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In Situ Imaging of the Endogenous CD8 T Cell Response to Infection

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Abstract

Mounting a protective immune response is critically dependent on the orchestrated movement of cells within lymphoid organs. We report here the visualization, using major histocompatibility complex class I tetramers, of the CD8-positive (CD8) T cell response in the spleens of mice to *Listeria monocytogenes* infection. A multistage pathway was revealed that included initial activation at the borders of the B and T cell zones followed by cluster formation with antigen-presenting cells leading to CD8 T cell exit to the red pulp via bridging channels. Strikingly, many memory CD8 T cells localized to the B cell zones and, when challenged, underwent rapid migration to the T cell zones where proliferation occurred, followed by egress via bridging channels in parallel with the primary response. Thus, the ability to track endogenous immune responses has uncovered both distinct and overlapping mechanisms and anatomical locations driving primary and secondary immune responses.

An effective immune response depends on the large-scale, but carefully regulated, movement of cells within and between lymphoid and peripheral tissues. In recent years, our understanding of events in secondary lymphoid tissues has been advanced by the use of multiphoton microscopy to visualize lymphocyte movement (1–4). Nevertheless, much remains to be elucidated about the microanatomy of antigen-specific primary and memory CD8 T cell responses, with relatively limited data currently available from in situ visualization of endogenous CD8 T cell responses (5–7). Indeed, because of technical difficulties with intravital imaging of the spleen, intravital microscopic analysis of immune responses has been limited to the lymph node and has only elucidated the properties of clonal, single-avidity T cell receptor (TCR) transgenic T cells after transfer of large numbers of cells. Because it is known that increasing naïve T cell precursor frequency affects immune responses (8) and that each TCR transgenic T cell exhibits distinct physiological characteristics (9), these data should be interpreted with these caveats in mind. Thus, determining the anatomical location and migration of endogenous antigen-specific T cells in lymphoid tissues during primary and secondary immune responses remains an important goal.

To achieve this objective, we used staining with major histocompatibility complex (MHC) class I tetramers, which allows in situ identification and localization of clonally diverse endogenous antigen-specific CD8 T cells (7). This approach avoids the complications associated with adoptive transfer of TCR transgenic T cells and challenge with model antigens. With this technique, we systematically examined the CD8 T cell response to primary and secondary infection with *Listeria monocytogenes* (LM), which is primarily induced in the spleen (10). C57BL/6 mice were infected intravenously with 1×10^6 colony-forming units (CFU) of an attenuated *actA*-deficient strain of LM that had been engineered to express the

exogenous antigen ovalbumin (LM-OVA) (11–13). This allowed generation of a robust ova-specific CD8 T cell response that can be readily followed by using tetramers. At days 3, 4, and 5 post infection (PI), the spleen from each mouse was cut in two equal halves with one half used for imaging studies and the other for flow cytometric comparison. Tetramer-positive (tet^+) CD8 T cells that displayed characteristics of activation were detected by 3 days (Fig. 1A), with tet^+ cells expanding almost 14-fold from day 3 to day 5 (Fig. 1A). Phenotypic analysis revealed up-regulation of the activation markers CD11a and PD1 by day 3 (Fig. 1A), while CD69 expression was elevated on a portion of tet^+ cells at day 3 PI, but was lost by day 4 (Fig. 1A). CD127 down-regulation on tet^+ cells was a late event, occurring at day 4 PI after the down-regulation of CD62L (Fig. 1A).

