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Dissecting T Cell Contraction In Vivo Using a Genetically Encoded Reporter of Apoptosis

Kym R. Garrod,[1,2] Hélène D. Moreau,[1,2,3] Zacarias Garcia,[1,2] Fabrice Lemaître,[1,2] Isabelle Bouvier,[4,5] Matthew L. Albert,[4,5] and Philippe Bousso[1,2,*]
[1]Institut Pasteur, Dynamics of Immune Responses Unit, 75015 Paris, France
[2]INSERM U668, 75015 Paris, France
[3]University Paris Diderot, Sorbonne Paris Cité, Cellule Pasteur, rue du Dr Roux, 75015 Paris, France
[4]Institut Pasteur, Immunobiology of Dendritic Cell Unit, 75015 Paris, France
[5]INSERM U818, 75015 Paris, France
*Correspondence: philippe.bousso@pasteur.fr
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SUMMARY

Contraction is a critical phase of immunity whereby the vast majority of effector T cells die by apoptosis, sparing a population of long-lived memory cells. Where, when, and why contraction occurs has been difficult to address directly due in large part to the rapid clearance of apoptotic T cells in vivo. To circumvent this issue, we introduced a genetically encoded reporter for caspase-3 activity into naive T cells to identify cells entering the contraction phase. Using two-photon imaging, we found that caspase-3 activity in T cells was maximal at the peak of the response and was associated with loss of motility followed minutes later by cell death. We demonstrated that contraction is a widespread process occurring uniformly in all organs tested and targeting phenotypically diverse T cells. Importantly, we identified a critical window of time during which antigen encounters act to antagonize T cell apoptosis, supporting a causal link between antigen clearance and T cell contraction. Our results offer insight into a poorly explored phase of immunity and provide a versatile methodology to study apoptosis during the development or function of a variety of immune cells in vivo.

INTRODUCTION

A core tenet in immunology is that protective T cell immunity develops in a triphasic manner, classically categorized into the three discrete phases of expansion, contraction, and memory (Sallusto et al., 2010). In brief, initial encounter with antigens derived from an infectious agent triggers the activation and robust proliferation of antigen-specific T cell clones. This expansion phase is followed by a precipitous decline in cell numbers (Badovinac and Harty, 2006; D'Cruz et al., 2009; Marrack et al., 2010; McKinstry et al., 2010). The remaining 5%–10% of activated T cells that survive the contraction phase go on to seed the memory pool (Kaech et al., 2002). Although much insight has been gained into the expansion and memory phases of the response, the mechanisms that underlie contraction remain to be clarified.

T cell contraction has been shown to operate through both the intrinsic and extrinsic pathways of apoptosis as a consequence of cytokine deprivation or engagement of death receptors, respectively (D'Cruz et al., 2009; Krammer et al., 2007; Marrack and Kappler, 2004). With the observation that the generation of greater memory T cell numbers following immunization correlates well with enhanced memory function (Harty and Badovinac, 2008), it is of critical importance for vaccine development to understand the mechanisms that regulate the process of effector T cell attrition. It was initially proposed that contraction was linked to antigen clearance, as the timing of these events often coincide (Sprent and Tough, 2001). Consistent with this possibility, in vitro experiments using highly activated T cells have shown that withdrawal of TCR stimulation can result in apoptosis (Harbertson et al., 2002; McKinstry et al., 2007). However, the role of antigen in regulating contraction in vivo has not directly been established. In fact, several studies have forced us to reexamine this assumption. For example, blunting the course of antigen presentation by antibiotic treatment following *Listeria monocytogenes* infection has been shown to have little impact on the onset of T cell contraction (Badovinac et al., 2002; Mercado et al., 2000). Moreover, it has been demonstrated that limiting antigen presentation through DTR-mediated ablation of dendritic cells does not interfere with the generation of normal memory T cell function (Prlic et al., 2006). These results have led to the suggestion that contraction is programmed early after initial antigen encounter, in line with the "autopilot" model of $CD8^+$ T cell immunity (Badovinac et al., 2004; Bevan and Fink, 2001; Kaech and Ahmed, 2001; van Stipdonk et al., 2001).

However, a limitation of previously reported experimental systems is that contraction was defined indirectly, as a decline in absolute T cell numbers, without specifically examining T cell apoptosis. In fact, although several methods are currently available to detect apoptosis in vitro (Elmore, 2007), it has been challenging to apply these approaches to an in vivo setting due in large part to the rapid clearance of dying cells by phagocytes (Bahl et al., 2010). This is illustrated by the finding that few

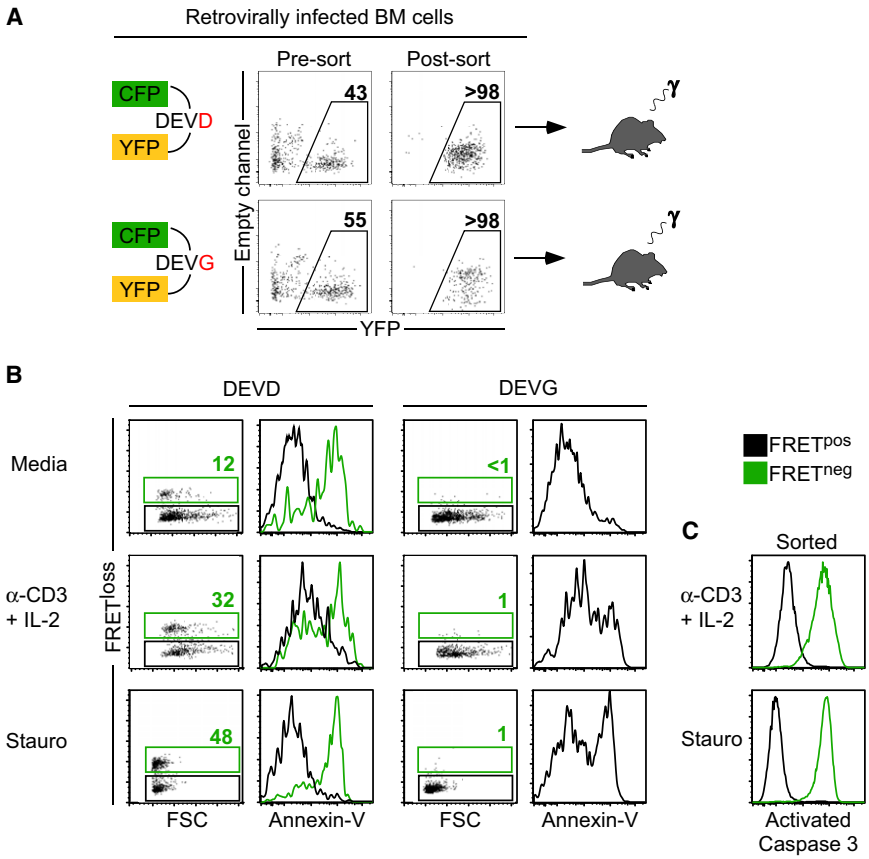

Figure 1. A Genetically-Encoded Reporter for Caspase-3 Activity Accurately Identifies Apoptotic T Cells

