Duration of the initial TCR stimulus controls the magnitude but not functionality of the CD8+ T cell response

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CD8+ T cells only require a brief stimulation with antigen in vitro to divide and differentiate into effector and memory cells upon transfer in vivo. The efficiency of clonal expansion and the functional characteristics of memory cells derived from briefly stimulated cells are poorly defined. We developed a system that allowed us to examine programming entirely in vivo. This was achieved by rapidly killing peptide-pulsed DCs carrying a diphtheria toxin receptor transgene with timed injections of diphtheria toxin without altering the course of an accompanying infection. The magnitude of clonal expansion, but not the functionality of the effector cells, correlated directly with the duration of antigen exposure. Furthermore, memory T cells were capable of mounting a secondary response, regardless of the length of antigen encounter during the primary response. These results indicate that the duration of initial antigen encounter influences the magnitude of the primary response, but does not program responsiveness during the secondary challenge.

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Abbreviations used: DT, diphtheria toxin; DTR, DT receptor; DTRtg, DTR transgene.

Distinct stages characterize the development of a naive CD8+ T cell into a memory T cell. A naive CD8+ T cell encounters its antigen, becomes activated, and while undergoing numerous rounds of cell division differentiates into an effector cell capable of killing infected target cells (1). At the peak of the CD8+ T cell response, antigen-specific cells may have increased as much as 50,000-fold (2), but 90–95% of these cells undergo apoptosis over the course of the next 7–14 d. The remaining cells continue to differentiate and establish an antigen-specific, long-lived memory CD8 T cell population (1).

The requirements to successfully prime a naive T cell and guide it into the memory cell development pathway have been of long standing interest. In particular, the impact individual activation requirements might have on the size of the memory pool and on the quality of a secondary response is crucial for the development of better vaccines. It has become clear that to be fully activated a CD8+ T cell needs to receive three distinct signals: antigen, costimulation, and a signal 3 cytokine which can be provided by IL-12 or type I interferon (3–5). Several key studies have introduced the concept of T cell programming (6–10), which describes the phenomenon that a brief encounter with antigen is sufficient to trigger a cell autonomous program leading to proliferation and differentiation into memory T cells. Ensuing studies further addressed the time frame necessary to ensure successful programming of a T cell (11–13). Stipdonk et al. (11) suggested that a very brief stimulation (4 h) would result in clonal abortion, whereas a somewhat longer stimulus (20 h) leads to expansion. Curtsinger et al. showed that 6 or 18 h of in vitro stimulation in the presence of IL-12 was not sufficient for optimal expansion and full development of effector function, as they observed a substantial increase in CD8+ numbers and CTL activity when antigen stimulation was prolonged to 64 h (14).

One caveat of these studies is the limitation of providing the initial timed antigenic stimulus in vitro before transferring the cells into an antigen-free environment. Other studies overcome this hurdle by controlling bacterial antigen presentation in vivo through various treatment patterns with antibiotics, thus reducing inflammatory stimuli and the antigen load (6, 12, 13). The enhancing effect of inflammation on effector T cells independently of antigen was documented by Busch et al. by

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demonstrating that in vivo–generated CD8$^+$ effector T cells do undergo further short-term expansion in response to bacterial infection even in the absence of antigen (15). Collectively, these studies suggest that in vivo programming of CD8$^+$ T cells is completed within 36–60 h (assuming antibiotic clearance of the pathogen within 12 h), but they also underline the necessity of studying programming in a system that allows dissecting the role of the TCR stimulus from inflammatory signals. Another complication of controlling bacterial antigen presentation by antibiotic treatment is that the timing cannot be precise. There is the potential of prolonged antigen presentation through cross-presentation, even in the absence of a detectable bacterial load. It is thus not clear if the T cells were indeed deprived of an antigen stimulus after clearance of the bacteria 12–24 h after onset of the antibiotic treatment.

We wanted to overcome the limitations of previous studies and examine the concept of CD8$^+$ programming in a defined in vivo environment with close to physiological conditions while matching the efficient removal of antigen possible in in vitro experiments. Here, we used a system that allowed us to isolate the role of the TCR in programming, i.e., vary antigen exposure time while keeping constant other variables such as cytokine environment (16–18), nature of the antigen-presenting cell and costimulatory molecules (19, 20), and signal strength (21, 22). In addition to studying programming in vivo, we examined whether parameters encountered during the priming phase would be imprinted in these cells and subsequently affect CD8$^+$ T cell behavior in a secondary challenge. We report here that cells stimulated with antigen for a limited time display a limited potential to accumulate in the primary response but become

