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Intercellular Adhesion Molecule-1-Dependent Stable Interactions between T Cells and Dendritic Cells Determine CD8$^+$ T Cell Memory

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SUMMARY

The initiation of cytotoxic immune responses requires the direct interaction between naive CD8$^+$ T lymphocytes and dendritic cells (DCs). Multiphoton imaging in intact lymph nodes (LNs) showed that during priming, naive T cells and DCs establish sequentially brief (i.e., minutes) and long (hours) antigen-specific contacts. We show here that the expression of the Intercellular Adhesion Molecule-1 (ICAM-1) by mature DCs is critical for long-lasting contacts with CD8$^+$ T cells but dispensable for short-lived antigen-specific interactions. Serial brief DC-T cell contacts induced early CD8$^+$ T cell activation, proliferation, and differentiation into effector cytotoxic T lymphocytes in the first few days after immunization. ICAM-1-deficient mature DCs, however, failed to induce fully effective priming, because CD8$^+$ T cells produced reduced amounts of interferon γ and were clonally depleted after 2 weeks. In addition, *Icam1*$^{-/-}$ mice failed to respond to rechallenge. We conclude that ICAM-1-dependent long-lasting interactions between mature DCs and naive CD8$^+$ T cells determine the survival of activated CD8$^+$ T cells and the establishment of effective memory.

INTRODUCTION

During T cell priming, the clonal selection of antigen-specific naive T lymphocytes is initiated by the engagement of the T cell receptor (TCR) by its cognate ligand on the surface of antigen-presenting mature dendritic cells (DCs). Engagement of the TCR occurs in secondary lymphoid organs, during a physical interaction between the naive T cells and DCs. The stability of these contacts determines the duration of the engagement of the TCR and, thereby, the intensity of TCR signaling. In vitro, the duration of TCR engagement is critical for T cell priming (Benvenuti et al., 2004b; Gett et al., 2003; van Stipdonk et al., 2001). However, studies using 3D collagen matrix as a substrate showed that multiple transient interactions with different DCs may add up to induce effective T cell stimulation (Gunzer et al., 2000).

Using multiphoton microscopy, several more recent studies have shown that T-DC interactions in lymph nodes (LNs) during the priming of both CD8$^+$ and CD4$^+$ naive T cells follow a complex choreography (Bousso and Robey, 2003; Hugues et al., 2004; Mempel et al., 2004; Miller et al., 2002; Shakhar et al., 2005). Whereas in the absence of antigen, no measurable interactions are seen during the first few hours after immunization, in the presence of antigen, T cells establish brief (i.e., minutes) contacts with multiple DCs. In the hours after immunization, T cells stop their migration and form long-lived (i.e., several hours) conjugates with single DCs. Finally, a few hours later, T cells regain motility and again establish brief antigen-dependent contacts for several hours before leaving the LNs.

The relative contribution of brief and prolonged interactions to T cell priming remains unclear. Several recent studies established a correlation between long-lasting DC-T cell contacts and effective priming on the one hand and tolerogenic or anergic T cell responses and short unstable contacts on the other hand. Indeed, in the absence of adjuvant, LN-resident DCs induced tolerance and only established brief contacts with T cells. After DC activation by lipopolysaccharides (LPS) or CD40 antibodies, the interactions were strongly prolonged and effective priming was observed (Hugues et al., 2004). Under several other conditions of induction of CD4$^+$ and CD8$^+$ peripheral T cell tolerance, either by clonal deletion or anergy or through regulatory T cells (Tregs), DC-T cell contacts were shorter than during the induction of priming (Tadokoro et al., 2006; Tang et al., 2006; Zinselmeyer et al., 2005). A more recent study shows two pathways of induction of T cell tolerance, depending on the avidity of the TCR. One of these pathways involves stable DC-T cell contacts, and the other does not (Skokos et al., 2007). Together, these studies suggest that multiple short DC-T cell contacts could participate in the induction of tolerance in vivo.

How, then, is the duration of DC-T cell contacts regulated? In vitro studies showed that stable DC-T cell interactions are antigen dependant, are influenced by chemokines (Molon et al., 2005), and require an active actin DC cytoskeleton (Benvenuti et al., 2004a). Adhesion molecules are also involved. Intercellular Adhesion Molecule-1 (ICAM-1), when associated with TCR ligands on planar lipid membranes, was sufficient to induce T cell arrest and polarization (Dustin et al., 2004). Indeed, TCR

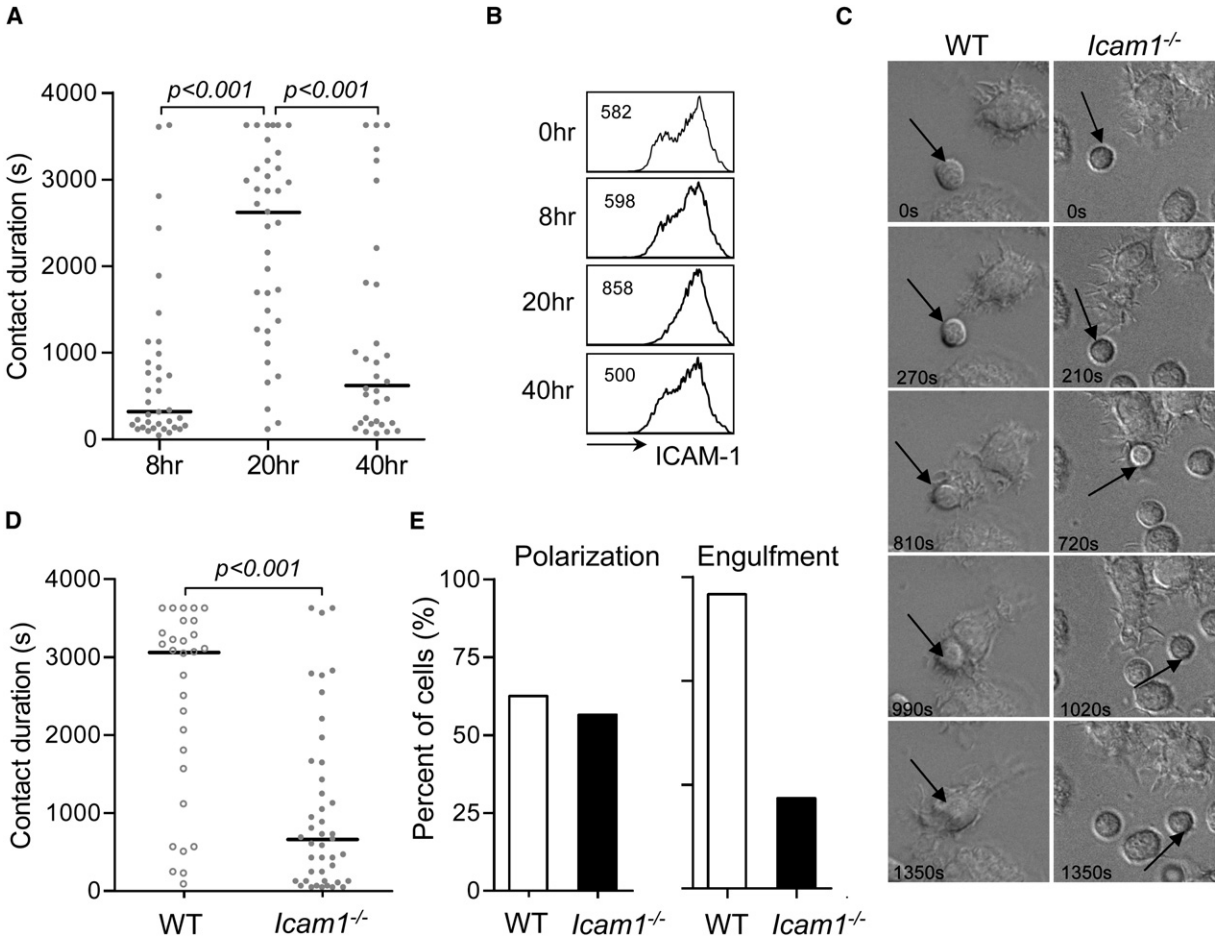

Figure 1. Long-lasting T-DC Interactions In Vitro Require ICAM-1 Expression by DCs

(A) WT mice were injected subcutaneously with αDEC205:OVA (5 μg) plus αCD40 (10 μg). $CD11c^+$ cells were purified from the draining LNs at indicated times and incubated with naive OT1 cells. DC-T cell contact duration was followed with time-lapse video microscopy. Black lines indicate medians; data were compared with the two-tailed unpaired Mann-Whitney test.

(B) WT mice were injected s.c. with αCD40 (10 μg), and draining LNs were collected at indicated times. Flow-cytometry histograms of ICAM-1 after gating on $CD11c^+$ cells are shown. Numbers in dots represent the mean fluorescence intensity. Data are representative of two independent experiments and were compared with the two-tailed unpaired Mann-Whitney test.