(A) BM cells transduced to express the capase-3 reporter DEVD or the control DEVG were FACS sorted and adoptively transferred into lethally irradiated hosts to generate chimeric donor mice.

(B) FRET loss and Annexin V reactivity for DEVD and control DEVG T cells differentiated for 7 days and stimulated overnight with α-CD3 + IL-2 or staurosporine.

(C) Intracellular staining for activated caspase-3 in sorted $FRET^{pos}$ (black) and $FRET^{neg}$ (green) T cells conditioned as in (B). Numbers adjacent to defined areas indicate percent cells in each gate. Data are representative of two experiments run in duplicate (B) or of three mice from two experiments (C).

thymocytes undergoing apoptosis can be detected at any given time point within the thymus, despite the fact that >95% of them are destined to die (Surh and Sprent, 1994). Inferring contraction from a decrease in absolute T cell numbers has additional drawbacks. For instance, the exact moment in which contraction begins is difficult to precisely define as T cell apoptosis and proliferation may occur simultaneously. In addition, although it has traditionally been thought that peripheral tissues, such as the liver and lungs, may represent the dominant sites of T cell death (Huang et al., 1994; McKinstry et al., 2010), the difficulty in directly identifying cells undergoing apoptosis in vivo has prevented a thorough assessment of where T cell contraction is occurring.

Herein, we established an in vivo approach to directly assess T cell contraction by visualizing cells undergoing apoptosis. Because both the extrinsic and intrinsic pathways of apoptosis converge to activate the same executioner caspase-3, we introduced a genetically encoded FRET-based reporter for caspase-3 activity into naive $CD8^+$ T cells (Breart et al., 2008; Tyas et al., 2000). We show that this probe identifies T cells as they enter the early phases of apoptosis. Using a combination of flow cytometry and two-photon imaging, we characterize the location, phenotype and dynamics of T cells undergoing contraction. In addition, we identify a critical window of time during which late antigen re-encounter antagonizes contraction, suggesting a causal link between antigen clearance and T cell contraction.

RESULTS

Detecting Caspase-3 Activation in T Cells Using a Genetically Encoded FRET-Based Reporter

Tracking T cell contraction in vivo has been hampered by the difficulty in detecting cells undergoing apoptosis. In particular, late markers of apoptosis such as Annexin V and TUNEL provide only partial quantitative information in vivo as apoptotic cells are rapidly scavenged by phagocytic cells (Bahl et al., 2010; Surh and Sprent, 1994). To circumvent this problem, we developed a system that allowed us to discriminate cells with caspase-3 activity, an early marker of apoptosis. A FRET-based probe (Breart et al., 2008; Tyas et al., 2000), composed of CFP and YFP molecules linked by the DEVD caspase-3 cleavage sequence (referred to as DEVD) was retrovirally transduced into cycling bone marrow (BM) cells. As a control, we also included a construct encoding the noncleavable DEVG linker (Figure 1A). Following reconstitution of lethally irradiated recipient mice, we validated the specificity and sensitivity of reporter activity (Figure 1). Specifically, we characterized the caspase-3 reporter activity in T cells using conventional inducers of apoptosis. T cells isolated from DEVD chimeras were activated in vitro and then treated with anti-CD3 + IL-2 to induce activation-induced cell death (AICD) or with staurosporine to pharmacologically induce apoptosis. After 7 days of culture, approximately 12% of DEVD T cells exhibited FRET loss (referred to as $FRET^{neg}$). This was elevated to 32% and 48% following treatment with anti-CD3 + IL-2 and staurosporine, respectively (Figure 1B). Importantly, Annexin V staining was restricted to DEVD T cells exhibiting FRET loss. Although control DEVG T cells were sensitive to the apoptotic stimuli, as detected by Annexin V reactivity, <2% were $FRET^{neg}$. Of note, $FRET^{neg}$ but not $FRET^{pos}$ T cells were stained with an antibody directed against activated caspase-3 (Figure 1C). These results establish the reliability of our system

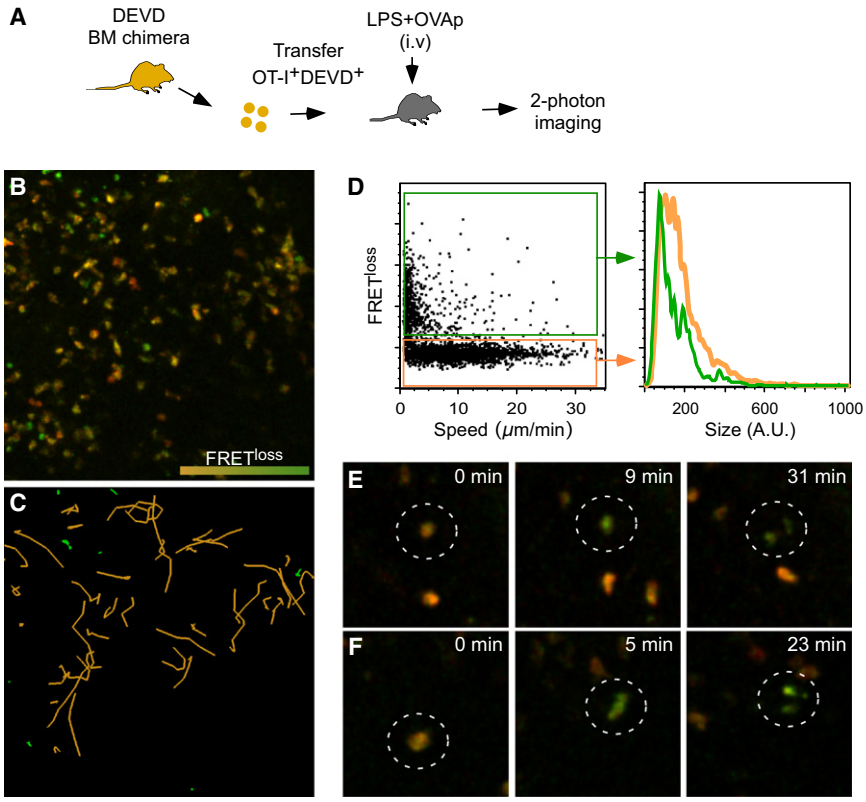

Figure 2. Caspase-3 Activity in T Cells Is Associated with Loss of Motility and Cell Death In Vivo

Recipients were adoptively transferred with OT-I DEVD T cells and immunized with peptide plus LPS. Intact lymph nodes were subjected to two-photon imaging at days 5–8.