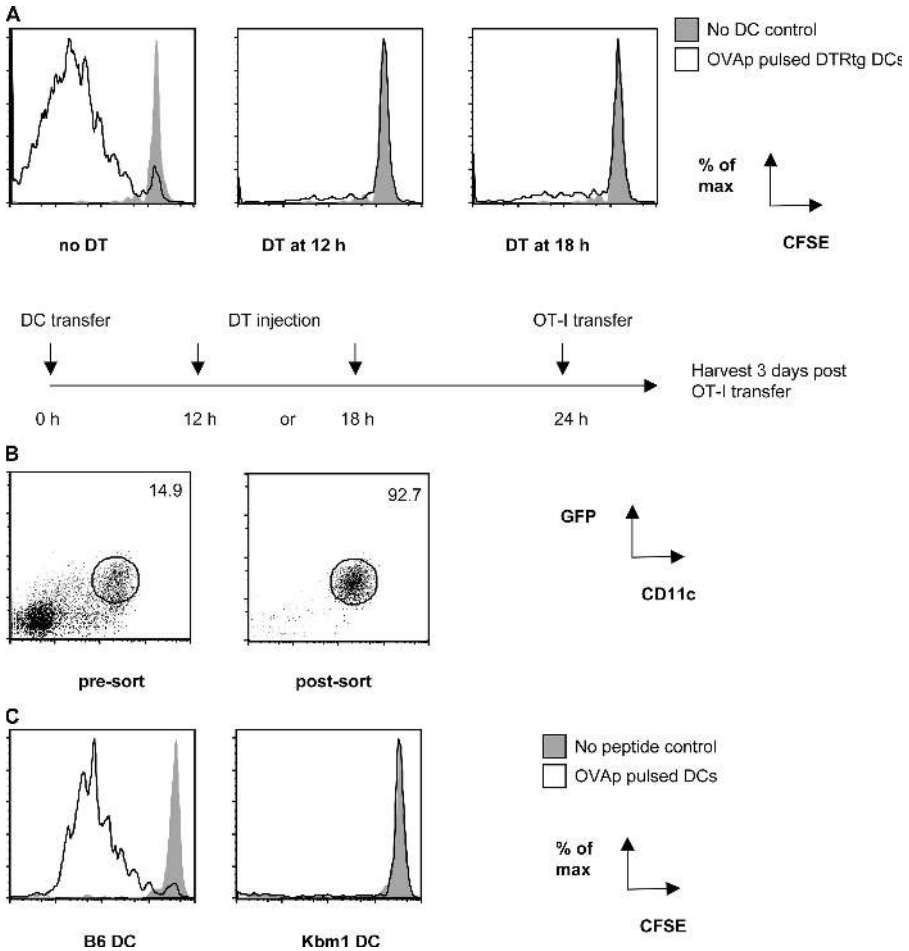

Figure 1. GFP^{hi} DTR transgenic DCs are rapidly deleted by diphtheria toxin. (A) 3.7 × 10^5 OVAp-pulsed, GFP-sorted DTRtg DCs were transferred i.v. and the mice were left untreated (left) or treated with DT at 12 h (middle) or 18 h (right) after transfer. 4 × 10^5 CFSE-labeled OT-I T cells were adoptively transferred 24 h after DC injection, and mice were harvested 3 d after OT-I transfer. Histograms shown are gated on OT-I T cells from an OT-I only control animal (gray) or the respective experimental groups (bold). (B) GFP^{hi} and thus DTR^{hi}-expressing DCs were sorted and used to assure responsiveness to the toxin. Pre-sort FACS plot shown is based on a spleen from a Flt3L-treated animal after depletion of T and B cells (see Material and methods). (C) 8 × 10^5 DCs from B6 or Kbm1 mice were pulsed with LPS (gray) or LPS + OVAp (bold) and transferred into B6 recipients that received 10^6 naive OT-I T cells 24 h earlier. 3 d after transfer of the DCs, the CFSE profile of the OT-I cells was used as a readout of T cell activation.

programmed to develop into memory cells that are fully functional in a rechallenge.

RESULTS

To study programming in vivo in an environment that mimics physiological conditions as closely as possible, we developed a system that allowed us to study the effects of varying the duration of antigen exposure to naive $CD8^+$ T cells without altering the inflammatory milieu in the host. This enabled us to distinguish between cytokine-mediated and antigen-mediated signals delivered to $CD8^+$ T cells. In our system, antigen is delivered via adoptive transfer of an excess number of peptide-pulsed DCs isolated from mice carrying a GFP-diphtheria toxin receptor transgene (DTRtg) expressed under the CD11c promoter (23). Since rodent cells do not express the receptor for diphtheria toxin (DT), the transgenic DCs are the only cells susceptible to the toxin (24).