(C–E) Interactions between bone-marrow-derived OVA-loaded, LPS-activated WT or *Icam1*$^{-/-}$ DCs and naive OT1 cells analyzed with time-lapse video imaging (Movies S1 and S2). Black lines indicate medians. (C) Sequential time series (20 × 20 μm) show a WT DC trapping a naive OT1 cell (left) and an *Icam1*$^{-/-}$ DC polarizing its dendrites toward a naive OT1 cell but without stabilizing the contact (right). (D) shows T-DC contact duration. (E) shows proportion of DCs showing dendrite polarization toward and engulfment of naive OT1 cells. Data are pooled from three independent experiments.

engagement on T cells activates Lymphocyte Function Antigen-1 (LFA-1), one of the main ICAM-1 ligands on T cells. High-affinity ICAM-1:LFA-1-mediated adhesion stabilizes DC-T cell interactions. LFA-1-mediated signaling, however, is not considered as a conventional T cell costimulation signal (signal 2) (Friedl et al., 2005). In contrast, very little is known so far about the regulation of DC-T cell interactions in vivo.

In this study, we show that the expression of ICAM-1 by mature DCs was required to establish long-lasting DC-T cell contacts. T cell priming in the absence of ICAM-1 expression resulted in normal activation, proliferation, and effector cytotoxic T lymphocyte (CTL) generation, but the effector $CD8^+$ T cells produced low amounts of interferon γ (IFNγ). $CD8^+$ T cells were then deleted, and the mice became unresponsive to antigenic rechallenge. We conclude that ICAM-1-dependent long-lasting DC-T cell interactions during priming were required for the survival of activated $CD8^+$ T cells and the establishment of effective memory.

RESULTS

ICAM-1 Expression by DC Controls DC-T Cell Contact Duration during Priming Induction In Vitro

DC maturation influences the duration of the interactions with naive $CD8^+$ T cells (Hugues et al., 2004). Indeed, as shown in Figure 1A with time-lapse video microscopy, the ability of mature DCs to establish long-lasting contacts with naive $CD8^+$ T cells ex vivo is first upregulated (20 hr after injection of αDEC205:OVA conjugates and CD40 antibodies) and then lost (after 30–48 hr). Interestingly ICAM-1 expression was increased 20 hr after

immunization, correlating with the phase of long-lasting DC-T cell interactions, and then decreased after 40 hr (Figure 1B).

We therefore analyzed the role of ICAM-1 on the stability of DC-T cell contacts in vitro. Mature wild-type (WT) or ICAM-1-deficient bone-marrow-derived DCs were loaded with ovalbumine (OVA) peptide (SINFEKL) and incubated with naive OT1 T cells. ICAM-1-deficient mature DCs established fewer long-lasting contacts than WT DCs (Figures 1C and 1D; Movies S1 and S2 available online). We showed previously that after an initial dendrite-mediated contact, mature DCs project numerous mobile membrane extensions, displace their cell bodies, and entrap the T lymphocyte to establish stable interactions (Benvenuti et al., 2004a). As shown in Figures 1C and 1E, ICAM-1-deficient DCs polarized normally toward $CD8^+$ T cells, but the entrapment phase did not occur, suggesting that ICAM-1-mediated adhesion is required for the entrapment and the establishment of stable contacts. These results show that ICAM-1 expression on the surface of mature DCs was required to entrap T cells within DC membrane extensions and to establish long-lasting interactions in vitro.

Absence of $CD8^+$ T Cell Arrests in *Icam1*$^{-/-}$ Mice

We and others (Hugues et al., 2004; Mempel et al., 2004) showed previously that during $CD8^+$ T cell priming, T cell motility follows three distinct phases: During phase I (5–10 hr after immunization, depending on the experimental systems), T cells repeatedly stop migrating for short periods of time (on the order of minutes), as a result of transient contacts with antigen-presenting DCs; during phase II (10–20 hr after immunization), T cells arrest completely for several hours in contact with individual DCs; and finally, during phase III (20–40 hr after immunization), T cell motility resumes partially, because transient T cell-DC interactions are observed again (as in phase I).

To determine the role of ICAM-1 in T cell dynamics within LNs during priming, we used two-photon microscopy. WT and *Icam1*$^{-/-}$ mice were injected with varying doses of αDEC205:OVA (0, 0.1, and 5 μg/mouse) in the presence of CD40 antibodies, after adoptive transfer of CFSE-labeled OT1 T cells (Figure 2). During phase II (15–20 hr after immunization) in LNs from WT mice, antigen-specific T cells migrated with lower mean velocity (Vmean: 5.1 ± 2.5 μm/min and 3.5 ± 1.4 μm/min after immunization with, respectively, 0.1 μg and 5 μg αDEC205:OVA) and performed long-lasting arrests (arrest coefficient: 26 ± 18% and 32 ± 14% after immunization with, respectively, 0.1 μg and 5 μg αDEC205:OVA) (Figure 2A). In LNs from *Icam1*$^{-/-}$ mice, T cell mean velocity was significantly higher (8.0 ± 1.9 μm/min, $p < 0.001$ and 6.7 ± 2.6 μm/min, $p < 0.001$ after immunization with, respectively, 0.1 μg and 5 μg αDEC205:OVA) and the T cell-arrest coefficient was decreased (9 ± 7%, $p < 0.001$ and 12 ± 11%, $p < 0.001$ after immunization with, respectively, 0.1 μg and 5 μg αDEC205:OVA) as compared to those from WT mice (Figures 2A and 2B, and Movies S3–S6). Accordingly, αDEC205:OVA and CD40 antibody injection induced a marked constriction of OT1 T cell trajectories in LNs from WT mice but not from *Icam1*$^{-/-}$ mice (Figure 2C). OT1 T cell motility in phases I and III (8–10 and 30–48 hr after immunization) was similar in LNs from WT and *Icam1*$^{-/-}$ mice (Figure S1, Movies S7–S10). In the absence of αDEC205:OVA (mice injected with CD40 antibodies alone), the motility of OT1 T cells was similar in LNs from WT and *Icam1*$^{-/-}$ mice, indicating that ICAM-1 is not involved in T cell motility in the absence of antigen (Figure 2A). Because regulatory T cells (Tregs) were shown to inhibit stable DC-T cell interactions (Tang et al., 2006), we analyzed the frequency of Tregs in *Icam1*$^{-/-}$ mice. We found that there was no significant difference in the percentage of $FoxP3^+$ LN T cells, as compared to WT animals (not shown).

To further characterize the differences in the migration dynamics, we classified $CD8^+$ T cells into four categories according to their migration characteristics, as described (Hugues et al., 2004): category I, T cells that did not pause or show constrained migration; category II, T cells that had multiple pauses or constrained migration at multiple points in the proximity of several DCs; category III, T cells that remained constrained in the proximity of the same DC throughout the imaging period; and category IV, T cells that remained arrested (instantaneous velocity < 2 μm/min) in close proximity to a DC during the entire observation period. In LNs from WT animals, the frequency of T cells arrested on a single DC (category IV) was high after 15 to 20 hr (over 40% and 70% after injection, respectively, with 0.1 μg or 5 μg αDEC205:OVA and CD40 antibodies), indicating that at these time points most T cells established long-lasting contacts with DCs (Figure 2D). In *Icam1*$^{-/-}$ mice, in contrast, very few cells fell into category IV (around 5% at both antigen doses) (Figure 2D). In these mice, T cells were in category II, indicating that DC-T cell interactions remained transient in LNs from *Icam1*$^{-/-}$ animals, even during phase II. Therefore, in the absence of ICAM-1 expression, the complete arrest in $CD8^+$ T cell migration, corresponding to phase II, was not observed.

ICAM-1 Expression by DCs Is Required for Long-Lasting DC-T Cell Contacts In Vivo

The arrests in $CD8^+$ T cell migration after immunization with αDEC205:OVA and CD40 antibodies probably correspond to direct interactions with antigen-bearing DCs, given that the only LN cells that cross present antigen to OT1 T cells under these experimental conditions are $CD11c^+$ cells (Bonifaz et al., 2002). Nevertheless, to confirm that the inability of $CD8^+$ T cells to arrest in *Icam1*$^{-/-}$ mice is due to the absence of ICAM-1 expression by DCs, we imaged in vivo OT1 T cell interactions with adoptively transferred WT or ICAM-1-deficient DCs in WT recipients (Figure 3). Mature WT and ICAM-1-deficient splenic DCs loaded with OVA peptide were injected respectively in the left and right footpad of WT mice after adoptive transfer of OT1 T cells (Movies S11 and S12). Individual interactions between OT1 T cells and WT or ICAM-1-deficient mature DCs were imaged and quantified (Figure 3A). $CD8^+$ T cells migrated with lower mean velocity in the presence of WT DCs as compared to *Icam1*$^{-/-}$ DCs (Vmean: 3.1 ± 1.1 μm/min and 4.6 ± 1.7 μm/min, $p < 0.0001$) (Figure 3B), showing that DC-T cell interactions were more stable in the presence of ICAM-1. Indeed, $CD8^+$ T cells established longer-lasting contacts with WT DCs than with *Icam1*$^{-/-}$ DCs (17.8 ± 14 min versus 6.8 ± 7.1 min, $p < 0.0001$) (Figure 3C). Because a large proportion of T cell-WT DC contacts lasted longer than the imaging period (30 min), the duration of interactions between $CD8^+$ T cells and WT DCs is probably underestimated in these experiments. We thus concluded that ICAM-1 expression by DCs controls T-DC contact duration although it was not required for T-DC contact initiation.