(A) Set-up.

(B and C) Time-lapse image (B) and tracked trajectories (C) of viable $FRET^{pos}$ (orange) and apoptotic $FRET^{neg}$ (green) DEVD T cells.

(D) DISC analyses of imaging data set correlating FRET negativity, cell arrest, and cell size.

(E and F) Time-lapse images illustrate two examples of viable DEVD T cell (0 min; orange) transitioning through apoptosis (green). Morphologic changes in cellular integrity are evident at time 31 min (E) and 23 min (F). Data are representative of eight movies obtained in two independent experiments.

See also Figures S1 and S2.

to detect, in a noninvasive manner, caspase-3 activity in primary T cells.

Caspase-3 Activity in T Cells at the Peak of the Response Is Associated with Loss of Motility and Cell Death

We next investigated the occurrence and fate of caspase-3 reporter positive T cells in vivo. To this end, we studied the response of adoptively transferred naive OT-I T cells expressing the DEVD probe following immunization with adjuvant and cognate peptide $OVA_{257–264}$ (OVAp) (Figure 2A). Consistent with the literature (Harty and Badovinac, 2008), we found the peak of expansion to be between days 5 and 8 (Figure S1). We used two-photon imaging of lymph nodes to relate changes in T cell behavior and morphology to FRET loss (Figure 2). As shown in Figure 2B, $FRET^{pos}$ and $FRET^{neg}$ T cells were clearly discernable. At the peak of the response, we found that $FRET^{pos}$ T cells were highly motile in the lymph node, displaying typical velocities (10–15 μm/min) characteristic of viable T cells (Figure 2C; Movie S1). Interestingly, both arrested $FRET^{neg}$ T cells and debris were evident throughout the imaging field (Movie S1), with only a minor fraction (<10%) of $FRET^{neg}$ T cells remaining motile throughout the duration of the movie. To further correlate T cell velocity and FRET levels, we took advantage of dynamic in situ cytometry (DISC), a methodology we have recently developed in which imaging data sets are processed, converted into flow cytometry-like files and subjected to multiparametric analysis using flow cytometry software (Moreau et al., 2012). As shown in Figure 2D, the majority of T cells exhibiting FRET loss displayed mean velocities <2 μm/min. Further gating of DISC data on $FRET^{neg}$ and $FRET^{pos}$ cells revealed that $FRET^{neg}$ cells displayed a smaller cell size compared to $FRET^{pos}$ cells (Figure 2D). We next focused our attention on T cells that transitioned from a reporter $FRET^{pos}$ to $FRET^{neg}$ state. Of all events analyzed (11/11), T cells arrested before or during the transition to $FRET^{neg}$ (Figure S2). Importantly, following arrest, cells exhibited morphological changes consistent with cell death (Figures 2E and 2F; Movie S2). Further movement of $FRET^{neg}$ debris was presumably the result of phagocytosis, suggesting rapid clearance after cell death. Taken together, these data provide strong evidence that caspase-3 reporter activity primarily marks T cells undergoing apoptosis. In addition, we demonstrate that induction of caspase-3 activity in T cells is associated with loss of motility followed within minutes by cell death.

T Cell Contraction Is a Systemic Process Initiated Prior to the Peak of Expansion

Although T cell contraction typically refers to the phase that immediately follows the peak of expansion, the precise timing as well as the exact location of contraction remains ill-defined. We therefore investigated the kinetics of caspase-3 reporter activity in the spleen of recipient mice transferred with DEVD OT-I T cells following immunization with LPS plus peptide using flow cytometry (Figure 3A). Caspase-3 activity, as detected by FRET loss, was negligible (<1% of T cells) prior to immunization, but reached 5%–6% by days 5–8 before declining to baseline levels by day 23 (Figures 3B and 3C). Importantly, OT-I T cells expressing the control DEVG probe did not exhibit FRET loss, confirming the specificity of measurements made with the DEVD probe (Figure 3C). Moreover, in contrast to $FRET^{pos}$ T cells,

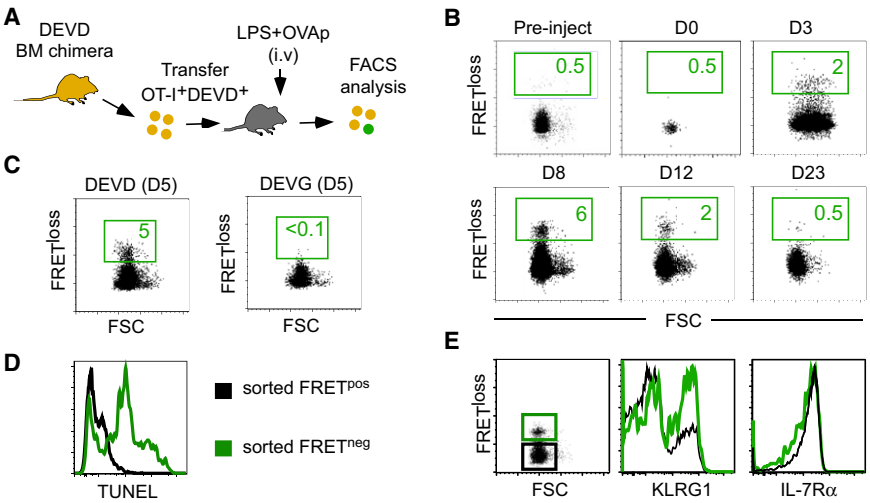

Figure 3. Identification of T Cells Undergoing Apoptosis during the Course of an Immune Response

Recipient mice adoptively transferred with OT-I DEVD T cells (or control OT-I DEVG T cells) were immunized with peptide plus LPS. At the indicated time, the spleen was harvested and analyzed by FACS.