First, we tested the efficiency and kinetics of toxin-mediated depletion of DCs by transferring 3.7×10^5 OVAp-pulsed DTRtg DCs, followed by DT injections at different time points before transfer of 4×10^5 OT-I T cells 24 h later (Fig. 1 A). CFSE dilution on day 3 after transfer of the OT-I cells was used as an indicator of DC presence. The vast majority of peptide-pulsed antigen-presenting DCs is eliminated within 6 h of DT injection (Fig. 1 A, right, DT at 18 h), and virtually all DCs are eliminated within 12 h of DT injection (Fig. 1 A, DT at 12 h, middle and reference 25). Such efficient removal of DTRtg DCs depended on transferring sorted GFP^{hi} $CD11c^+$ cells (Fig. 1 B), i.e., DCs which are most sensitive to the toxin. In accord with reports in the literature (26–29) we did not observe cross-presentation of the peptide, as Kbm1 DCs pulsed with OVAp did not trigger OT-I proliferation (Fig. 1 C). Thus, antigen presentation is aborted with deletion of the DC, as also shown by the lack of

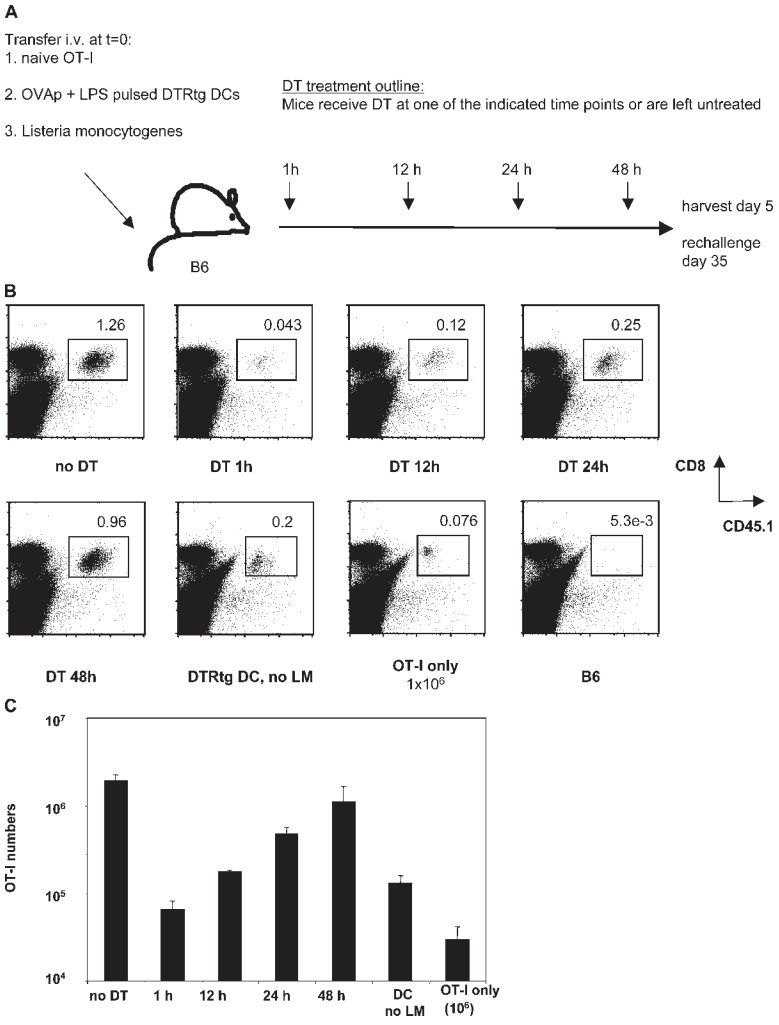

Figure 2. Varying the antigen exposure time affects the magnitude of the primary $CD8^+$ T cell response. (A) A schematic of the adoptive transfer system. (B) 10^4 CD45.1 congenic, CFSE-labeled OT-I T cells were transferred with 3.7×10^5 SIINFEKL-pulsed DTRtg DCs, and DT was administered at the indicated time points. All experimental groups apart from the DTRtg DC, OT-I only, and B6 control groups received 2,000 CFU WT-LM. The percentage of OT-I T cells in the spleen is shown. (C) Absolute numbers of OT-I T cells on day 5 of the groups shown in B. Note that the OT-I only animals received 100× more OT-I T cells than animals in the experimental groups.

antigen-specific T cell proliferation after DT treatment (Fig. 1 A). We furthermore confirmed that the priming ability of DTRtg DCs is not altered compared with WT DCs and that the toxin itself does not interfere with the $CD8^+$ T cell response (unpublished data).

As outlined in Fig. 2 A, we transferred 10^4 OT-I T cells together with 3.7×10^5 OVAp-pulsed DTRtg DCs and infected the mice with WT-LM to provide a general inflammatory environment (30). DT was administered 1, 12, 24, or 48 h after OT-I/DC transfer, and mice were harvested on day 5 after challenge (Fig. 2 B). Although the 48 h DT group showed no difference compared with the no toxin control, a reduction in OT-I numbers was observed in the 1-, 12-, and 24-h groups in percentage and absolute numbers (Fig. 2, B and C). Thus, the longer antigen is presented the more OT-I T cells accumulate. Interestingly, mice that were not treated with DT and did not receive WT-LM (Fig. 2 B, DTRtg DC, no LM group) resemble the 12-h DT group in terms of