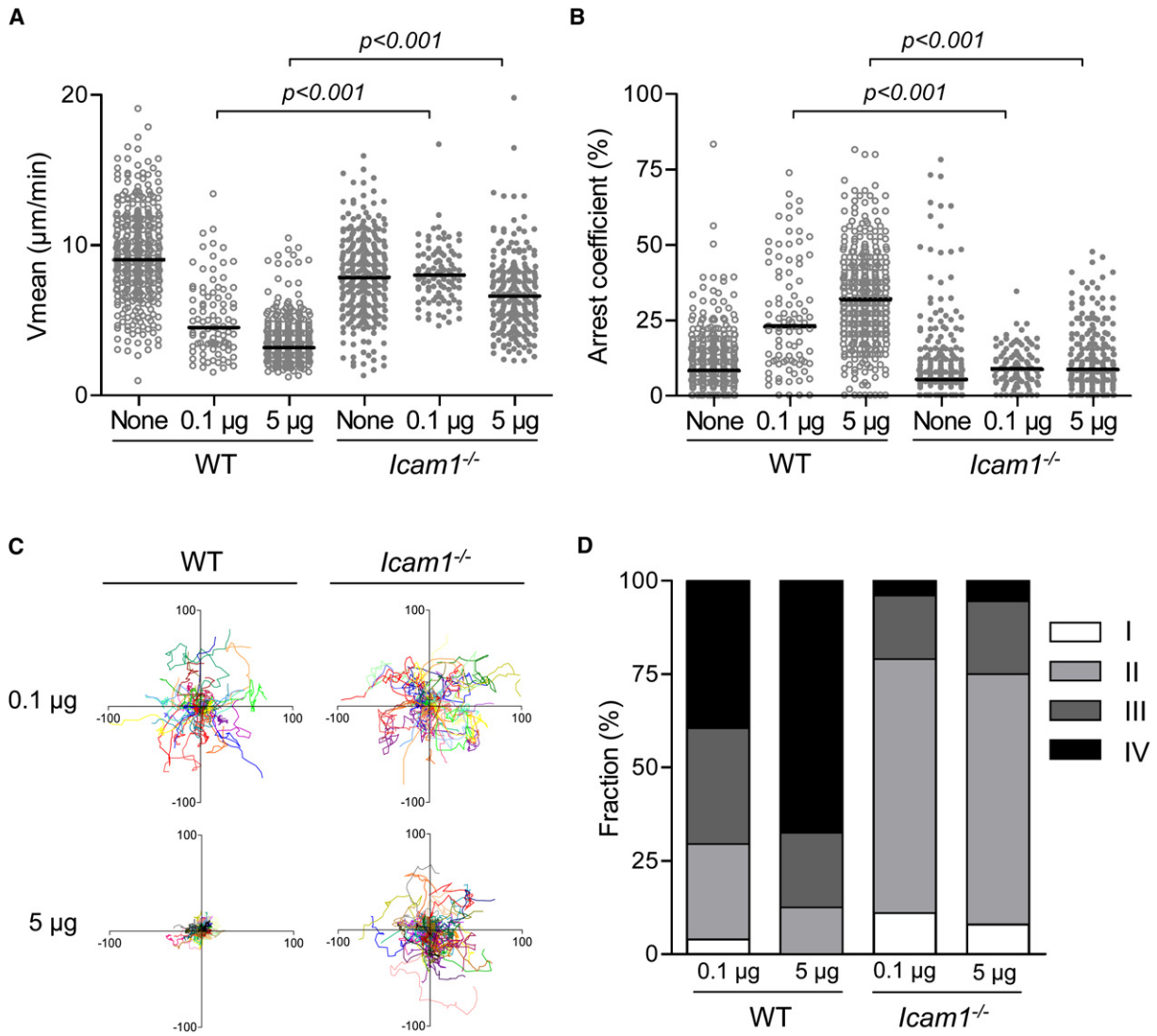

Figure 2. DC-T Cell Interaction Dynamics in LNs from WT and *Icam1*$^{-/-}$ Mice during Priming

(A–D) CMTMR-labeled OT1 T cells were injected intravenously into WT and *Icam1*$^{-/-}$ mice before they were injected s.c. 4 hr later with PBS (none) and indicated doses of αDEC205:OVA and αCD40 (10 μg). Draining LNs were collected 15–20 hr later and imaged by time lapse TPLSM. See Movies S3–S6.

(A) T cell mean velocity (Vmean) in LNs from indicated mice.

(B) T cell arrest coefficient in LNs from indicated mice. Black lines indicate medians. Data were compared with the two-tailed unpaired Mann-Whitney test.

(C) Representative migratory paths of 50 T cells per condition are shown normalized to the same departure point.

(D) Classification of DC-T cell interactions. T cell arrests on DCs were arranged in classes I–IV, as described (Hugues et al., 2004). Data represent the proportion of each category. Data are pooled from three independent experiments.

As expected from these results, the frequency of initial encounters between the T cells and DCs (K_{on} or hit ratio obtained after normalization by the imaging volume, time, and cell densities; see Experimental Procedures) was higher in the absence of ICAM-1 (Figure 3D). We conclude that the individual in vivo contacts between antigen-presenting mature DCs and CD8$^+$ T cells were shorter in the absence of ICAM-1.

ICAM-1 Expression by DCs Is Dispensable for CD8$^+$ T Cell Activation, Proliferation, and Cytotoxicity

What are the functional consequences of a reduction of the frequency of long-lasting, ICAM-1-dependent contacts between antigen-presenting mature DCs and naive CD8$^+$ T cells? To investigate this question, we adoptively transferred OT1 T cells to WT and *Icam1*$^{-/-}$ mice immunized with varying doses of αDEC205:OVA and CD40 antibodies. CD8$^+$ T cell activation and proliferation (CSFE dilution) were analyzed in the draining LNs. The upregulation of CD69 by OT1 T cells is partially inhibited in *Icam1*$^{-/-}$ mice, as compared to WT mice, at low doses of αDEC205:OVA (0.1 or 0.5 μg), but recovered at high antigen doses (Figure 4A). Over three independent experiments, the mean inhibition of early T cell activation was around 30% at low and 10% at high antigen doses (Figure S2A). We conclude that efficient early CD8$^+$ T cell activation (at 18 hr) can occur in

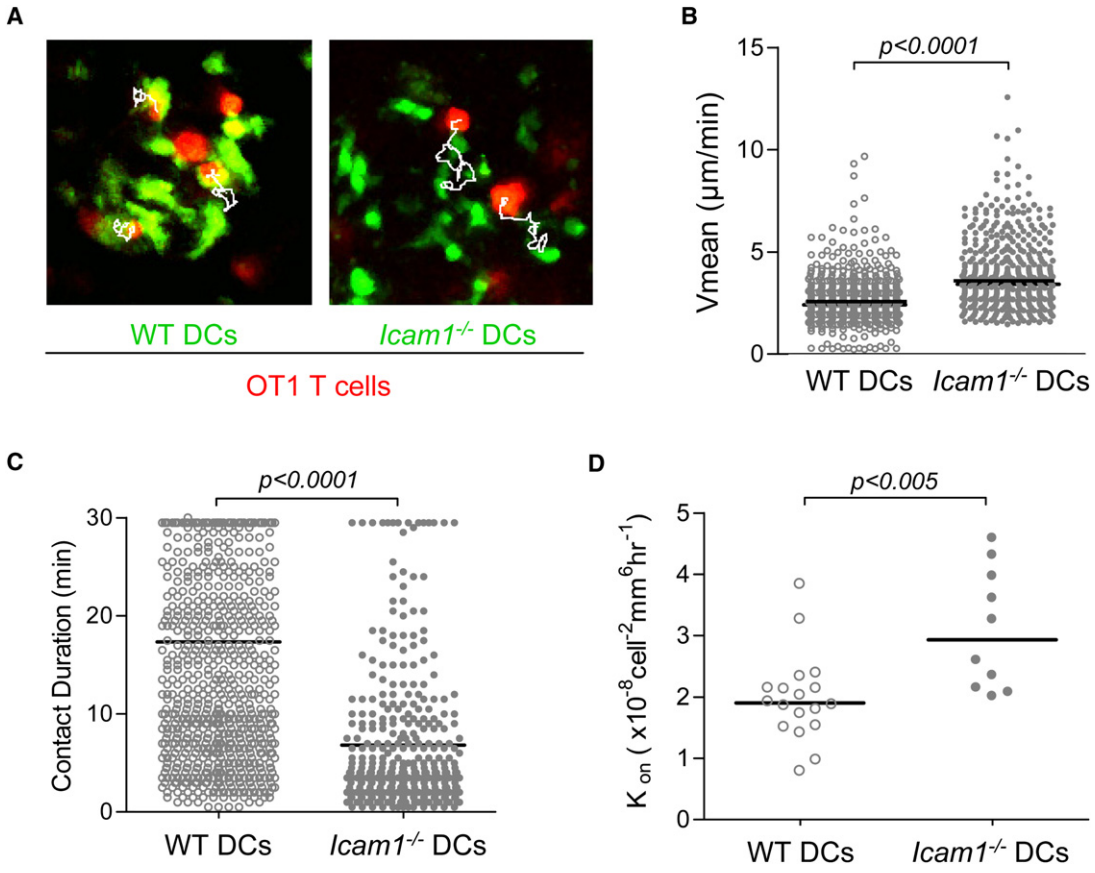

Figure 3. ICAM-1 Expression by DCs Controls T-DC Contact Duration

(A–D) Labeled WT and *Icam1*$^{-/-}$ splenic DCs were injected s.c., respectively, in the left and right footpad of WT recipients that received GFP^{+} OT1 T cells transfer. Popliteal LNs were collected 15–20 hr later. Interactions between CD8^{+} T cells and DCs were imaged by time-lapse TPLSM during 30 min.