(A) Set-up.

(B) Frequency of FRET^{neg} T cells prior to injection (pre-inject) or in the spleen at the indicated time postimmunization. Data are gated on OT-I T cells.

(C) FRET^{loss} measured in the spleen at day 5 during the response of OT-I DEVD or OT-I DEVG T cells.

(D) FRET^{neg} (green line) and FRET^{pos} (black line) OT-I T cells from the spleen were sorted 5 days after immunization and DNA fragmentation was assessed using a TUNEL assay. Representative of three animals from two experiments.

(E) Phenotype of gated splenic FRET^{neg} (green line) and FRET^{pos} (black lines) OT-I T cells on day 12 following staining with KLRG1 and CD127 (IL-7Rα) mAb. Data are representative of three animals from two experiments.

See also Figure S3.

most OT-I T cells displaying FRET loss were undergoing apoptosis, as detected by TUNEL staining (4% versus 82% TUNEL$^+$ T cells, respectively) (Figure 3D). These results establish that T cells undergoing apoptosis can be directly identified using a genetically encoded reporter for caspase-3 and reveal that the rate of contraction is maximal at the peak of the response, i.e., before a substantial decrease in total OT-I T cell numbers could be appreciated.

It has been proposed that effector CD8$^+$ T cells can be subdivided into KLRG1$^+$IL-7Rα$^-$ short-lived effector cells and KLRG1$^-$IL-7Rα$^+$ memory precursor cells (Joshi et al., 2007). Our reporter allowed us to readily examine the phenotype of T cells undergoing apoptosis. Although KLRG1$^+$ cells were overrepresented in the fraction of apoptotic T cells at day 12, contraction targeted both KLRG1$^+$ and KLRG1$^-$ T cell subsets and the majority of these cells also expressed IL-7Rα (Figure 3E). At an earlier time point (d5), we note that both IL-7Rα$^-$ and IL-7Rα$^+$ T cells undergo contraction although apoptosis was more frequent in the IL-7Rα$^-$ subset (Figure S3). Our results therefore indicate that T cell contraction, in our set-up, is not uniquely restricted to the KLRG1$^+$IL-7Rα$^-$ population, but rather targets phenotypically diverse populations of activated T cells.

We next extended our analysis to different tissues. We were consistently able to detect reporter positive cells in all locations analyzed, including spleen, lymph node, PBL, BM, liver, lungs, and thymus (Figure 4A). The rate of apoptosis was remarkably similar in all tissues analyzed, thus revealing contraction is a systemic process. As a consequence, when absolute numbers were calculated, the spleen (containing the largest pool of OT-I effector T cells [Figure S1]) was found to be the major site for contraction (Figure 4B). Similar results were obtained in the context of a bacterial infection using OVA-expressing *Listeria monocytogenes* (LM-OVA) (Figure S4). To examine T cell contraction in the context of a local immunization, we followed the rate of T cell apoptosis upon subcutaneous injection of adjuvant plus cognate peptide (Figure 4C). The rate of contraction was identical in the draining and nondraining lymph nodes as well as in the spleen (Figure 4D). Altogether, our results indicate that T cell contraction occurs systemically, irrespective of the site of priming. The fact that the rate of apoptosis is similar in all organs tested despite extensive variations in macrophages frequencies (Figure S5), suggest that the phagocytic activity is not limiting in vivo.

Monitoring T Cell Susceptibility to Apoptosis over the Course of an Immune Response

Our results demonstrate that the frequency of apoptotic T cells measured in vivo varied over the course of the response, peaking at days 5–8. We reasoned that by removing contracting T cells from their intact environment (and possibly preventing their rapid phagocytosis), we should be able to measure, with a higher sensitivity, their underlying propensity to die. To this end, we cultured T cells at various stages of the response and measured the induction of apoptosis following a short (2–3 hr) incubation period (Figure 5A). As shown in Figure 5B, activated OT-I T cells isolated at day 8 progressively accumulated within the FRET^{neg} population over the 3 hr incubation. This induction of T cell apoptosis was consistently detected in all ex vivo organ cultures analyzed (Figure 5C). Mirroring our in vivo observations, T cell susceptibly to apoptosis in vitro varied over the course of the response, with maximal death occurring at day 5 and substantially decreasing by day 15 (Figure 5C). Collectively, our results demonstrate that the observed changes in the frequency of apoptotic T cells during the response accurately reflects differences in T cell susceptibility to apoptosis over the course of the response.

Sustained Antigen Recognition Antagonizes T Cell Contraction

Antigen clearance and T cell contraction often coincide; therefore it has been proposed that these events are linked. More