Having framed the overall kinetics of the early CD8 T cell response, we undertook imaging of the splenic response in situ. Splenic architecture is organized into two distinct compartments: white pulp (WP) and red pulp (RP) (Fig. 1B) (14). The WP includes the B cell follicles and a T cell area, the periarteriolar lymphoid sheath (PALS). The RP is a blood-filled space between each WP lymphoid follicle and the next; it contains a complex venous system, reticular fibroblasts, macrophages, and some lymphocytes. The marginal zone (MZ) separates the WP from the RP, surrounds the B cell follicles and is populated with B cells expressing high levels of surface immunoglobulin IgM, dendritic cells (DCs), and macrophages (14). Tet^+ CD8 T cells were essentially undetectable in the spleen of uninfected mice (Fig. 1B). However, at 3 days PI, but not earlier, small numbers of tet^+ CD8 T cells could be readily detected (Fig. 1C) as clusters, primarily at the border of the T cell–B cell zones of the splenic WP and in the MZ (Fig. 1C and inset). These tet^+ cells were also in close contact with CD11c $^+$ DC (Fig. 1D), with many exhibiting apparent polarization of TCR and CD8 co-receptors toward the contact areas with DC, consistent with an immunological synapse (Fig. 1, D and E; see movie S1). Some tet^+ cells were also detected in the RP, MZ (white arrows) or the B cell areas [yellow arrow in (fig. S1A)]. Four days PI (24 hours later), a substantial increase in tet^+ CD8 T cells was noted, with a majority (>80%) now located in the PALS (Fig. 2A and fig. S2). Tet^+ CD8 T cells were localized in only a small number of lymphoid follicles; other follicles appeared devoid of proliferating tet^+ CD8 T cells (fig. S3), which suggested that CD8 T cell activation and proliferation occurred in a limited number of foci.

By 5 days PI, tet^+ CD8 T cells continued to proliferate (Fig. 1A) and were located along the border of the T cell and B cell zones (>40%) or in the MZ (>50%) in discrete clusters (Fig. 2, B and C, and fig. S2). CD11c $^+$ DCs were concentrated within these clusters (Fig. 2C) and, in many cases, formed apparent synapses with tet^+ CD8 T cells [(Fig. 2C, bottom), arrowheads in magnifications of boxed region above, and (fig. S4)]. Because antigen presentation occurs for ~10 days after infection (fig. S5), we tested whether the clustering and TCR reorganization of CD8 T cells relatively late in the response was antigen-dependent. To do so, we used the 25D-1.16 monoclonal antibody (mAb) (15) that recognizes SIINFEKL bound to H-2K b to block antigen recognition. mAb treatment on days 0 or 3 PI blocked cluster formation (fig. S6A) and expansion to some extent (fig. S6C). Although some tet^+ CD8 T cell clusters were present in the spleens of mice treated with mAb at 4 days PI, TCR or CD8 co-receptor polarization was not evident [(fig. S6B), arrowheads, left versus right]. These data demonstrated that secondary antigen-dependent interactions occurred between CD8 T cells and DCs.

The route that antigen-specific CD8 T cells follow to exit the WP is not known (14,16) but examination of the location of tet^+ CD8 T cells at days 5 and 6 PI clearly revealed this pathway (Fig. 3 and figs. S7 and S8, and movie S2). At 5 days PI, clusters as well as individual tet^+ cells were located in regions originally described as bridging channels (16,17) that apparently connect the PALS to the MZ/RP (Fig. 3, A and B) and are often associated with the central arteriole and its branches (fig. S7 and movie S2). tet^+ CD8 T cells egressed the PALS via the

bridging channels and followed a relatively uniform path in the MZ (Fig. 3, A and B, right, and Fig. 3B, white arrows, magnified lower panels). By 6 days PI, most of the tet⁺ population (>80%) had exited the PALS and was localized in the RP and/or the MZ (fig. S8, A and B), and by 7 days PI, nearly all (>90%) of the cells were located in the RP. Although a more detailed analysis is required, the CD8 T cell response to VSV infection was characterized by similar anatomical events, though at a more rapid pace (fig. S9, A, B, and C). Thus, although the kinetics of the CD8 response may be distinct between different infections, responding splenic CD8 T cells appear to follow a prescribed pathway driving immune response initiation, expansion, and exit.