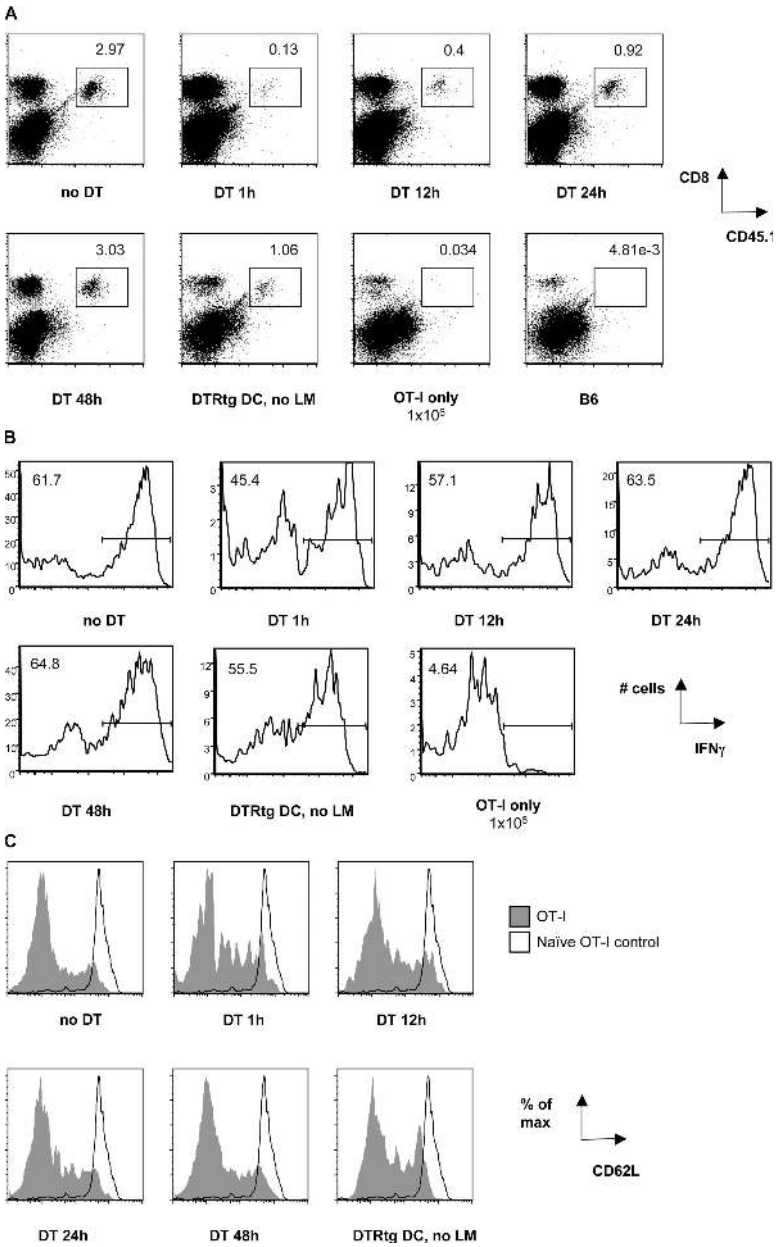

Figure 3. Varying the antigen exposure time does not affect $CD8^+$ T cell functionality. (A) To determine trafficking properties, lungs of mice were harvested after perfusion of the animal and analyzed for the presence of OT-I T cells. The percentage of OT-I T cells in the lung is shown. (B) Splenocytes were tested for their ability to produce IFNγ in a 4-h assay. Histograms are based on a gate specific for the congenic OT-I T cells. (C) Splenic OT-I T cells were analyzed for CD62L surface expression on day 5. Naive OT-I control cells are shown in white, and OT-I T cells from the experimental groups are shown in gray.

OT-I numbers (Fig. 2 C), thus illustrating the enhancing effect provided by WT-LM infection. As a control, B6 mice received 10^6 OT-I T cells alone to determine the efficiency of the take, or no OT-I T cells at all to establish the staining background (Fig. 2, B and C). We confirmed that the efficiency of the take is comparable between 10^4 and 10^6 cells (unpublished data) and calculated the fold expansion for the various experimental groups. OT-I T cells in animals that received no DT expanded more than 6,000-fold, whereas treatment with DT 1 h after transfer resulted in ~200-fold expansion (Fig. 2 C). Waiting for 12 or 24 h before injecting DT increased OT-I numbers 3–9-fold, respectively, compared with the 1-h DT-treated group.

Despite this reduction in OT-I numbers by DT treatment up to 24 h, we did not observe major functional differences between the groups. OT-I T cells showed no impairment in their ability to traffic to the lung (Fig. 3 A), maintaining the accumulation pattern among the groups that was noted in the spleen. Similarly, their ability to produce IFNγ is comparable (Fig. 3 B), though OT-I cells from the 1 h DT group tend to produce less IFNγ and express higher levels of CD62L (Fig. 3 C) than OT-I cells from the other groups.