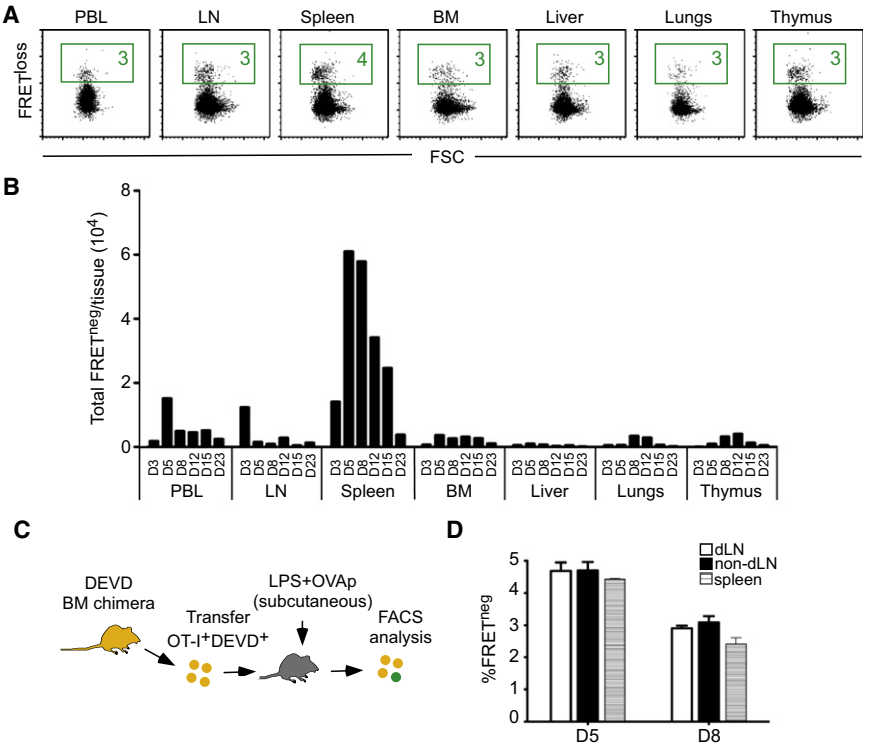

Figure 4. T Cell Contraction Is a Systemic Process

(A and B) Recipient mice adoptively transferred with OT-I DEVD T cells were immunized i.v with peptide plus LPS. (A) Frequency of FRET^{neg} T cells in the indicated tissue 5 days postimmunization. Numbers represent percent cells in the indicated gate. (B) Total FRET^{neg} T cells in distinct tissues at the indicated time points. Data are representative of five experiments.

(C) Set-up. Recipient mice adoptively transferred with OT-I DEVD T cells were immunized subcutaneously with peptide plus LPS.

(D) Frequency of FRET^{neg} T cells 5 or 8 days following subcutaneous immunization was assessed in the draining LN (dLN), nondraining LN (non-dLN), and spleen. Representative of three to four animals from two experiments. Data are presented as mean ± SEM.

See also Figures S4 and S5.

recent work has challenged this belief and suggested that the kinetics of T cell contraction is independent of antigen persistence (Harty and Badovinac, 2008). To resolve whether the decision to undergo apoptosis is imprinted upon priming or whether late antigen recognition events could influence T cell sensitivity to apoptosis, we first assessed antigen availability over the course of the response. By transferring a cohort of CFSE-labeled OT-I T cells at various time points following immunization, we noted that antigen availability sharply decreased by days 5–8, coinciding with the bulk of contraction (Figure S6). To assess whether decreasing levels of antigen were the underlying mechanism for enhanced susceptibility to apoptosis as measured in our in vitro assay, we incubated T cells isolated at various time points postimmunization with cognate peptide (Figure 6A). Strikingly, TCR stimulation provided by OVAp during these brief 3 hr cultures profoundly limited T cell sensitivity to apoptosis. This effect was apparent up to 12 days postimmunization (Figure 6B). To test whether TCR stimulation could also antagonize T cell contraction in vivo, we provided a cohort of immunized animals with an antigen bolus at day 4 and assessed T cell apoptosis the following day (Figures 6C–6F). Increasing antigen availability in vivo sharply reduced T cell susceptibility to apoptosis (Figures 6D and 6F). Moreover, this protection from apoptosis was reflected in enhanced levels of the prosurvival factor Bcl-xL (Figure 6E). Interestingly, augmenting antigen availability at later time points in the response (day 7) had the opposite effect (Figure 6G), presumably reflecting the well-characterized activation-induced cell death (Krammer et al., 2007). The differential modulation of caspase-3 activity by antigen at day 4 compared to day 7 was reflected in alterations in the frequency of T cells recovered (Figure 6H). Thus, our data identify a window of time during which T cell reencounter with antigen antagonizes contraction. In particular, the finding that T cell susceptibility to apoptosis could be manipulated implies that T cells can actively sense changes within their microenvironment and therefore, that T cell contraction is not fully programmed upon initial antigen recognition.

Although our data demonstrate a role for antigen in antagonizing contraction, it remained to be determined whether late TCR engagement was acting in a cell autonomous manner to promote survival or whether the effects were mediated through the production of prosurvival factors, or both. To address these questions, mice were transferred with OT-I DEVD reporter T cells plus CD8$^+$ T cells expressing the pmel-1 TCR (specific for gp100$_{25–33}$ peptide) admixture and immunized with both cognate peptides (Figure 6I). We reasoned that if late TCR recognition was reducing T cell contraction through the production of prosurvival factors, then prolonging the availability of gp100$_{25–33}$ to pmel-1 T cells should also antagonize the contraction of bystander OT-I T cells. Supporting this hypothesis, we found that injection of a bolus of gp100$_{25–33}$ peptide at day 4 substantially decreased OT-I T cell susceptibility to apoptosis (Figure 6J). Of note, the protective effect provided by injection of OVAp was even more profound than that provided by gp100$_{25–33}$ (Figure 6J), possibly revealing a cell-intrinsic consequence of TCR reengagement. In sum, our results support the idea that T cell contraction is governed by antigen availability and that late TCR engagement can antagonize T cell apoptosis, at least in part, through prosurvival factors acting in *trans*.

DISCUSSION

In the present article, we have characterized the anatomy of T cell contraction during the course of CD8$^+$ T cell responses. We report that contraction is a systemic process, occurring at

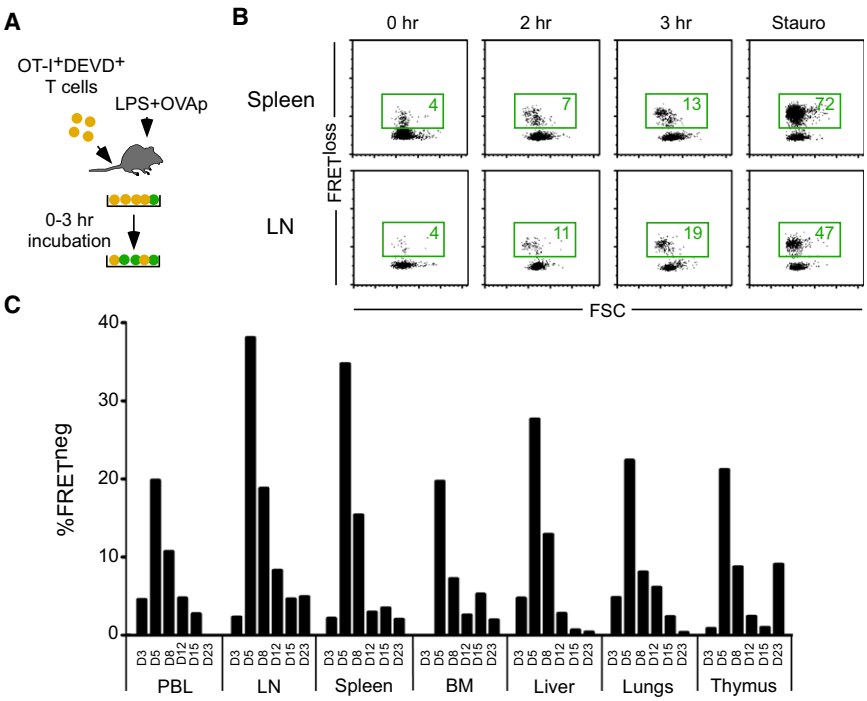

Figure 5. T Cell Susceptibility to Apoptosis Monitored over the Course of an Immune Response

(A) Schematic depicts OT-I DEVD T cell transfer and peptide immunization of recipient mice. On the specified day, the indicated tissue was harvested and after a brief in vitro incubation analyzed by FACS.

