that DCs established no more than one stable interaction with CD4 T cells at 48 h, independent of T cell number (Fig. 4). This result supports the idea that the number of sites on DCs permitting the establishment of a stable interaction at 48 h is the limiting factor in this system.

Clonal Abundance Regulates the Efficiency of T Cell Activation on a Per-Cell Basis. Next, we assessed whether the distinct patterns of late interactions observed in conditions of high versus low T cell frequency were reflected at the level of T cell activation. Recipient mice were adoptively transferred with 1 or 10×10^6 CD4 T cells bearing the Marilyn TCR and immunized by footpad injection of 1.5×10^6 Dby-pulsed DCs. As shown in Fig. 5, T cell activation (as detected by CD69 up-regulation) and proliferation (as detected by the loss of the CFSE dye) were less efficient on a per-cell basis in the presence of higher number of responding T cells. Similar results were obtained whether DCs were pulsed with 10 or 100 nM Dby peptide (Fig. 5). Competition was largely reduced when we used higher peptide concentrations (data not

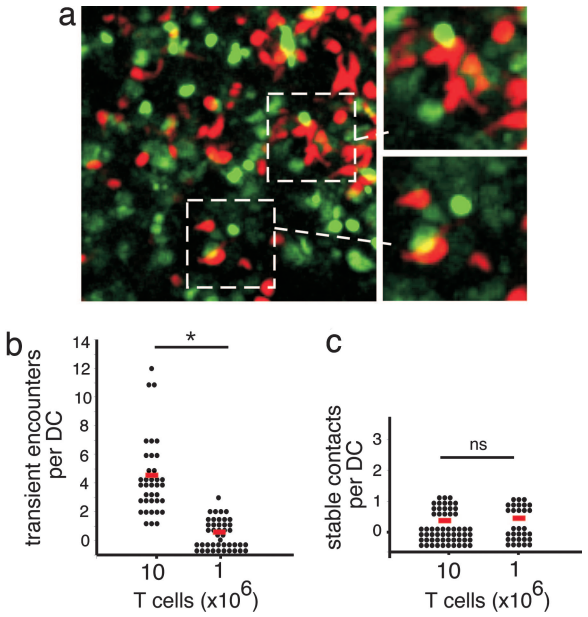

Fig. 4. T cell competition is not due to steric hindrance but to the lack of sites on DCs allowing the formation of stable interactions. Recipient mice were immunized by footpad injection of 1.5×10^6 SNARF-labeled DCs pulsed with 100 nM Dby peptide. After 2–4 h, recipients were adoptively transferred with 1×10^6 or 10×10^6 CFSE-labeled naïve Marilyn CD4 T cells. Intact popliteal lymph nodes were subjected to two-photon real-time imaging at 48 h after DC injection. (*a*) Representative images showing the density of CD4 T cells 48 h after transfer of 10×10^6 T cells. (*b* and *c*) Stable interactions but not transient encounters are limiting in the presence of high T cell numbers. Individual DCs were analyzed from two-photon time-lapse movies, and the number of transient encounters (<5 min) was recorded (over a fixed period of 25 min) as well as the number of stable interactions (>20 min). Mean values are indicated by a red bar. *, $P < 0.001$; ns, not significant ($P = 0.32$). Results are representative of at least three time-lapse movies obtained in two independent experiments.

shown). Thus, the lack of stable interactions at 48 h between CD4 T cells and DCs, at high T cell precursor frequency, was reflected by a less efficient activation process on a per-cell basis.

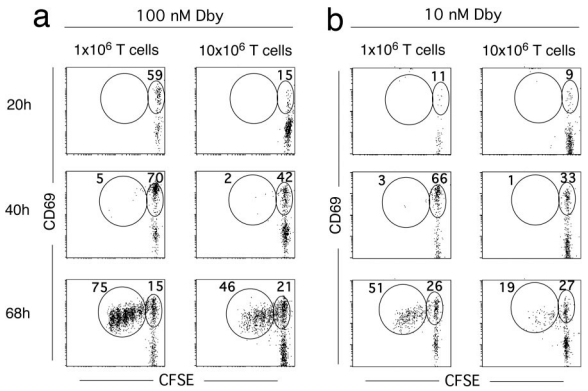

Fig. 5. T cell activation is reduced in the presence of high numbers of T cells. Recipient mice were immunized by footpad injection of 1.5×10^6 SNARF-labeled DCs pulsed with either 100 nM (*a*) or 10 nM (*b*) Dby peptide. After 2–4 h, recipients were adoptively transferred with 1×10^6 or 10×10^6 CFSE-labeled naïve Marilyn CD4 T cells. At various time points, popliteal lymph nodes were harvested and lymph node cells were analyzed by flow cytometry. Data are gated on $CD4^+CD45.1^+$ cells. At high T cell precursor frequency, T cell activation and proliferation were markedly reduced on a per-cell basis. Results are representative of three independent experiments.