We conclude from these results that to guarantee optimal expansion of $CD8^+$ T cells, they need to be able to encounter their antigen for up to 54 h (treatment at 48 h after priming plus 6 h to eliminate the vast majority of OVAp-presenting DCs). In contrast, less than 7 h (treatment at 1 h past priming and 6 h to eliminate DCs) is sufficient to establish functionality of effectors as assessed by cytokine production and tissue migration.

We went on to address which mechanisms contribute to the increased accumulation in mice treated with DT at later time points. We analyzed the CFSE profile of transferred OT-I T cells in all groups, but no apparent differences were visible (Fig. 4 A), as all cells were CFSE negative, suggesting that cells in all treatment groups underwent at least seven rounds of division. However, we were concerned that some of our transferred T cells in the 1- and 12-h groups did not get recruited into the response, which could be masked by the bulk of expanding cells. To determine if recruitment of cells was a major factor, we took advantage of the pull-down assay, a recently published approach to harvest the majority of adoptively transferred cells (31). After enrichment of the transferred OT-I T cells (Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20060928/DC1), we analyzed the retrieved cells by FACS and found that only a small fraction did not fully dilute their CFSE in the 1- and 12-h group (Fig. 4 B). The small fraction of CFSE-containing cells cannot explain the difference between the 1- and 12-h group, and cannot account for the ~30-fold difference in numbers between the untreated and 1-h group (Fig. 2 C). We also confirmed that infection with 2×10^3 CFUs of WT-LM did not cause apoptosis of unprimed T cells (unpublished data), thus excluding the possibility that nonrecruited OT-I T cells are not detected because they were eliminated from the host (32).

We considered the possibility that $CD8^+$ T cells encountering antigen only briefly might undergo seven rounds of division and thus fully dilute the CFSE dye (Fig. 4 A), but then stop or slow down proliferation. To address this issue, we pulsed mice with BrdU for 2 h before harvesting to obtain a snapshot of their turnover on day 5. Although the cells of the 1-h group incorporate substantial amounts of BrdU, we did observe a reduction in BrdU uptake in the 1- and 12-h groups compared with the untreated control group (Fig. 4 C). Although the difference in turnover is likely a contributing factor, we cannot rule out that survival of OT-I T cells increases with longer antigen exposure, thus further potentiating the effect of increased cell turnover.

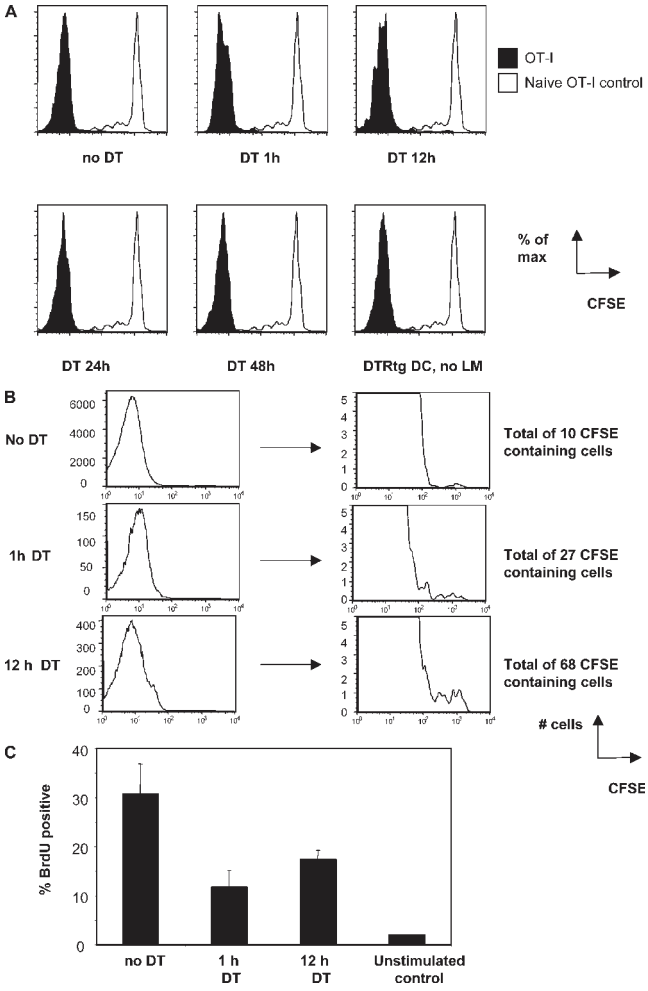

Figure 4. Proliferation but not recruitment contributes to the different size of the OT-I pool in DT-treated animals. (A) The CFSE profile of OT-I T cells in all groups was analyzed on day 5 after priming (black histogram) and compared with an unprimed control (white histogram). (B) To further assess whether unrecruited, CFSE high cells are present in our early DT-treated groups, a pull-down assay was performed (see Materials and methods). The panels on the left are gated on OT-I T cells from untreated (top), 1 h–treated (middle), or 12 h–treated (bottom) mice. On the right, the same data are shown on a bigger scale to facilitate detection of low numbers of cells. (C) Mice were pulsed with BrdU 2 h before harvesting on day 5. Congenic OT-I T cells were analyzed for their BrdU content.