(B) Frequency of FRET loss after a 2–3 hr incubation in OT-I DEVD T cells 8 days after immunization. Three-hour incubation with staurosporine was included as a positive control for apoptosis. Numbers within defined areas indicate percent cells in each gate.

(C) Frequency of FRET^{neg} T cells following a 3 hr incubation. Data are representative of five experiments.

the same rate in lymphoid and nonlymphoid compartments. Moreover, we provide evidence that T cell contraction is regulated by antigen availability. Specifically, we identify a critical window of time during which T cell fate remains flexible and sensitive to changes in the microenvironment. Taken together, our results suggest that in contrast to the notion that contraction is a hard-wired program, the decision to undergo apoptosis is a stochastic process largely influenced by cell-extrinsic factors, with late antigen-dependent signals acting as apical mediators of effector T cell survival.

We have introduced a methodology for identifying in real-time the onset of apoptosis using a genetically encoded reporter for caspase-3 activity. Our technique offers additional advantages over conventional approaches for detection of apoptosis. First, our approach allows the direct identification of apoptotic cells without further in vitro manipulation. Second, viable T cells transitioning to the early phase of apoptosis can be isolated for further ex vivo interrogation. Finally, when combined with imaging, our approach permits the visualization of contraction as it occurs over the course of an immune response.

Noteworthy, although caspase-3 activation is a classic hallmark of both the intrinsic and extrinsic pathways of apoptosis, recent work has indicated that caspase-3 activation can also be associated with nonapoptotic processes (Alam et al., 1999; McComb et al., 2010; Puga et al., 2008) and may even be dispensable for contraction (Murakami et al., 2010; Nussbaum and Whitton, 2004). In our system, however, caspase-3 reporter activity corresponded to apoptosis as measured by TUNEL. Furthermore, our two-photon imaging data provided additional evidence that induction of caspase-3 reporter activity resulted in behavioral and morphological changes associated with apoptotic cell death in the vast majority of T cells analyzed. Dying T cells were cleared within minutes, presumably explaining why the frequency of FRET^{neg} cells never exceeded 10%, even at the peak of contraction. Collectively, our methodology is broadly suited to study apoptosis in vivo in many cell types under a variety of immunological processes including thymocyte development, dendritic cell activation and immune cell homeostasis.

The course of a T cell response is traditionally divided into discrete phases of proliferation, contraction, and subsequent memory formation. Such a three-phase model would predict that the rate of contraction would progressively increase after the peak of the response. In contrast, our results revealed that the maximal rate of T cell apoptosis occurred concurrently with the peak of expansion. This observation implies a substantial degree of overlap between proliferation and death. Furthermore, the location of T cell contraction has been controversial and may well vary based on the experimental model. For example, LCMV-specific CD8$^+$ T cells from lymphoid organs have been shown to be more susceptible to apoptosis than their peripheral counterparts (Wang et al., 2003). Conversely, influenza-specific T cells preferentially die in infected tissue rather than lymphoid organs (McKinstry et al., 2010). We performed a thorough quantification of T cell apoptosis during local and systemic immunization. We found the frequency of dying T cells to be similar in all organs and tissues analyzed indicating that contraction is by large a systemic process. Furthermore, it has been suggested that T cells harboring a KLRG1$^+$ IL7Rα^- phenotype were fated to die in contrast to KLRG1$^-$IL-7Rα^+ memory precursor cells. Although the proportion of KLRG1$^+$ T cells was indeed increased in T cells undergoing apoptosis, KLRG1$^-$ T cells still represented approximately half of the subset of dying T cells and most of these cells expressed IL-7Rα. Thus, in our settings, T cell contraction targets phenotypically diverse T cell subsets.

A critical question is whether the timing of T cell apoptosis is dictated early on during the priming phase or at later stages as T cells perceive changes in their environment. In particular, the role of antigen clearance in initiating contraction has remained