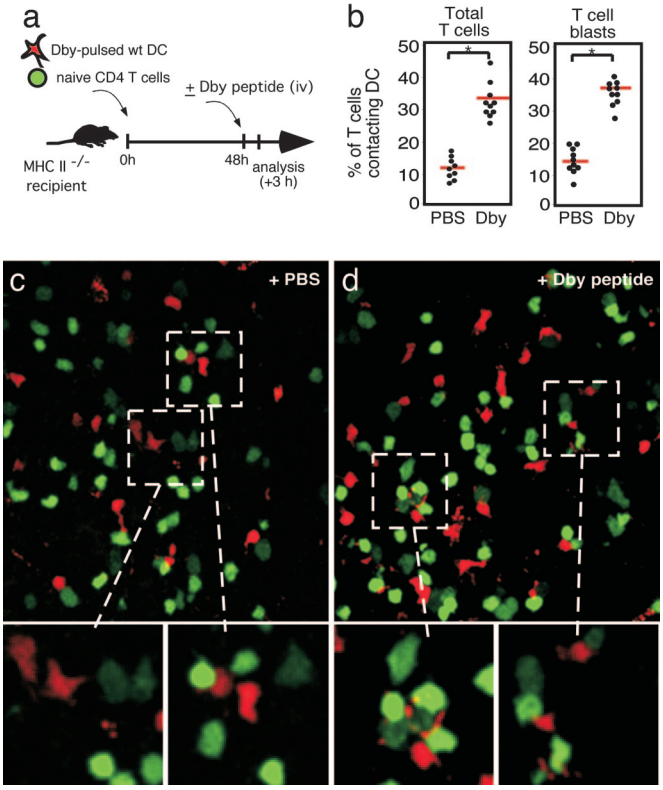

Fig. 6. Recovery of late T cell–DC interactions upon restoration of pMHC on DCs. (*a*) Experimental setup. MHC class II-deficient mice were injected in the footpad with DCs pulsed with 100 nM Dby peptide. After 2–4 h, recipients were adoptively transferred with 10×10^6 CFSE-labeled naïve Marilyn T cells. Recipient mice were injected i.v. with 50 μg of Dby peptide at 48 h after DC injection to restore pMHC levels at the surface of the transferred DCs. Control animals were injected with PBS. T cell–DC contacts were quantified 3 h after peptide (or PBS) injection. (*b*) The fraction of Marilyn CD4 T cells found to interact with a transferred DC after peptide or PBS injection was quantified on lymph node sections. Each dot represents the value derived from an individual confocal image of a lymph node section. The corresponding value was calculated for those T cells displaying the hallmark of T cell activation (large cells with dim CFSE intensity). Mean values are indicated by a red bar. (*c* and *d*) Representative images of lymph node section after PBS (*c*) or Dby (*d*) injection. *, $P < 0.001$. Results are representative of two independent experiments.

Recovery of Late T Cell–DC Interactions upon Restoration of Peptide–MHC (pMHC) on DCs. Finally, we assessed whether increasing the availability of pMHC complexes at the DC surface at 48 h, was sufficient to restore late T cell–DC contacts. Dby-pulsed DCs were labeled with the SNARF dye and injected in MHC class II-deficient recipients that were also adoptively transferred with 10×10^6 naïve CFSE-labeled Marilyn CD4 T cells. At 48 h, recipient mice were injected i.v. with Dby peptide to re-load the transferred DCs or with PBS as a control (Fig. 6*a*). The frequency of Ag-specific T cells interacting with DCs was then evaluated on lymph node sections 3 h after injection. In PBS injected animals, only few (12.3%) of T cells were found to contact DCs as expected. Importantly, large numbers of T cell–DC contacts were restored upon peptide injection, with 33% of Marilyn T cells contacting DCs (Fig. 6). As shown in Fig. 6*b*, this observation also applied to those T cells that displayed the hallmarks of activated cells (large cells with dim CFSE staining). The findings that restoration of pMHC is sufficient to restore late contacts *in vivo* further support the view that the lack of stable T cell–DC contacts at high T cell precursor frequency is linked to the limited availability of pMHC complexes.

Discussion

In the present article, we provide evidence that the formation of stable T cell–DC interactions *in vivo* is largely regulated by T cell precursor frequency. At low precursor frequency, T cells have the opportunity to establish one or several stable interactions with DCs during at least 2 days. When the number of responding T cells is too high, however, most activated CD4 T cells fail to stably interact with DCs. These results establish a cellular basis for intraclonal T cell competition and provide a feedback mechanism for the termination of T cell responses.