Initial experiments on CD8 programming have addressed the primary response of in vitro–stimulated cells and their ability to respond 43 d later in an IFNγ assay (7). In light of our finding that cells that were only briefly stimulated display a decrease in accumulation and turnover, we wanted to examine the potential of these cells to respond to a rechallenge more closely. We chose to use a recombinant LM strain that expresses OVA (LM-OVA) for the rechallenge. Since all mice were injected with WT-LM during the primary response, the difference in OT-I T cell numbers between our experimental groups will not be a decisive factor in clearing LM-OVA. This rechallenge strategy provides a very similar environment in our host mice, yet allows for expansion of the OT-I T cells, thus ensuring a fair comparison. Mice were infected with 2×10^5 LM-OVA on day 35 after the primary infection with WT-LM and injection with OT-I cells and peptide-pulsed DCs, and harvested on day 3 after rechallenge. We observed an expansion of OT-I T cells in all groups (Fig. 5, A and B). Significantly, the proportion of the OT-I population within the groups remained constant between the primary and secondary response (Fig. 2 C and Fig. 5 B), suggesting that all T cells proliferated and survived at similar rates in the secondary response (Fig. S2, available at http://www.jem.org/cgi/content/full/jem.20060928/DC1). OT-I T cells of all groups were capable of responding in an IFNγ assay (Fig. 5 C) and were found in nonlymphoid tissues at ratios comparable to the spleen (unpublished data). It is noteworthy that at this point the OT-I T cells from the 1-h DT-treated group produce as much IFNγ as OT-I T cells from all other groups, hence the tendency to produce less IFNγ that was observed during the primary response is not imprinted in the memory cell response to rechallenge. Our data indicate that prolonged antigen exposure is required for an optimal primary response, but a shortened exposure in the primary response does not impair T cells in their ability to respond to a rechallenge.

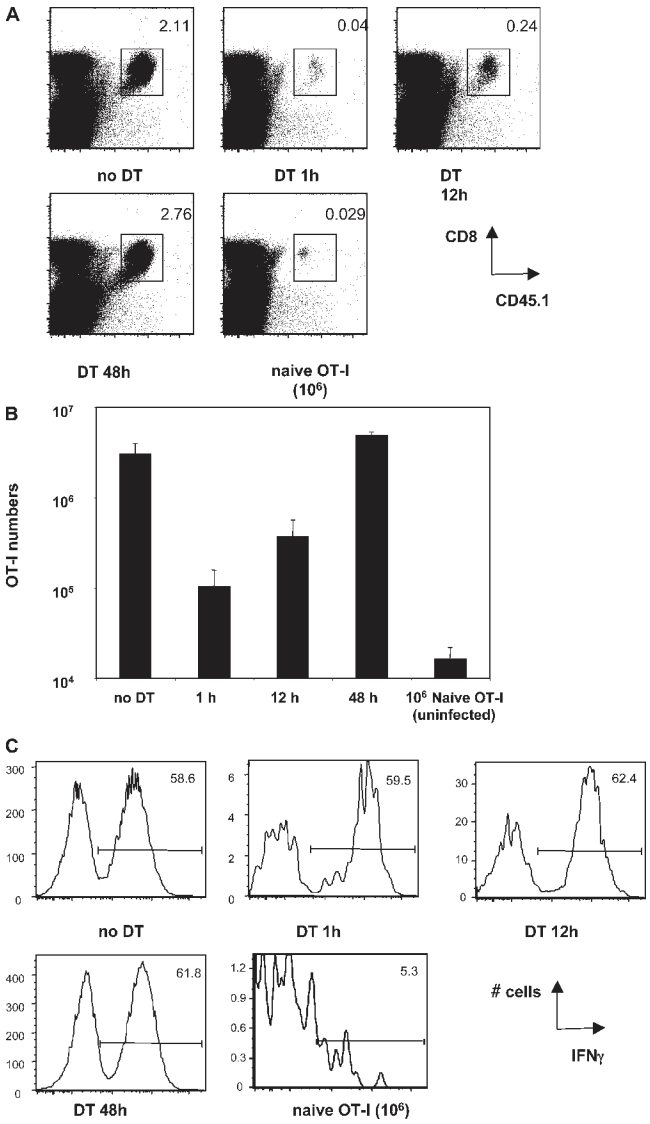

Figure 5. Shortening antigen exposure time in the primary response does not affect the efficiency of the secondary response. (A) 35 d after priming, mice were rechallenged with a dose of 2×10^5 LM-OVA, harvested 3 d after rechallenge, and the magnitude of the recall response was assessed by analyzing the size of the OT-I pool. The naive OT-I group that had received 10^6 OT-I on day 0 remained uninfected. The percentage of OT-I T cells in the spleen is shown. (B) Based on the data shown in A, the absolute number of OT-I T cells was calculated. (C) Splenocytes were tested for their ability to produce IFNγ in a 4-h in vitro assay. Histograms are based on a gate specific for the congenic OT-I T cells.