(A) Time-lapse TPLSM images (100 × 100 μm) of WT DCs (green, left panel) or *Icam1*$^{-/-}$ DCs (green, right panel) and OT1 T cells (red), presented as an average intensity projection along the z axis (top view). See Movies S11 and S12. Thirty minute lasting paths of T cells are shown (white lines).

(B) T cell mean velocity (Vmean) in the LN-draining WT or *Icam1*$^{-/-}$ DCs as indicated.

(C) T-DC contact duration.

(D) K_{on} or Hit rate constants. Black lines indicate medians. Data are pooled from three independent experiments and were compared with the two-tailed unpaired Mann-Whitney test.

the absence of ICAM-1 when the amount of antigen is not limiting.

In order to analyze the role of ICAM-1-dependent stable DC-T cell contacts in T cell proliferation, we labeled OT1 T cells with CSFE before adoptive transfer. The mice were then immunized with varying doses of αDEC205:OVA and CD40, and proliferation in LNs was assayed with CSFE dilution on day 3. As shown in Figure 4B and Figure S2B, no difference in the rate of OT1 T cell proliferation was observed between WT and *Icam1*$^{-/-}$ mice. Therefore, ICAM-1-dependent stable T cell-DC contacts are not required for the induction of CD8^{+} T cell proliferation. Interestingly, proliferation of CD8^{+} T cells primed in *Icam1*$^{-/-}$ mice was normal, even at low antigen doses that induced impaired CD69 upregulation at earlier time points. This suggests that the accumulation of sequential short signals in *Icam1*$^{-/-}$ mice over a few days (but not 18 hr) could result in normal CD8^{+} T cell activation when the amount of antigen is limiting.

The acquisition of OT1 T cell effector functions, cytotoxicity, and IFNγ secretion in WT and *Icam1*$^{-/-}$ mice was assessed at day 5. IFNγ production, as assayed with intracellular FACs staining after ex vivo restimulation, was decreased in *Icam1*$^{-/-}$ mice, as compared to WT mice (Figure 4C), even at high antigen concentrations. ICAM-1-dependent stable DC-T cell contacts were therefore required for optimal IFNγ production by effector CD8^{+} T cells.

Cytotoxicity was evaluated with a 4 hr in vivo clearance FACS assay. As shown in Figure S3, injected peptide-bearing target cells were cleared in WT mice at day 5 more efficiently and at lower antigen doses than in *Icam1*$^{-/-}$ mice. Reduced clearance may be the result of either a reduced number of cytotoxic cells or a reduced cytotoxicity on a per-cell basis. To distinguish between these two possibilities, we sorted transferred OT1 T cells by flow cytometry 5 days after immunization and assayed ex vivo both cytotoxicity and IFNγ production. As expected from the results presented in Figure 4C, the production of IFNγ after ex vivo restimulation was reduced in the OT1 T cells primed in *Icam1*$^{-/-}$ mice (Figure 4D, left panel). In contrast, no difference in the cytotoxicity was observed between the OT1 T cells sorted

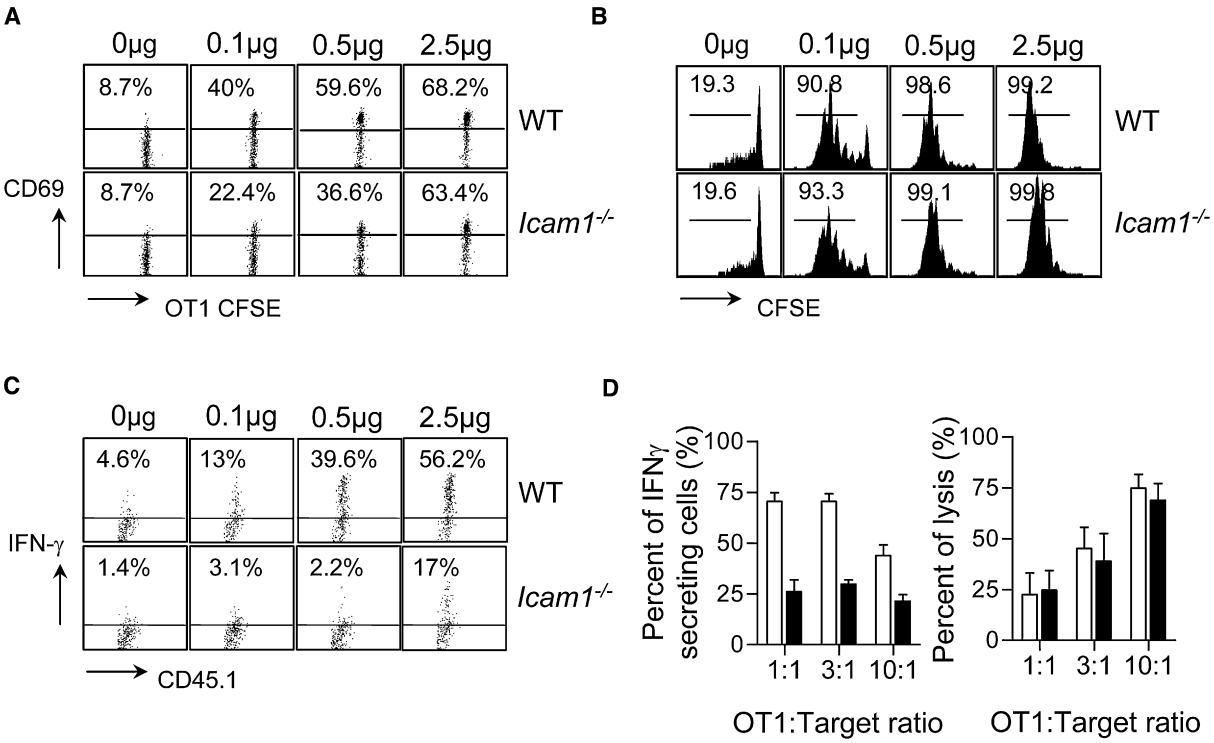

Figure 4. Activation and Effector Functions of CD8+ T Cells in WT and *Icam1*$^{-/-}$ Mice

(A and B) CFSE-labeled OT1 T cells were adoptively transferred into WT and *Icam1*$^{-/-}$ mice that were injected 4 hr later with indicated doses of αDEC205:OVA plus αCD40 (10 μg). (A) Profiles of CD69 expression on OT1 T cells (gated on CFSE^{+} cells) in draining LNs collected 18 hr later. Numbers indicate the percentage of cells with high expression of CD69. Data are representative of three independent experiments. (B) OT1 T cell proliferation profiles in draining LNs collected at day 3. Numbers indicate the percentage of dividing cells after gating on CFSE^{+} cells. Data are representative of three independent experiments.

(C) CD45.1^{+} OT1 T cells were adoptively transferred into WT and *Icam1*$^{-/-}$ mice that were injected 4 hr later with indicated doses of αDEC205:OVA plus αCD40 (10 μg). At day 5, IFNγ production by OT1 T cells was assessed after in vitro restimulation. Numbers indicate the percentage of IFNγ secreting OT1 T cells after gating on CD45.1^{+} cells. Data are representative of three independent experiments.

(D) GFP^{+} OT1 T cells were adoptively transferred in WT or *Icam1*$^{-/-}$ mice that were injected s.c. with αDEC205:OVA (2.5 μg) plus αCD40 (10 μg). At day 5, GFP^{+} OT1 T cells were sorted by flow cytometry and coculture with CD45.1^{+} splenocytes pulsed (CFSE^{hi}) or not (CFSE^{lo}) with OVA peptide to measure intrinsic CTL activity of OT1 T cells primed in *Icam1*$^{-/-}$ (black bars) or WT mice (white bars). Histograms indicate the percentage of IFNγ-secreting OT1 T cells (left panel, data are representative of two independent experiments) and percentage of target cell lysis (right panel, data are representative of three independent experiments). Error bars represent mean ± SD.

from WT and *Icam1*$^{-/-}$ mice independently of the antigen dose (Figure 4D, right panel and Figure S4). We conclude that although OT1 T cells primed in *Icam1*$^{-/-}$ mice present an intrinsic defect in IFNγ production, the clearance defect observed in vivo might be due to a reduced number of effector OT1 T cells (see below). Taken together, these results indicate that ICAM-1-dependent long-lasting T-DC contacts were dispensable for early CD8^{+} T cell activation, proliferation, and differentiation into cytotoxic cells. However, naive CD8^{+} T cells primed in ICAM-1-deficient mice exhibited a reduced ability to differentiate into high-IFNγ-secreting cells.