The recent use of dynamic imaging to track T cell–DC contacts in intact lymph nodes has deepened our understanding of T cell activation *in vivo*. These experiments revealed numerous stable T cell–DC contacts a few hours after the initiation of the T cell response (13–15). Surprisingly, in most cases, stable conjugates were lost after 24 h and activated T cells only transiently contacted DCs in a manner very similar to nonspecific interactions (2–4). What dictated the changes in T cell–DC contact stability over time? One possibility is that recently activated T cells exhibit a defect in forming tight contacts with Ag-bearing DCs. TCR down-modulation (16, 17) or TCR unresponsiveness after an initial stimulation (18) have been well documented and could potentially reduce the ability of activated T cells to interact with Ag-bearing DCs. Using several approaches, we found, on the contrary, that recently activated T cells were fully competent for engaging Ag-bearing DCs *in vitro* and *in vivo* (Figs. 1 and 2). On the other hand, we found that the probability that an individual T cell undergoes a stable interaction with an Ag-bearing DC was largely dependent on T cell precursor frequency (Fig. 3). In light of this observation, it is important to consider that one caveat of most two-photon experiments is the requirement for adoptive transfer of large number of T cells of known specificity (typically $5–10 \times 10^6$) so that enough events can be visualized and analyzed. In fact, and in contrast to previous observation, at lower precursor frequency, stable T cell–DC contacts are frequent, even at 48 h. This finding is likely to be relevant to the observation that optimal CD4 T cell expansion requires exposure to Ag for several days (12). We have previously shown that CD4 T cells can engage multiple DCs successively and integrate activation signals delivered during successive DCs encounters *in vivo* (11). Together with the result of the present study, this suggests that, when Ag-specific T cells are present at physiological frequency, they can establish repeated stable contacts during a period that last several days.

Our results extend previous studies that have shown a potential bias introduced by the transfer of large number of T cells bearing a given TCR (19, 20). With respect to two-photon imaging, care should be taken to minimize the effect of T cell competition for example by reducing the number of T cell transferred or increasing the level of Ag and number of APCs.

Why is T cell activation less efficient (on a per-cell basis) when the number of responding T cells is elevated? Several studies have reported different levels of T cell expansion, differentiation and survival based on the number of responding T cells used (19–25) and in some cases, competition for Ag have been demonstrated (21, 23, 26). However, the cellular events underlying intraclonal T cell competition have remained unclear. One possibility is that T cells compete for physical access to the APC. This was not the case in our study as numerous transient T cell–DC encounters were observed in the context of high T cell number. In addition, DCs failed to stably engage more than one Ag-specific T cells at 48 h, leaving most of the DC surface accessible to other T cells. A second possibility is that DCs are killed when surrounded by an excessive number of responding CD4 T cells. This was not the case either, because we did not observe obvious differences in the DCs number under different T cell precursor frequency. Another hypothesis is that T cells compete for a limited amount of a soluble factor. Although, our results do not exclude this possibility, this would be unlikely to affect the formation of T cell–DC conjugates. Rather, the bulk of our data suggests that the "niches" for which T cells compete during clonal expansion are sites on DCs still harboring enough Ag for the establishment of a long-lived interaction. This interpretation is supported by our observation that DCs were unable to bind more than one Ag-specific T cell at 48 h and is consistent with our previous finding that DCs displaying low levels of cognate pMHC do not induce the formation of large T cell clusters (13). Three phenomena likely contribute to the overall decrease of available pMHC during the course of T cell responses. First, pMHC half-life is finite, resulting in a decrease of pMHC density at the APC surface overtime in the absence of additional Ag delivery. Second, the first T cells engaging DCs could sequester cognate pMHC at the immunological synapse, reducing the probability that additional T cells sharing the same specificity get recruited by the same DC. Third, Ag-specific T cells can remove pMHC from the APC surface upon Ag recognition and therefore actively reduce Ag availability (27–29). Of note, although our study focused on T competing for pMHC, additional factors could also be limiting, as demonstrated in the case of competition between T cells of distinct specificities (30).

Although our experiments have identified a major factor controlling the stability of T cell–DC interactions over time, it is important to ask how the present data pertain to an endogenous T cell response. In the preimmune repertoire, the frequency of T cells specific for a given epitope is low (1×10^5 to 1×10^6) and T cell competition is unlikely to occur in the first 48 h during which only a few rounds of cell division occurs. Instead, during this stage, T cells have the opportunity to engage in multiple stable interactions with DCs and integrate signals for efficient activation. At later time points, T cell expansion and the decrease in Ag level promote T cell competition for the formation of stable contacts with DCs. As shown here, this would result in the prevention of most additional long-lived T cell–DC interactions. In addition, the diminution of stable T cell–DC contacts over time could be responsible for the weaker stimulation received by Ag-specific T cells arriving at late-time points in the lymph node which could promote the generation of central-memory cells (23). Overall, the dependence of T cell–DC contact dynamics on T cell frequency represents a safeguard mechanism for controlling the extent of CD4 T cells responses.