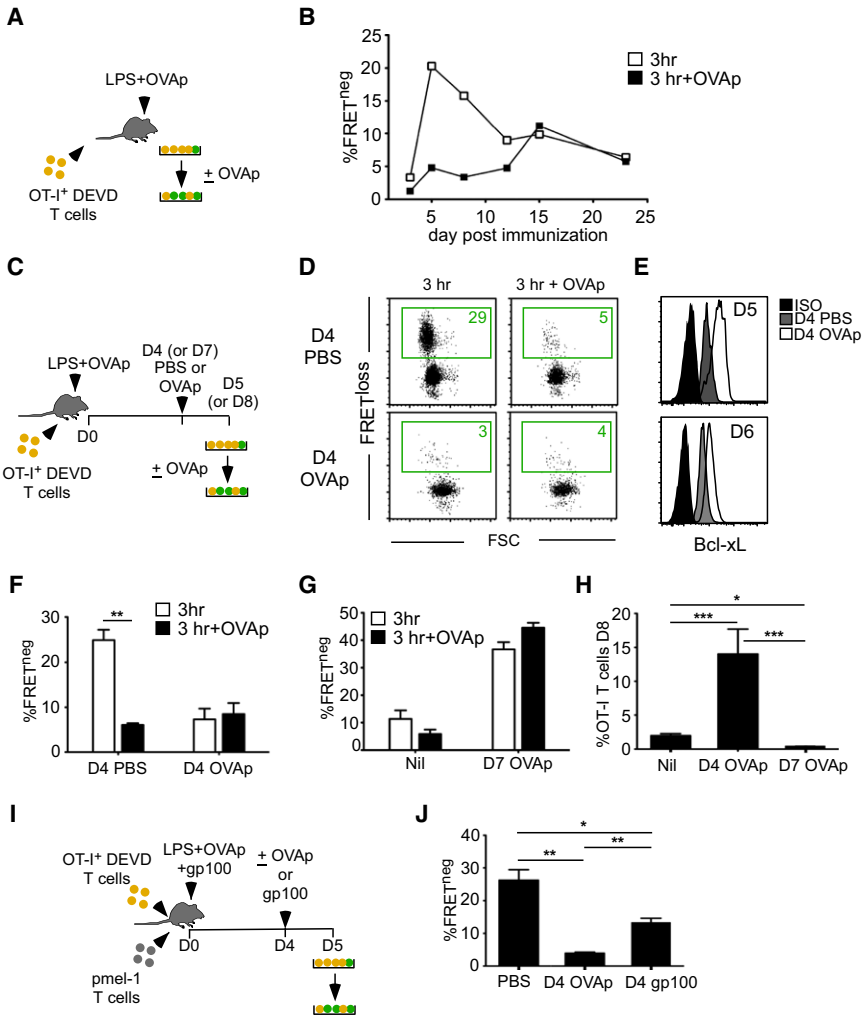

Figure 6. Late Antigen Encounters Antagonize T Cell Contraction

(A and B) OT-I DEVD T cells were adoptively transferred into recipient mice and immunized with peptide plus LPS the following day. At the indicated time, splenocytes were cultured for 3 hr in the presence (3 hr + OVAp) or absence of OVA peptide (3 hr).

(C–H) Recipients received OT-I DEVD T cells the day prior to immunization. Four (D–F, H) or 7 days (G, H) later, animals received an additional peptide bolus (or PBS) and the following day splenocytes were analyzed by FACS. (D, F) Frequency of $FRET^{neg}$ T cells following a 3 hr incubation in media alone (3 hr) or in the presence of OVA peptide (3 hr + OVAp) in animals that had received a bolus of antigen or PBS at day 4 (n = 3–4; each group). Numbers within defined areas indicate percent cells in each gate (D). Data shown are derived from three animals representing one of three experiments. (E) Expression of Bcl-xL at 5 and 6 days postimmunization. Animals received either a peptide bolus (white) or PBS (gray shaded) at day 4 (n = 2–3; each group). Black filled histogram depicts isotype control staining. (G) Frequency of $FRET^{neg}$ T cells following a 3 hr incubation in media alone (3 hr) or in the presence of OVA peptide (3 hr + OVAp) in animals that had received or not a bolus of antigen at day 7 (n = 3–4; each group). Samples were analyzed 8 days postimmunization. (H) Frequency of $OT\text{-}I^+$ T cells recovered from spleen 8 days postimmunization (Nil) or after day 4 or day 7 antigen bolus.

(I and J) Animals received a 1:1 OT-I DEVD:pmel-1 $CD8^+$ T cell admixture. The following day animals were immunized with LPS plus each respective peptide and, at day 4, received an additional antigen bolus. Splenocytes were analyzed at day 5 by FACS. Set-up (I) and frequency of $FRET^{neg}$ T cells following a 3 hr in vitro incubation (n = 3; each group) (J). *p < 0.05, **p < 0.005, and ***p < 0.0005 (two-tailed unpaired t test). Data are representative of four (B) or two (E, F, J) experiments. Data are presented as mean ± SEM.

See also Figure S6.

controversial (Badovinac and Harty, 2002; D'Cruz et al., 2009; Marrack et al., 2010; McKinstry et al., 2010). With the ability to dissect, at the single cell level, the effect of antigen re-encounter on T cell survival, we provide evidence that sustained antigen availability antagonizes contraction. This was unexpected given that TCR re-engagement has been shown to induce AICD. In fact, we found that antigen re-encounter has divergent effects on T cell survival and could induce AICD as the response weans. Specifically, we identified a window of time around the peak of the response during which additional TCR signals dramatically reduce caspase-3 activity, induce the anti-apoptotic mediator Bcl-xL and increase the overall size of the surviving T cell pool. We propose that this temporal window provides a mechanism to maintain effector T cell viability until infection has been cleared. The finding that T cells can be rescued from apoptosis by antigen re-encounter supports a model in which a T cell's decision to undergo apoptosis is not fully imprinted during priming, but rather, the result of continuous signals received from the microenvironment. In contrast to $CD4^+$ T cells, activated $CD8^+$ T cells have been shown to proliferate and differentiate in the absence of continued antigenic stimulation (van Stipdonk et al., 2001). However, recent work has shown that prolonged antigen availability may be required for optimal $CD8^+$ T cell responses (Blair et al., 2011; Hovav et al., 2007; Jusforgues-Saklani et al., 2008). By suggesting a causal link between antigen availability and T cell contraction, our work further challenges the idea that the fate of $CD8^+$ T cells is completely hard-wired following initial priming.

Our results would also be consistent with the observation that stimulation by high-affinity peptide may limit T cell apoptosis (Malherbe et al., 2004; Wensveen et al., 2010). While an optimal priming could explain this observation, it is interesting to consider that when antigen load becomes limiting in the late phase of the response, T cells may only remain responsive to

high but not low-affinity ligands. High affinity TCR ligands may act to limit the extent of contraction through sustained TCR stimulation. This would be consistent with the observation that activation by low-affinity peptide induces an earlier onset of contraction than their high-affinity counterparts (Zehn et al., 2009) and together with our work, suggests that contraction may be a default pathway triggered in antigen poor conditions.

It has been well established that T cell contraction is greatly influenced by cytokine signals (Badovinac et al., 2000; Joshi et al., 2007; Rubinstein et al., 2008; Sanjabi et al., 2009; Schluns and Lefrançois, 2003). Our work may help provide a link between cytokine- and antigen-dependent processes during the contraction phase. Indeed, we found that the protective effect of antigen re-encounter was extended to bystander effector T cells. Our results thus imply a role for antigen-dependent soluble factors that, acting in both an autocrine as well as paracrine manner, promote T cell survival. In support, we have found the addition of IL-2, IL-7, or IL-15 to our in vitro cultures antagonizes T cell contraction, albeit to a lesser degree than cognate peptide (K.R.G and P.B., unpublished data). Although the present work has specifically focused on understanding factors underlying the contraction phase of T cell responses, our approach could readily be applied in future studies to help dissect the mechanisms driving memory T cell differentiation.