Lack of Effective Memory T Cell Induction in the Absence of ICAM-1 Expression

The clearance results presented in Figure 4D suggest that at day 5, in spite of the similar proliferation rates observed at day 3, the actual number of effector OT1 T cells is decreased after priming in the absence of ICAM-1. We therefore analyzed the proportion of OT1 T cells in the draining LNs after immunization in WT and *Icam1*$^{-/-}$ mice. As shown in Figure 5A, at day 3 after adoptive transfer the proportion of OT1 T cells was similar in WT and *Icam1*$^{-/-}$ mice immunized with αDEC205:OVA (2.5 μg) and αCD40 antibodies. At days 5 and 7, in contrast, the proportion of OT1 T cells decreased more rapidly in *Icam1*$^{-/-}$ than in WT mice. At day 12, the proportion of OT1 T cells in *Icam1*$^{-/-}$ mice was around 0.1%, much lower than the proportion of OT1 T cells in immunized WT animals (around 4%, Figure 5B). Actually, the proportion of OT1 T cells in immunized *Icam1*$^{-/-}$ mice was similar to the proportion of OT1 T cells in nonimmunized WT mice and in WT mice injected with αDEC205:OVA in the absence of CD40 antibodies (Figures 5A and 5B). In the absence of antigen, however, OT1 T cells persisted similarly in WT and *Icam1*$^{-/-}$ mice, suggesting that their deletion is not due to an immune response against ICAM-1 expressed by OT1 T cells in *Icam1*$^{-/-}$ mice (Figure S5). These results suggest that αDEC205:OVA- and CD40-antibody immunization in *Icam1*$^{-/-}$ mice induced effective T cell accumulation followed by a more drastic retraction phase, suggesting that effective memory may not have been induced.

To address this possibility, we injected WT and *Icam1*$^{-/-}$ mice with αDEC205:OVA in the absence or in the presence of CD40

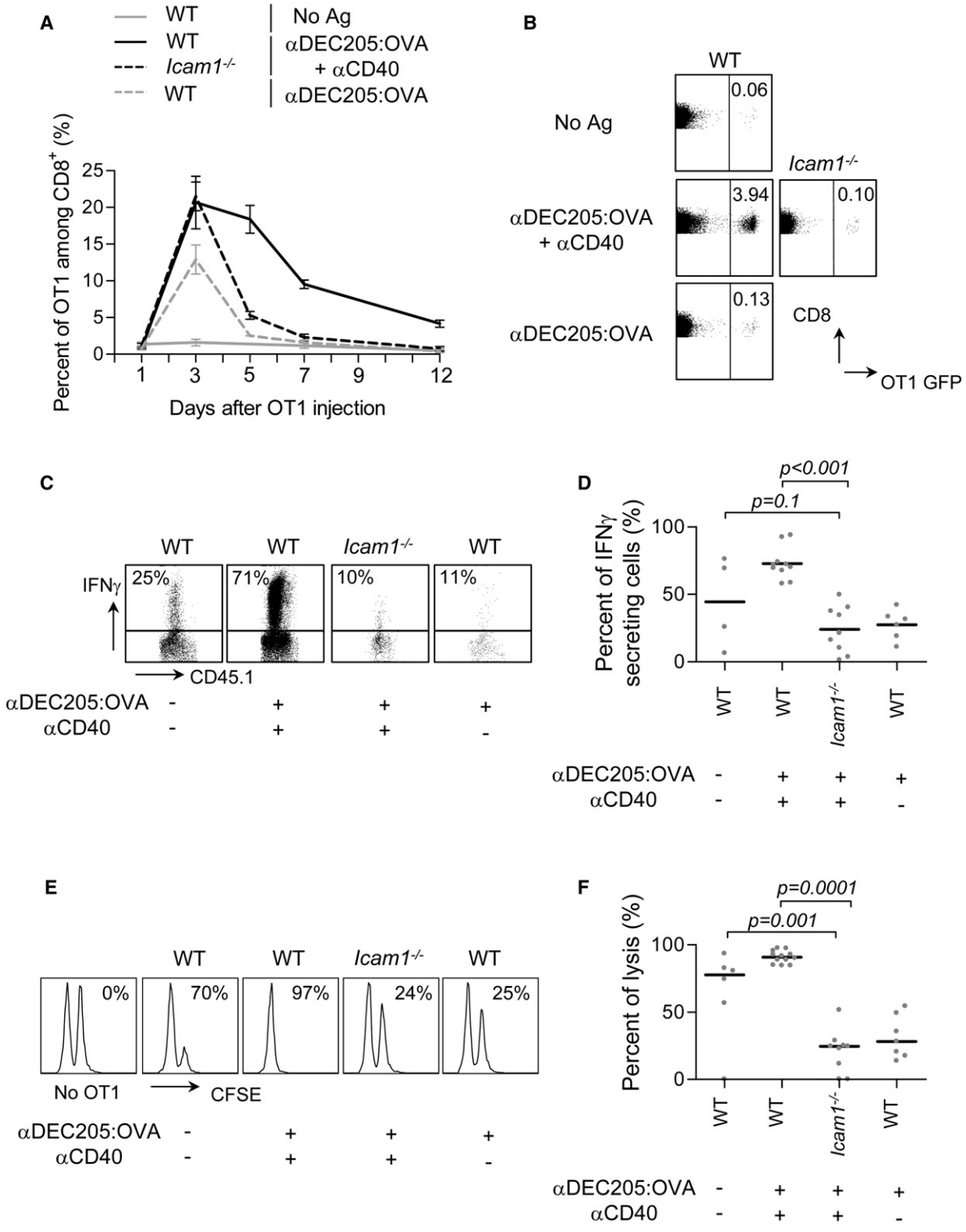

Figure 5. Impaired CD8+ T Cell Memory Induction in Absence of ICAM-1 Expression by DCs

GFP+ (A and B) or CD45.1+ (C–F) OT1 T cells were adoptively transferred into WT and *Icam1*$^{-/-}$ mice that were injected 4 hr later with indicated doses of αDEC205:OVA in the presence or not of αCD40 (10 μg).

(A) Percentage of OT1 T cells among CD8+ T cells in draining LNs over time. Results are pooled from three independent experiments.

(B) OT1 T cell accumulation in draining LNs at d12 in *Icam1*$^{-/-}$ and WT mice. Numbers indicate percentage of OT1 T cells among CD8+ T cells.

(C–F) Twelve days after immunization with 2.5 μg of αDEC205:OVA in the presence or not of 10 μg of αCD40, mice were boosted with 40 μg of OVA protein in complete Freund's adjuvant (CFA). (C) shows representative flow-cytometry profiles of IFNγ-secreting cells after gating on CD45.1+ OT1 T cells 4 days after

antibodies. These mice were rechallenged with OVA in CFA 12 days later. Three days after rechallenge, OT1 cells in WT mice injected with phosphate-buffered saline (PBS) or with αDEC205:OVA and CD40 antibodies produced strong CTL responses (as detected by the in vivo clearance assay, Figures 5E and 5F) and IFNγ secretion after ex vivo restimulation (Figures 5C and 5D). In contrast, in both WT mice pretreated with αDEC205:OVA alone and *Icam1*$^{-/-}$ mice injected with αDEC205:OVA and CD40 antibodies, no effective recall responses were observed. Therefore, after injection of αDEC205:OVA and CD40 antibodies in *Icam1*$^{-/-}$ mice, CD8$^+$ T cells proliferate normally but do not survive the retraction phase as efficiently as in WT mice. The few cells that survived priming in ICAM-1-defective mice at day 12 still produced IFNγ ex vivo, but they displayed reduced cytotoxicity, as compared to unprimed OT1 cells (Figures 5D and 5F). We conclude that short, transient interactions of naive OT1 T cells with ICAM-1-deficient mature DCs sufficed for primary CD8$^+$ T cell expansion and cytotoxicity, but failed to induce effective T cell memory.

Lack of Effective Memory T Cell Induction to Tumoral Antigen in the Absence of ICAM-1 Expression

In the experiments presented so far, the antigen was always delivered to DCs by targeting with the αDEC205:OVA antibody. After targeting with this antibody, antigen is selectively cross-presented by a large number of LN-resident DCs, mainly of the CD8$^+$ subtype (Dudziak et al., 2007). Although very efficient, this mode of antigen delivery does not reflect physiological situations in which antigen arrives from the periphery in limiting amounts, probably carried by few DCs. Therefore, in order to mimic a more physiologic situation in terms of antigen delivery, we next investigated the role of ICAM-1 expression by DCs during priming in mice bearing an immunogenic tumor. *Icam1*$^{-/-}$ and WT mice were injected subcutaneously (s.c.) with EL4 or EG7 (OVA-expressing EL4) thymomas. OT1 T cells were then adoptively transferred to the mice when the tumor sizes were 500–1000 mm^3 (around day 10). The cell populations presenting tumor antigen in the tumor-draining LNs of WT mice was analyzed by purification of CD11c$^+$ and CD11c$^-$ cells and incubation of these two populations with naive OT1 T cells ex vivo. As shown in Figure 6A, only CD11c$^+$ cells activated OT1 T cells, attesting that DCs cross-present the tumor antigen preferentially in this system.