DISCUSSION

Several reports established the concept of $CD8^+$ T cell programming after short-term antigen presentation relying on delivering the priming stimulus in vitro (7–9). Here we report the design of a system that allowed us to study $CD8^+$ T cell programming entirely in vivo. We examined the relevance of the duration of TCR engagement in programming the primary response and its consequences for the secondary response.

We chose to deliver antigen through peptide-pulsed DCs carrying a transgene encoding the DTR. The DTRtg DCs can be efficiently removed by administration of the toxin without passing on the antigen to host APCs. This was done in the context of a WT-LM infection, providing a physiological environment encountered during infection (Fig. 2 A). In this system, we found that OT-I T cells that encountered antigen for only 7 h receive all necessary signals to develop effector function, including proper trafficking to nonlymphoid tissues and the ability to differentiate into functional memory cells. These 7 h are the maximum interaction time possible, based on the assumption that the OT-I T cell gets primed by the antigen-presenting DC immediately after adoptive transfer and stays in contact with DCs until the latter are eliminated 6 h after administration of the toxin (Fig. 1) at 1 h after transfer. It is thus very likely that the actual interaction time between OT-I T cells and DCs is considerably shorter, implying that the required time span to program

effector function and subsequent differentiation into a memory cell is less than 7 h.

Some insight regarding the requirements for T cell programming was provided by previous in vivo studies, though these were limited in their ability to terminate antigen presentation at defined time points. Termination of antigen presentation could not be achieved without altering other environmental conditions and was further complicated by the possibility that antigen continued to be presented through cross-presentation even after antibiotic mediated removal of a pathogen, thus making it difficult to define when APCs cease to present antigen. Collectively, this means that thus far we have lacked a clear definition for the role of TCR-derived signals in promoting $CD8^+$ T cell programming in vivo. Another in vivo study indeed reports data that seem to contradict the programming hypothesis. Storni et al. observed that $CD8^+$ T cells require antigen for several days in vivo to produce IFNγ (33). However, interpretation of their data is complicated by the fact that in their system using virus-like particles, inflammation is rather limited, which further underlines the importance of studying programming in a system that dissects the roles of these two stimuli. Although we define the role of duration of antigen presentation, we do not directly address to what extent cytokines and other inflammatory mediators contribute to $CD8^+$ T cell programming other than describing the enhancing effect of cytokines and other mediators on T cell accumulation (Fig. 2, B and C, no DT vs. DTRtg DC, no LM).

The ability to discontinue antigen presentation within a defined time period allows us to discuss our findings in regard to a 2-photon microscopy study in live animals that showed that T cell priming occurs in three distinct phases (34). The first phase is characterized by short-lived T cell–DC interactions and lasts for ∼8 h. The subsequent 12 h are defined by slower migration of the T cell and longer T–DC conjugates, before shortening the contact time again on the second day after priming. Our data suggest that the early interactions are sufficient to instruct the T cell for functionality, but longer contact with DCs is needed for optimal long-term proliferation and survival. Deleting DCs with diphtheria toxin 48 h after priming results in removal of the antigen within 54 h or less. This time span is sufficient to equip the T cell with its full functional and proliferative potential. Our 24-h DT experimental group (being equal to or less than 30 h of TCR signals), on the other hand, showed consistently lower T cell accumulation, indicating that the third phase of priming as described by Mempel et al., though not essential for functionality, does play a role in further boosting the T cell response (34). We considered the possibility that these later phases of priming might be important to equip OT-I T cells with the ability to home to nonlymphoid tissues. Examination of the lungs failed to show any evidence of an impaired trafficking pattern (Fig. 3 A) regardless of the DT treatment protocol. OT-I T cells of all groups were found in the lung in proportion to their splenic abundance, illustrating that the completion of the first two phases of priming, as described with two photon microscopy, is not essential for effector T cell development itself.

The potential of $CD8^+$ T cells to accumulate depends on the antigen exposure time (Fig. 2, B and C). Although we have evidence that a slower turnover in cells that received a shorter antigen stimulus is a contributing factor (Fig. 4 C), recruitment of cells into the response does not play a role at the T cell to DC ratio used in our study. It is likely that cell survival is involved as well, though preliminary experiments using annexin V staining did not shed light on this issue (unpublished data).

Interestingly, the differences in OT-I T cell accumulation after the primary response between the different groups are still reflected after a rechallenge of the memory cells. One would expect a larger difference in OT-I cell numbers between the groups after rechallenge, if OT-I memory cells from the 1-, 12-, and 24-h DT groups were behaving similarly as they did during the primary response. Importantly, the differences observed after the primary response are maintained at unaltered ratios, indicating that during the secondary challenge OT-I T cells from the 1-h DT group are as potent in proliferating and accumulating as their DT untreated counterparts. Prolonged TCR-mediated signals are thus not essential for triggering the instructional program to become a functional memory cell. This is confirmed by our finding that OT-I T cells from untreated and 1-h DT-treated mice responded equally well in an IFNγ assay after rechallenge (Fig. 5 C) despite the tendency of 1-h DT-treated mice to produce less IFNγ in the primary response. This finding appears to be in contrast with studies that correlate a short antigen interaction time with induction of tolerance (11, 14, 33). We believe that the different outcome is caused by the experimental setups applied and suggest that T cells that encounter an antigen briefly during an infection develop into fully functional memory cells, though their initial contribution to the size of the T cell pool will be limited.