In sum, by employing a genetically based strategy to track effector T cell apoptosis, we have provided insights into an enigmatic phase of adaptive immunity. Specifically, we have demonstrated that contraction occurs concurrently with the expansion phase and is detected at similar rates in both the lymphoid and nonlymphoid compartments. In contrast to the belief that contraction is a hard-wired program, our results highlight an inherent plasticity in effector T cell fate during contraction and suggest a prominent role for antigen availability in antagonizing T cell apoptosis. Exploiting this plasticity and the prosurvival effects of late antigen encounter may provide promising targets for boosting immune responses.

EXPERIMENTAL PROCEDURES

Mice

C57BL/6 and pmel-1 ($gp100_{25–33}$ peptide-specific $CD8^+$ TCR-transgenic) mice were purchased from Charles River and The Jackson Laboratory, respectively. OT-I ($OVA_{257–264}$ peptide-specific $CD8^+$ TCR-transgenic) mice were bred in our animal facility. Mice were cared for in accordance with Pasteur Institute guidelines in compliance with European animal welfare regulations, and all animal studies were approved by the Pasteur Institute Safety Committee in accordance with French and European guidelines.

Bone Marrow Chimeras

DEVD and DEVG constructs (provided by J.M. Tavaré [Tyas et al., 2000]) were cloned into a murine stem cell virus (MSCV) vector using standard techniques. Transduction of cycling C57BL/6 and OT-I BM cells was done as described (Dahl et al., 2000; Pear et al., 1998) with the exception that Platinum-E packaging cell line was used for production of retrovirus. Transgene$^+$ cells were selected on a FACSAria II (Becton Dickenson) and adoptively transferred into lethally irradiated (two doses of 625 rads given at a 3 hr interval) recipients. Mice were rested >8 weeks following BM reconstitution.

In Vitro Detection of Apoptotic Cells

$CD4^+$ T cells from C57BL/6 BM chimeras were purified from lymphoid organs using Dynabead negative selection and activated with α-CD3/CD28 beads (25 μl/10^6 T cells; Invitrogen) under Th1 skewing (10 ng/ml IL-12 and 10 U/ml IL-2; Invitrogen and Roche, respectively) conditions. After 6 days, cells were left untreated or treated overnight with either staurosporine (1 μM; Sigma-Aldrich) or soluble α-CD3 (1 μg/ml; eBioscience) plus IL-2 (50 U/ml) to induce AICD. The following day, samples were stained for Annexin V as per manufacturer's instructions (eBioscience) and analyzed by a BD FACSCanto II. In similar experiments, day 7 samples were sorted into $FRET^{pos}$ and $FRET^{neg}$ populations and stained for intracellular activated caspase-3 (C92-605; BD PharMingen) expression as per manufacturer's instructions (Cytofix/Cytoperm kit; BD Bioscience).

Monitoring Contraction In Vivo

$CD8^+$ T cells from OT-I BM chimeras were purified from lymphoid organs using Dynabead negative selection and 0.5-1 × 10^6 OT-I DEVD T cells were transferred to recipient mice. The following day, animals were immunized intravenously (i.v.) with $OVA_{257–264}$ plus $OVA_{323–339}$ (100 μg each peptide; PolyPeptide Group) in LPS (40 μg *Escherichia coli* 026:B6; Sigma-Aldrich) in a single dose i.v. or in two equal doses subcutaneously. The immunodominant $OVA_{323-339}$ was included to promote endogenous $CD4^+$ T cell help (Williams and Bevan, 2007). Other recipients were infected i.v. with 2 × 10^4 CFU OVA-expressing *Listeria monocytogenes* (LM-OVA). For some peptide-immunized animals, a bolus of either 100 μg $OVA_{257-264}$ or an equal volume of PBS was administered at the indicated time. In additional experiments, animals received a 1:1 mixture of 1 × 10^6 pmel-1:OT-I DEVD $CD8^+$ T cells and were immunized the following day with $OVA_{257–264}$, $OVA_{323–339}$, and $gp100_{25–33}$ (100 μg each peptide; AnaSpec) in LPS. Four days later, animals received a 100 μg bolus of either $OVA_{257–264}$, $gp100_{25–33}$, or an equal volume of PBS. At the indicated time, single cell suspensions were prepared from nonlymphoid and/or lymphoid organs and RBC-lysis (eBioscience) was performed. Lymphocytes from the liver were enriched using a density gradient (Histopaque-1077; Sigma-Aldrich). All samples were extensively washed, resuspended in complete T cell media[46]and either directly analyzed by FACS or cultured 2–3 hr at 37°C. Some 3 hr cultures received 1 μg/ml $OVA_{257–264}$.

Quantification of Apoptosis

FACS samples were gated with the following strategy to quantify apoptotic events: doublet exclusion, FSC/SSC viable lymphocytes, SSC/YFP transgene expression, and FRET gate. Greater than 99% of the adoptively transferred OT-I DEVD reporter T cells were double positive for CD8 and Vβ5. Samples were analyzed on a BD FACSCanto II (peptide immunized mice) or a LSR II (LM-OVA infected mice). The parameter $FRET^{loss}$ was defined using the FlowJo software as the ratio of CFP to FRET fluorescence. DNA fragmentation was assessed on $FRET^{neg}$ and $FRET^{pos}$ OT-I DEVD T cells that were FACS sorted from the spleen 5 days after immunization using an Apo-BrdU Apoptosis Detection kit (eBioscience) with a BrdU monoclonal antibody (MoBU-1; Invitrogen).

Two-Photon Imaging and Analysis

Five to eight days after peptide immunization of mice transferred with OT-I DEVD T cells, LN were harvested and two-photon imaging was performed using an upright microscope (DM6000B, Leica Microsystems) with a 20×/0.95 NA water-dipping objective (Olympus). Excitation was provided by a Chameleon Ultra Ti:Sapphire (Coherent) tuned at 880 nm. Data sets were processed and analyzed using Imaris (Bitplane) and the custom designed DISCit software for conversion into FCS files (Moreau et al., 2012).

Statistical Analysis

Statistical significance was calculated using a Student's t test. p values < 0.05 were considered significant. Data are presented as mean ± SEM.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures and two movies and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2012.10.015.

LICENSING INFORMATION

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