Regarding T cell activation, and consistent with our previous results, proliferation of OT1 T cells primed in EG7-bearing recipients is not altered in *Icam1*$^{-/-}$ mice as compared to WT mice (Figure 6B). The survival of OT1 T cells, in contrast, is dramatically compromised when the CD8$^+$ T cells are primed by tumor antigen in the absence of ICAM-1 (Figures 6C and 6D). The proportion of OT1 T cells that survived in EG7-bearing *Icam1*$^{-/-}$ mice is actually much lower than in WT mice bearing EL4 (antigen negative) tumors, indicating an active mechanism of clonal deletion. To determine whether the few OT1 T cells that have survived this clonal deletion may respond to antigen challenge, we rechallenged the EG7-bearing WT and *Icam1*$^{-/-}$ mice with OVA-CFA, and IFNγ secretion was analyzed with intracellular FACs after ex vivo rechallenge. As shown in Figures 6E and 6F, in WT mice bearing either EL4 or EG7 tumors, OT1 T cells produced IFNγ upon rechallenge. In EG7-bearing *Icam1*$^{-/-}$ mice, in contrast, rechallenge with OVA-CFA resulted in low amounts of IFNγ production. These results indicate that in the absence of ICAM-1 expression, CD8$^+$ tumor-specific T cells first proliferate and are then clonally deleted. The remaining cells failed to respond efficiently to antigen rechallenge, indicating a defect in the differentiation into memory cells. Previous studies, like ours, showed that LFA-1 is critical for CD8$^+$ T cell activation at low antigen concentrations (Bachmann et al., 1997). In our experimental system, however, T cell activation in the tumor-draining lymph nodes was strong (up to 70% of CD69^{hi} T cells, Figure S6) and was not affected in the *Icam1*$^{-/-}$ mice, suggesting that the defect in the anti-tumor immune response was not related to the limiting of antigen transfer from the tumor.

ICAM-1-Dependent Long-Lasting T-DC Contacts in Tumor-Draining LNs

We next sought to investigate whether the priming defect observed in tumor-bearing *Icam1*$^{-/-}$ mice correlated with the absence of stable DC-T cell interactions in the tumor-draining LNs. We first analyzed OT1 T cell dynamics in EG7 tumor-draining and nondraining LNs from WT mice by using two-photon microscopy. EG7-bearing WT mice were injected with OT1 T cells, and LNs were imaged 15 to 20 hr later. In tumor-draining LNs, OT1 T cells migrated with a reduced mean velocity (Vmean: 5.6 ± 3.9 μm/min) as compared to nondraining LNs from the same mice (Vmean: 7.5 ± 3.4 μm/min, $p < 0.0001$). T cells from draining LNs performed longer-lasting arrests (arrest coefficient: 23 ± 17% in tumor-draining LNs versus 13 ± 13% in nondraining LNs, $p < 0.0001$), suggesting that they establish stable contacts with antigen-bearing DCs (Figures 7A and 7B, Movies S13 and S14).

In contrast, in *Icam1*$^{-/-}$ mice, OT1 T cells migrated with similar mean velocities (5.8 ± 3.0 μm/min in tumor-draining LNs versus 5.7 ± 2.4 μm/min in nondraining LNs, $p = 0.46$) and arrest coefficients (19 ± 15% in tumor-draining LNs versus 18 ± 14% in nondraining LNs, $p = 0.26$) (Figures 7C and 7D, Movies S15 and S16) in tumor-draining and nondraining LNs, indicating that DCs presenting the tumor antigen in tumor-draining LNs from *Icam1*$^{-/-}$ mice do not establish stable contacts with OT1 T cells. The lack of CD8$^+$ T cell arrests in EG7-draining LNs from *Icam1*$^{-/-}$ mice was not due to lower levels of antigen delivery, because OT1 T cell activation (as detected with CD69 expression, see Figure S6) was similar in WT and *Icam1*$^{-/-}$ mice. We conclude that antigen-presenting DCs in tumor-draining LNs from *Icam1*$^{-/-}$ mice fail to establish stable contacts with OT1 T cells, resulting in the induction of clonal deletion and the failure to mount effective memory T cell responses to the tumor antigen.

rechallenge. (D) Pooled results of three independent experiments are expressed as the percentage of IFNγ-secreting cells among CD45.1$^+$ OT1 T cells. (E and F) Three days after rechallenge, mice were injected with mixture of CFSE-labeled CD45.2 splenocytes pulsed (CFSE^{hi}) or not (CFSE^{lo}) with OVA peptide. Cytotoxicity was assessed 18 hr later. (E) Representative dot plots are shown; numbers indicate the percentage of specific lysis. (F) Results are pooled from three independent experiments. Data were compared with the two-tailed unpaired Mann-Whitney test.

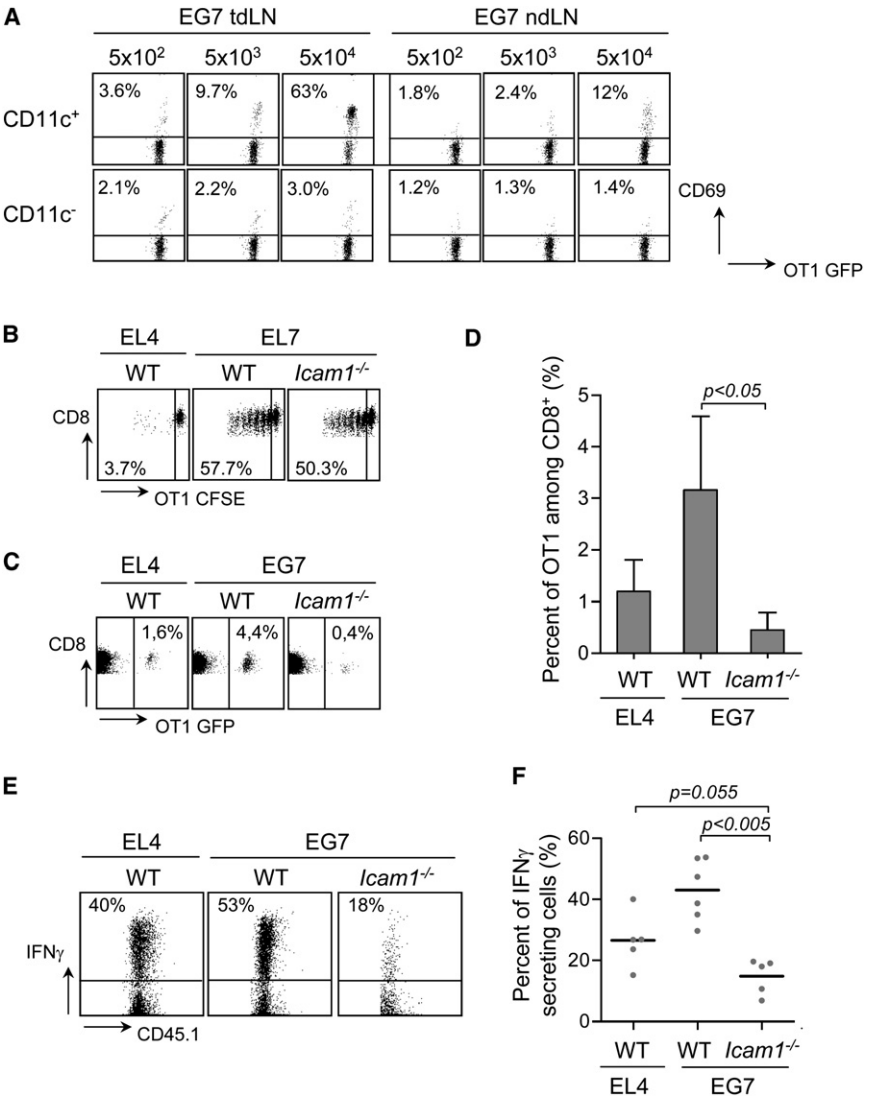

Figure 6. Impaired CD8⁺ T Cell Memory to Tumoral Antigen in the Absence of ICAM-1 Expression by DCs

(A) CD11c⁺ and CD11c⁻ cells were purified from tumor-draining LNs (tdLNs) and nondraining LNs (ndLNs) of EG7-bearing WT mice. Indicated numbers of cells were incubated with 10^5 GFP⁺ OT1 T cells over 18 hr. CD69 expression was assessed by flow cytometry. Numbers in dot plots indicate the percentage of OT1 T cells with high CD69 expression. Data are representative from three independent experiments.

(B–F) CFSE-labeled (B), GFP-labeled (C and D), or CD45.1-labeled (E and F) OT1 T cells were adoptively transferred into WT and *Icam1*$^{-/-}$ mice bearing EG7 or EL4. (B) At day 3, OT1 T cell proliferation was analyzed. Numbers indicate the percentage of proliferating OT1 T cells after gating on CFSE⁺ cells. (C and D) OT1 T cell accumulation at day 12 in EG7- or EL4-bearing WT and *Icam1*$^{-/-}$ mice. (C) Representative dot plots are shown; numbers indicate the percentage of OT1 T cells among CD8⁺ T cells. (D) Results are pooled from three independent experiments. Error bars represent mean ± SD. (E and F) Twelve days after OT1 T cell injection, mice were boosted with 40 μg of OVA protein in CFA. (E) Representative flow-cytometry profiles of IFNγ-secreting cells after gating on CD45.1⁺ OT1 T cells 4 days after rechallenge are shown. (F) Pooled results of two independent experiments expressed as the percentage of IFNγ-secreting cells among CD45.1⁺ OT1 T cells. Data were compared with the two-tailed unpaired Mann-Whitney test.

DISCUSSION

In naive T lymphocytes, signaling through the TCR occurs during the interaction with DCs presenting the cognate TCR ligand at their surface. The duration and frequency of TCR engagement is therefore not exclusively determined by the affinity of the TCR for its peptide-MHC ligand but will also depend on the dynamics of the interaction between the two cells. This interaction is controlled by the engagement of multiple adhesion receptor-ligand interactions on the surface of the two cell types. Adhesion receptors are themselves activated by T cell activation. Recent two-photon microscopy studies of T-DC interactions in intact lymph nodes showed a complex regulation of the duration of the interactions (Bousso and Robey, 2003; Hugues et al., 2004; Mempel et al., 2004; Miller et al., 2002; Shakhar et al., 2005). In this study, we identify ICAM-1 as a critical regulator of the duration of DC-CD8⁺ T cell interactions in intact LNs: In the absence of ICAM-1 expression, stable, long-lasting interactions were less frequent than in WT mice. Instead, DCs and naive CD8⁺ T cells established mostly short antigen-dependent interactions during the two first days after immunization.