Materials and Methods

Mice. C57BL/6 (B6) mice were purchased from Charles River Laboratories (l'Arbresle, France). Six- to 9-week-old female Marilyn (anti-HY) TCR transgenic CD45.1$^{+/+}$ RAG-2$^{-/-}$ mice (31) and MHC class II-deficient mice were obtained from the Centre de Distribution, Typage et Archivage Animal. Transgenic mice expressing the enhanced GFP under the human ubiquitin C promoter (32) were purchased from The Jackson Laboratory (Bar Harbor, ME). All animal experiments were performed in accordance with the institutional guidelines for animal care.

Cell Preparation and Transfer. Splenic DCs were purified by using anti-CD11c-conjugated microbeads (Miltenyi Biotec, Auburn, CA) and an AutoMacs system as described (11). Cell purity was 80–90%. DCs were pulsed with the Dby (NAGFNSNRANSSRSS) peptide (NeoMPS, Strasbourg, France) for 1 h at room temperature and injected in the footpads of recipient female mice. Unless otherwise stated, DCs were pulsed with 100 nM peptide. Naïve CD4 T cells were isolated from lymph nodes of female Marilyn TCR transgenic RAG-2$^{-/-}$ mice and injected i.v. For preparation of activated T cells, naïve Marilyn CD4 T cells were cultured with anti-CD3/CD28 beads (Dynal, Carlsbad, CA) in the pres-

ence of 25 units/ml IL-2 at a ratio of one bead for four T cells. After 48 h, beads were removed by gentle pipetting. In most experiments, DCs and T cells were labeled before injection by using the indicated concentration of SNARF and/or CFSE dyes (Invitrogen, Carlsbad, CA) for 10 min at 37°C. T cells were adoptively transferred 2–4 h after DC injections.

Cell Conjugation Assay and FACS Analysis. Cell conjugates were analyzed as follows. Splenic DCs were pulsed with the indicated concentration of Dby peptide for 1 h at room temperature and then labeled with 1 μM SNARF for 15 min at 37°C. DCs were then mixed with either naïve or activated Marilyn T cells labeled with 0.5 μM CFSE at a ratio of four DCs per T cell. Cells were incubated for 20 or 60 min at 37°C and then directly analyzed by flow cytometry. For analyzing T cell activation *in vivo*, lymph nodes were harvested and incubated at 37°C for 15 min in RPMI medium 1640 containing 1 mg/ml collagenase. Lymph node cells were stained with the following antibodies: allophycocyanin-labeled anti-CD4, phycoerythrin-labeled anti-CD69, and anti-CD45.1. Samples were analyzed on a FACSCalibur (BD Biosciences, San Jose, CA).

Confocal and Two-Photon Microscopy. Popliteal lymph nodes were fixed in 4% PFA for 15 min at 4°C, washed in PBS, incubated in PBS 20% sucrose for 1 h at 4°C, and frozen in OCT compound (Tissue-Tek Sakura Finetek, Zoeterwoude, The Netherlands). Eight-micrometer-thick cryosections were mounted by using Vectashield mounting medium (Vector Laboratories, Burlingame, CA) and analyzed with a confocal microscope (Carl Zeiss MicroImaging). Z-stacks of images spaced 1 μm apart were acquired. Maximum intensity projection were obtained by using ImageJ software. Two-photon imaging was performed by using an upright DM6000 microscope with a SP5 confocal head (Leica Microsystems, Wetzlar, Germany) and a Chameleon Ultra Ti-:sapphire laser (Coherent) tuned at 900 nm. Emitted fluorescence was split with a 560-nm dichroic filter and passed through 525/50 and 610/75 filters (Chroma Technology, Rockingham, VT) to nondescanned detectors (Leica Microsystems).

Lymph nodes were maintained at 37°C and superfused with RPMI medium 1640 without phenol red bubbled with a gas mixture containing 95% O_2 and 5% CO_2. Typically, eight *z*-planes spaced 7 μm apart were acquired every 40s. All images were acquired at least 150 μm below the lymph node surface. Time-lapse movies were processed by using ImageJ software. Velocities and trajectories were analyzed by using the Mtrack2 plug-in for ImageJ. The confinement ratio represents the ratio of the distance between the first and last positions of the T cell to that of the length of the trajectory. T cells with constrained trajectories typically displayed low confinement ratios.

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