Knowing the anatomy of the primary response to LM infection, we set out to define the location of resting and reactivated memory cells derived from that response. Image analysis 30 days after infection revealed that over 60% of tet⁺ memory CD8 cells were embedded in the B cell follicles (Fig. 4A and figs. S2 and S10, and movie S3). In addition, memory cells were also present in the MZ and RP (~30%; fig. S2), as previously suggested by adoptive transfer studies (18,19). Intranasal influenza virus infection also resulted in the appearance of flu-specific memory cells in B cell follicles (Fig. 4B), which suggested that this was a general characteristic of early memory CD8 T cells. To determine the effect of secondary antigen encounter on memory cells, LM-primed mice were reinfected with LM, and spleens were analyzed (Fig. 4, C to G). At 5 (Fig. S11A) and 15 hours (Fig. 4, C and D) post-challenge, the tet⁺ memory cells remained in the RP and B cell areas. In contrast, 24 hours post-challenge, tet⁺ cells had relocated to the margins of the PALS (Fig. 4E), and by 48 hours, cells were centrally located in the T cell zones (Fig. 4F). Note that unlike the cells in the primary infection, virtually all lymphoid follicles (PALS) were populated with responding tet⁺ CD8 T cells (compare Fig. 2A with fig. S11B), which suggests that precursor frequency may dictate the extent of follicle involvement. Events up to this point occurred in the absence of T cell expansion (Fig. 4C), an unexpected result given the rapidity with which memory T cells are believed to be reactivated. By 65 hours post recall, the tet⁺ CD8 cells had proliferated (Fig. 4C), and most had exited the PALS and were localized to RP and MZ (Fig. 4G). Movement of memory T cells into the PALS after secondary infection was antigen-specific because infection with wild-type LM had no effect (fig. S12, A and B).

Although previous studies have examined splenic lymphoid architecture, the anatomical events driving primary and secondary CD8 T cell responses to infection have not been clearly delineated. The technique we used did not allow us to quantify movement of individual cells, but allowed us to examine large areas of tissue with relative ease, which is difficult to achieve using other imaging procedures. At the population level, our kinetic analysis clearly revealed a stepwise progression of cellular movements leading to CD8 T cell expansion, exit from the spleen, and localization of resulting memory CD8 T cells in discrete splenic locales. Before this, these events had not been visualized. Imaging also revealed previously unappreciated secondary encounters of daughter CD8 T cells with antigen-bearing DC in large clusters. These findings align with a recent study that concluded that prolonged interactions between CD4 T cells and antigen-presenting cells (APCs) can occur at lower T cell frequencies (4). Thus, the contention that only a single brief encounter with an APC is needed to drive CD8 T cell activation (20,21), although it occurs in experimental systems using TCR transgenic T cells, may not represent in situ events during infection. In contrast, memory CD8 T cells appeared not to undergo secondary activation events and large cluster formation, but upon reactivation, rapidly moved from the B cell follicles to the RP via bridging channels. Therefore, these results lend evidence for a novel mechanism in which B cells or other follicular APCs induced memory CD8 T cell activation. It will be of considerable interest to determine the localization of memory cells as the population undergoes development and maturation.

Overall, the methodical examination of large areas of tissue without disturbing the integrity of structures or the localization of cellular compartments within the organ added a new dimension to the analysis of immune responses. These studies will set the stage for identification of the factors, such as chemokines and other inflammatory mediators, that control the processes driving each anatomical phase of the response. In addition, by comparing the anatomy of different types of immunizations the importance of each step in mounting a protective immune response can be determined. Thus, by monitoring how anatomical relations change during the initiation, expansion, and memory phases of an antimicrobial immune response, we have obtained an understanding of how a productive immune response takes place in vivo, and this information will provide clues to improving vaccine design.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Time course of the early splenic response to LM infection and imaging of early events in situ. (A) Flow cytometric and microscopic analysis of mouse spleen at 3, 4, and 5 days PI. Dot plots represent gated CD8⁺ T cells and histograms, gated tet⁺ CD8⁺ cells. The values represent tet⁺ cells as a percentage of CD8⁺ T cells. Gated CD8⁺CD11a^{lo}CD62L^{hi} represent naive cells. (B to E) Thick sections were stained with K^b-OVA tetramer and the indicated mAb, and multiple sections from each spleen were analyzed by confocal microscopy. B220, a CD45 isoform preferentially expressed by B cells. (B) Uninfected spleen with regions marked. MZ, marginal zone. The image was acquired by using a 20× 0.75 numerical aperture (NA) objective; a 24-μm merged z-stack is shown. (C) Splenic tissue 3 days PI. Note small clusters of tet⁺ CD8 T cells. (Magnified cluster of tet⁺ CD8 T cells in the MZ shown in inset.) A 20-μm merged z-stack is shown. (D) Cluster of tet⁺ CD8 T cells interacting with CD11c⁺ DC 3 days PI. Image acquired by using a 40× 1.2 NA water objective; a 12-μm merged z-stack is shown. [Gallery of individual z-sections shown in (fig. S1B).] (E) Magnified view of a tet⁺ CD8 T cell in contact with a CD11c⁺ DC. Image acquired by using a 40× 1.2 NA water objective (also see movie S1). The data are representative of three different experiments with two or three mice each.