We took advantage of this situation to analyze the functional contributions of short and long individual DC-T cell contacts to CD8⁺ T cell function. Of course, in the absence of ICAM-1 expression on DCs, ICAM-1 ligands—such as LFA-1—on T cells are not engaged. Our conclusions on the functional contribution of stable interactions are therefore limited to ICAM-1-dependent stable interactions (the interactions that engage ICAM-1 and its ligands on T cells). Because most adhesion molecules also signal, and because adhesion is activated through signaling by other receptors, it is extremely difficult to distinguish the direct effects of individual receptors when analyzing this type of complex cell-cell interaction. Nevertheless, in the absence of ICAM-1, the interactions between mature DCs and naive T cells were briefer than in the presence of ICAM-1.

Several studies in the literature analyzed the requirements for the duration of DC-naive T cells contacts for T cell priming and memory, both in vitro and in vivo (Benvenuti et al., 2004b; Gett et al., 2003; Gunzer et al., 2000; Prlic et al., 2006; van Stipdonk et al., 2001). These studies, however, analyzed the duration of the contacts between the two cell populations and not between individual cells. During the periods of contact between the two

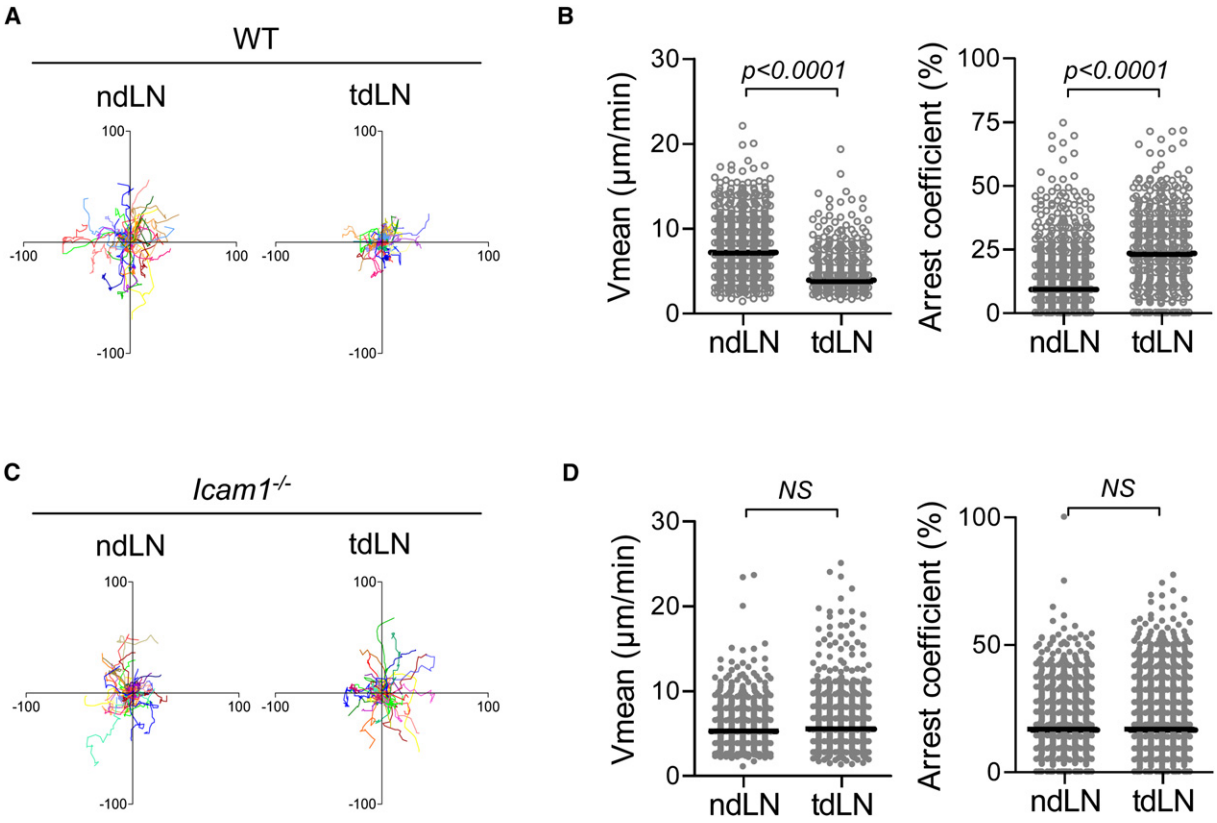

Figure 7. DC-T Cell Interaction Dynamics in Tumor-Draining LNs from WT and *Icam1*$^{-/-}$ Mice

(A–D) GFP$^+$ OT1 T cells were injected intravenously into WT and *Icam1*$^{-/-}$ mice bearing EG7 tumors. TdLNs and ndLNs were collected 15–20 hr later and imaged by time lapse TPLSM. See Movies S13–S16. (A) Representative migratory paths of 50 T cells in LNs from WT mice are shown. Paths are represented normalized to the same departure point. (B) T cell mean velocity (Vmean) (left panel), and T cell arrest coefficient (right panel) in LNs from WT mice. (C) Representative migratory paths of 50 T cells in LNs from *Icam1*$^{-/-}$ mice. (D) T cell mean velocity (Vmean) (left panel), and T cell arrest coefficient (right panel) in LNs from *Icam1*$^{-/-}$ mice. Black lines indicate medians. Data are from three independent experiments and were compared with the two-tailed unpaired Mann-Whitney test.

populations, the individual contacts may have been brief or long. Our study, in contrast, attempts to correlate the duration of individual T cell-DC contacts with the functional outcome of the CD8$^+$ T cell response. Several previous studies correlated brief DC-T cell interactions with the induction of tolerance (Hugues et al., 2004; Tadokoro et al., 2006; Tang et al., 2006) and stable long-lasting mature DC-T cell contacts with the induction of CD8$^+$ T cell priming (Hugues et al., 2004; Mempel et al., 2004; Miller et al., 2004). We now show that the genetic ablation of *Icam1* prevents the establishment of long-lasting interactions and fails to induce effective memory. Whether or not the priming of the CD8$^+$ T cells in ICAM-1-deficient mice resulted in the induction of tolerance was less clear and will need to be addressed in further studies.

Our results, however, differ from the results of the group of M. Bevan, who showed that if DCs are ablated from the mice 7 hr after immunization, the induction of CD8$^+$ T cell memory is normal (Prlic et al., 2006). These authors, on the basis of two-photon results by other groups showing transient interactions in the first hours after immunization, propose that short DC-T cell interactions suffice for the induction of CD8$^+$ T cell memory. This discrepancy between their conclusions and ours could be accounted for by at least two different explanations. First, stable interactions can be observed very early (a few hours) after immunization, depending on the experimental systems used (Celli et al., 2007; Shakhar et al., 2005), and it could be that stable interactions (lasting several hours) occurred during the first 7 hr in their experiments. Second, in our experimental system, transient interactions continue for several days, whereas DC ablation stops any further interaction. It is also possible that repeated, transient TCR stimulations inhibit the induction of memory cells. Further studies will address these questions in more details. A role for ICAM-1 in the induction of CD8$^+$ T cell memory had been suggested previously (Parameswaran et al., 2005), but the actual mechanisms have not been elucidated. A role for the stability of DC-T cell interactions in the induction of CD8$^+$ T cell memory, however, has not been proposed before. Decreased memory in the absence of stable DC-T cell interactions could be related to a lack of formation of mature immune synapses. During the brief (minutes) DC-T cell interactions observed in the absence of ICAM-1, receptor polarization and effective cytokine secretion through the intercellular space may not occur. Consistent with this scenario, a recent study showed that ICAM-1-dependent stable DC-T cell contacts, and the resulting initial asymmetrical division, may affect IFNγ secretion by effector CTLs (Chang et al., 2007). IFNγ is also involved in CD8$^+$ T cell

survival in different experimental systems (Badovinac et al., 2000; Hollenbaugh and Dutton, 2006; Sercan et al., 2006; Whitmire et al., 2005). Our results are fully consistent with this possibility, because IFNγ production was the most affected T cell function, together with T cell accumulation (a consequence of T cell survival), during the primary T cell response in *Icam1*$^{-/-}$ mice. The effect of IFNγ on the establishment of CD8$^+$ T cell memory remains controversial (Haring et al., 2006; Pearce and Shen, 2007; Whitmire et al., 2007). However, Whitmire et al. report that direct IFNγ signaling enhances the development of CD8$^+$ T cell memory. One could thus speculate that impaired CD8$^+$ T cell memory in *Icam1*$^{-/-}$ mice may be due to reduced IFNγ production by CD8$^+$ T cells during the primary response.