Although our data add to and refine studies that addressed $CD8^+$ T cell programming, more work is needed to shape a clearer picture of $CD4^+$ T cell programming. A recent study suggests that $CD4^+$ T cells are critically dependent on continuous presentation of antigen in vivo to sustain proliferation and mediate effector cell differentiation in an environment with no or limited inflammation (35). This would imply that programming requirements for $CD4^+$ and $CD8^+$ T cells are intrinsically different. However, another study concluded that $CD4^+$ T cells do undergo programming after short-term antigen stimulation in vitro and can proliferate in the absence of antigen (36). Further experiments are required to elucidate differences between the $CD8^+$ and $CD4^+$ T cell subsets and the underlying mechanisms.

In summary, the duration of the TCR stimulus in vivo determines the magnitude of the primary $CD8^+$ T cell response, but the progeny of cells exposed to antigen for as short as 7 h differentiate to effectors, enter nonlymphoid tissue, and produce memory cells. Notably, $CD8^+$ T cells of all experimental groups display identical characteristics during the secondary response indicating that differences observed during the primary response in accumulation (Fig. 2 C),

IFNγ production (Fig. 3 B), and turnover (Fig. 4 C) are not maintained in the memory stage.

MATERIALS AND METHODS

Mice. CD11c DTRtg and C57BL/6 mice were obtained from The Jackson Laboratory and housed in specific pathogen-free conditions in the animal facilities at the University of Washington. OT-I TCR transgenic mice congenic for Thy1.1 and CD45.1 were bred and maintained in the same facilities. Mice were infected at 8–12 wk of age. All experiments were performed in compliance with the University of Washington Institutional Animal Care and Use Committee regulations.

Dendritic cell isolation. DCs were expanded in CD11c DTRtg mice with a Flt-3L–secreting mouse melanoma cell line as previously described (34). Before FACS sorting, $CD3^+$ and $CD19^+$ cells were depleted with magnetic beads (Miltenyi) to increase the percentage of $CD11c^+$ cells. $CD11c^+$ GFP^{hi} cells were obtained by sorting on a FACSAria. $CD11c^+$ GFP^{hi} cells were pulsed with 1 μg/ml LPS and 1 μg/ml SIINFEKL (OVAp) for 1 h at 37°C, washed twice, and resuspended in PBS.

DT-mediated depletion. 100 ng DT (Sigma-Aldrich) was injected i.v. and another 100 ng DT was injected i.p. at the time points indicated.

Adoptive transfer and cell sorting. Naive $CD44^{low}$ OT-I T cells were isolated from lymph nodes using the Miltenyi CD8 isolation kit plus anti-CD44bio (IM-7) and CFSE labeled after the protocol previously described (37). OT-I T cells were resuspended in PBS and mixed with DCs immediately before i.v. adoptive transfer into recipient mice.

Bacterial infections. LM-OVA (38) and WT-LM were grown as previously described (13). For primary infections, mice were injected i.v. with 2×10^3 CFU WT-LM after adoptive transfer of OT-I T cells and DCs. For secondary infections, mice received 2×10^5 CFU LM-OVA and were killed 3 d later.

Flow cytometry. Recipient mice were killed at the time points indicated and single cell suspensions were prepared from the spleen, lymph nodes, and lungs after perfusion of the animal. Red blood cell–depleted splenocytes were treated with 2.4G2.1 (Fc-block) before further staining. Cells were typically stained with anti-CD8, anti-CD62L, anti-IL-7Rα, anti-Thy1.1, and anti-CD45.1. Experiments using the pull-down assay followed the previously published protocol (31).

For intracellular staining, cells were prepared with the Cytofix/Cytoperm kit in the presence of brefeldin A (BD Biosciences) and stained with anti-IFNγ PE (XMG1.2; eBioscience), anti-Thy1.1, or CD45.1 APC and anti-CD8 PerCP. For BrdU incorporation, BrdU was injected i.p. 2 h before harvesting. Cells were stained using anti–BrdU-APC antibodies according to manufacturer's protocol (BrdU Flow kit; BD Biosciences).

Cells were analyzed using a FACSCanto and FACSCalibur (BD Biosciences) and analyzed using FLOWJO (TreeStar) software.

Online supplemental material. Fig. S1 illustrates the increase of OT-I T cells available for analysis by using the pull-down assay. Fig. S2 shows the number of OT-I memory T cells before rechallenge with LM-OVA on day 35. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20060928/DC1.

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