Fig. 2.

Localization of antigen-specific CD8 T cells 4 and 5 days PI. **(A)** CD8 T cells localize to the PALS 4 days PI. **(B and C)** Clusters of antigen-specific CD8 T cells localize along the border of the T and B cell zones 5 days PI in multiple thick sections from each spleen. **(B)** Cluster of tet⁺ CD8 T cells on the T cell–B cell zone border. **(C)** Tet⁺ CD8 T cells cluster with CD11c⁺ DC. (Top) A 32-μm merged z-stack. (Bottom) Three-dimensional (3D) reconstruction of a 24-μm z-stack represents the magnified view of the boxed region in the top middle. Arrowheads indicate TCR and CD8 co-receptor polarization toward the adjacent CD11c⁺ DCs. (Bottom, far right) A rendered 3D-reconstruction. T, T cell zone; B, B cell zone. All images acquired by using a 20× 0.75 NA objective. The data are representative of five different experiments with two or three mice each.

Fig. 3.

Antigen-specific CD8 T cells exit the white pulp via bridging channels. Multiple thick sections from each spleen, 5 days PI, were stained with K^b-OVA tetramer and the indicated mAb. **(A)** Low-power view of spleen. Rendered 3D reconstruction (right) of the 110-μm merged z-stack (left) of a spleen fragment shows multiple clusters of tet⁺ CD8 T cells along the border of the B–T cell zones and the MZ. **(B)** Spleen section stained (CD31-specific, green) for blood vessels (arrows) to identify the central arteriole (CA) and associated branches (see also fig. S6). The image shows two bridging channels (BC, yellow arrows) through which tet⁺ CD8 cells exit the PALS into the MZ (top). (Bottom) Magnified views of the BC boxed top left. (Top and bottom, far right) Rendered 3D reconstructions are shown. (Top) Images acquired by using a 10× 0.45 NA water objective and (bottom) by using a 40× 1.2 NA water objective; both are 30-μm merged z-stacks. MZ, marginal zone; RP, red pulp. Also see fig. S6 and movie S3. The data are representative of five different experiments with two or three mice each.

Fig. 4.

Memory tet⁺ CD8 T cells localized to the B cell follicles, MZ and RP rapidly undergo local migration after infection. Multiple thick sections from each spleen, 30 days PI, were stained with K^b-OVA tetramer and the indicated mAb. (A) (Top left) A 36-μm merged z-stack image acquired by using a 20× 0.75 NA objective. The data are representative of six different experiments. (Top right) A 3D reconstruction of the z-stack shown at left. The SpotCheck function of Imaris was used to quantify the number of OVA-specific memory CD8 T cells (red spots, 41 cells) in the 37.1-mm³ volume of spleen shown. (Bottom) Magnified view of a B cell follicle of the splenic white pulp, an 18-μm merged z-stack. The data are representative of six different experiments with two or three mice each. (B) NP-specific memory CD8 T cells also

localize to the B cell follicles. Spleen sections from mice infected 35 days earlier with 300 times the 50% egg infective dose (EID₅₀) of HKx31 influenza virus intranasally were stained with NP-tetramer and the indicated mAb. (Right) A 3D reconstruction of the 35- μ m z-stack shown at left and acquired by using a 20 $\times$ 0.75 NA objective. SpotCheck analysis revealed 104 NP-specific memory CD8 T cells in the 40.4 mm³ volume of spleen shown. The data are representative of two different experiments with two mice each. (C to G) Mice infected 30 days previously were infected with 1×10^4 CFU of LM-OVA. At the indicated times post recall (PR), halves of the spleen were used for flow cytometry (C) or imaging [(D) and (E)]. Dot plots represent gated CD8 T cells. The values represent OVA-tet⁺ cells as a percentage of CD8 T cells. (D) At 15 hours PR. (E) At 24 hours PR. (F) At 48 hours PR. (G) At 65 hours PR. The data are representative of two different experiments with two mice each.