Our results in mice bearing antigenic tumors are also fully consistent with a critical role for the duration of DC-T cell contacts in the functional outcome of CD8$^+$ T cell responses. In tumor-draining LNs, CD8$^+$ T cells displayed reduced motility, as compared to nondraining LNs. These T cell arrests occur in contact to tumor-antigen-bearing DCs, as only CD11c$^+$ cells present the tumor antigen in the draining LNs. These stable DC-T cell interactions result in T cell accumulation, effector differentiation, and effective tumor rejection in WT mice (Boissonnas et al. [2007] and data not shown). In *Icam1*$^{-/-}$ animals, CD8$^+$ T cell motility was not reduced in tumor-draining as compared to nondraining LNs, indicating that DC-T cell contacts were not stabilized in absence of ICAM-1. The phenotype of the CD8$^+$ T cells in the tumor-draining LNs was consistent with our results from the use of αDEC205:OVA as an antigen-delivery system: no T cell accumulation and lower IFNγ production. In addition, the clonal deletion observed in absence of ICAM-1 may account for the poor CD8$^+$ T cell response against tumor antigen described in LFA-1^{ko} mice (Schmits et al., 1996). Furthermore, the OT1 T cells in these animals did not respond to a rechallenge with OVA in CFA, suggesting a defective differentiation of memory T cells.

To conclude, our results indicate that the dynamics of T-DC interactions alter the antigenic signal delivered to T cells by the DCs, as well as their implication for tolerance versus programming of effector responses. Because this statement is also valid for antitumor immune responses, it is tempting to speculate that nonantigenic tumors may somehow regulate ICAM-1 expression on DCs, resulting in tumor-antigen presentation without stable DC-T cell contacts and the induction of tolerance to tumor antigens. Our results also suggest that it may be important to consider the levels of expression of adhesion molecules on the in vitro-generated DCs used for immunotherapy.

EXPERIMENTAL PROCEDURES

Mice

C57BL/6 (CD45.1 or CD45.2) mice were obtained from Charles River Labs. C57BL/6 *Rag2*$^{-/-}$ OT1 TCR (Vα2, Vβ5)-transgenic (C. Reis e Sousa, ICRF, UK), UBI-GFP/BL6, and UBI-CFP/BL6 mice were maintained in the Curie Institute animal facility. C57BL/6 *Rag2*$^{-/-}$ OT1-TCR-transgenic mice were crossed with UBI-GFP/BL6 or CD45.1/BL6 mice to obtain the F1 progeny. C57BL/6 *Icam1*$^{-/-}$ mice were obtained from Jackson Laboratory. Indicated doses of αDEC205:OVA were injected subcutaneously (flank) in the absence or presence of CD40 mAbs (10 μg). Live-animal experiments were performed in accordance to the guidelines of the French Veterinary Department.

Reagents

$OVA_{257–264}$ peptide (SIINFEKL) (Neosystem) was dissolved in PBS. αDEC205:OVA conjugates (Bonifaz et al., 2002), CD40 (1C10), and CD11c-Alexa488 were produced in the laboratory. All other monoclonal antibodies were purchased from Becton Dickinson.

Cells

At specified times after αDEC205:OVA injection, DCs were purified from draining LNs as previously described (Hugues et al., 2002). CD8$^+$ T cells specific for OVA peptide ($OVA_{257–264}$ in a H2-K^b MHC class I context) were obtained from LNs from *Rag2*$^{-/-}$ OT1-TCR-transgenic mice (purity around 96%) or OT1-GFP-transgenic mice, adequately labeled with 5 μM CMTMR or CFSE (Molecular Probes), and injected intravenously into 4–5-week-old female C57BL/6 recipient mice. In some experiments, DCs were obtained as described (Thery et al., 2002) from the bone marrow of WT and *Icam1*$^{-/-}$ mice, matured with LPS, and pulsed with OVA peptide (5 μM). Splenic DCs were isolated from mouse spleens adapting described protocols (Vremec et al., 2000). In brief, mice were intravenously injected with 10 μg of plasmid pNGVL-hFLex (a gift from Kris Thielemans) to increase the absolute number of spleen DCs (He et al., 2000). The DNA was diluted in 1.6 ml of sterile 0.9% NaCl solution and injected into mice through the tail vein over 10 s via a $27^{1/2}$ gauge needle (Liu et al., 1999). Eight to ten days after injection, spleens were harvested from mice and DCs were purified with mouse CD11c Miltenyi Biotech kit according to the manufacturer's conditions.

Flow Cytometry

Phenotypic characterization of DCs and T cells was performed on a FACSCalibur (Becton Dickinson). The activation level of DCs was quantified through IAb, Kb, CD40, CD80, CD86, and ICAM-1 expression after gating on CD11c$^+$ cells. Labeled OT1 T cell activation was measured as a CD69 upregulation after appropriate gating. OT1 T cell proliferation was assessed by CFSE dilution after appropriate gating. IFNγ production by OT1 T cells was measured after incubation for 4 hr at 37°C in complete Iscove's modified Dulcecco's medium (IMDM) containing 4 μg/ml of Brefeldin A (Sigma) in the presence of 5 μM OVA peptide. Cells were fixed with a solution of paraformaldehyde (PAF) 2% for 30 min at room temperature in the dark, permeabilized in a BD Perm/Wash solution (Becton Dickinson), and stained with an αIFNγ. In vivo CTL activity was evaluated in LNs after injection of a mixture (1:1) of CFSE (1 μM)-labeled, unloaded splenocytes (7×10^6) and CFSE (10 μM)-labeled, OVA peptide (5 μM)-loaded splenocytes (7×10^6). Similarly, in vitro CTL activity was analyzed by incubated indicated ratios of GFP$^+$ OT1 T cells sorted by flow cytometry with CFSE (0.1 μM)-labeled unloaded splenocytes (2×10^4) and CFSE (1 μM)-labeled OVA peptide (5 μM)-loaded splenocytes (2×10^4). The percentage of antigen-specific lysis was calculated (1 − ((%CFSE^{hi} cells)/(%CFSE^{lo} cells)).

Time-Lapse Video Microscopy

Poly L Lysine-treated coverslips coated with 2×10^5 DCs were placed into an imaging chamber at 37°C in a 5% CO2 atmosphere. Contact duration between individual T cells and DCs was quantified on sequential differential interference contrast (DIC) images acquired with a 63× 1.32 NA objective and a cooled CCD camera (Micromax, Princeton Instruments) controlled through Metamorph software (Universal Imaging Corporation). Beginning one minute after the addition of 2×10^5 OT1 CD8$^+$ T cells (t = 0), images were collected every 10 s over 30 min.

Two-Photon Laser Scanning Microscopy

Ten million CMTMR-labeled (5 μM) or GFP$^+$ OT1 T cells were transferred to 4–5-week-old female C57BL/6 recipients by injection into the tail vein. Four hours later, recipient mice received a subcutaneous injection (flank) of the appropriate dose of αDEC205:OVA, in the absence or presence of αCD40. At various time points during the following 48 hr, draining LNs (inguinal, axillary, and brachial) were carefully collected. Labeling of endogenous LN-resident DCs was achieved by injection via a microelectrode of 0.2 μl of αCD11c-Alexa488 (50 μg/ml in PBS, 0.5% BSA) in the LN and further incubation of the LN at 4°C for 3 hr in this solution, as described (Hugues et al., 2004). In some experiments, mice were injected subcutaneously with 2×10^6 of CFP$^+$- or CMTMR-labeled (5 μM) splenic DCs, which were previously incubated with

LPS (100 nM) in the presence of OVA peptide (5 μM). CMTMR labeling of splenic DCs did not alter CD8+ T cell interaction dynamics (data not shown). LNs were immobilized in an imaging chamber and perfused with oxygenated (95% O2 and 5% CO2) Roswell Park Memorial Institute (RPMI) medium containing 10% FCS. Local temperature was monitored and maintained at 37°C. Measurements were performed in at least three independent experiments. The two-photon laser scanning microscopy (TPLSM) setups used were either (1) a Fluoview200 (Olympus) in a descanned configuration, equipped with a 40× 0.8 NA dipping objective (Olympus) and coupled to a Tsunami femtosecond laser (Spectra Physics), with a 540 nm dichroic mirror to separate GFP and CMTMR emission spectra, or (2) an LSM510 Meta (Zeiss) coupled to a Maitai HP DeepSee laser (690–1020 nm) (Spectra-Physics). The excitation wavelength was 870 nm. Every 30 s over the course of 30 to 60 min, four consecutive 280 × 280 μm or 330 × 330 μm images with 6 μm *z* spacing with the 40× objective were acquired. Images were despeckled and z-average-projected with Image J (NIH), and manual tracking of individual cells was performed with Metamorph software. Fluorescent CD8+ T cells were detected in the paracortical region up to 300 μm below the LN capsule. The arrest coefficient is defined as the proportion of time each cell's instantaneous velocity (calculated between every 30 s intervals) is lower than 2 μm/min. The number of individual new contacts formed during the imaging session between a DC and CD8+ T cells in the same LN were quantified in the 4D imaging data set. A T-DC hit rate was then calculated with the equation $K_{on} = A / [T] [DC] t$, where K_{on} is the calculated hit rate ($cell^{-2}\ mm^6 \cdot h^{-1}$), A is the total observed number of DC-T cell contacts, [T] and [DC] are densities of T cells and DC in the data imaging volume, and t represents the duration of imaging session.

Statistical Analysis

Nonnormally distributed data were compared with the two-tailed unpaired Mann-Whitney test. Data are shown as medians ± standard deviation (SD).

Supplemental Data

Six figures are available at http://www.immunity.com/cgi/content/full/28/2/258/DC1/.

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