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Immunity, Vol. 16, 365–377, March, 2002, Copyright ©2002 by Cell Press

Tracking *Salmonella*-Specific CD4 T Cells In Vivo Reveals a Local Mucosal Response to a Disseminated Infection

Stephen J. McSorley,[1,2] Sarah Asch, Massimo Costalonga, R. Lee Reinhardt, and Marc K. Jenkins
Department of Microbiology
and Center for Immunology
University of Minnesota Medical School
Minneapolis, Minnesota 55455

Summary

A novel adoptive transfer system was used to track the fate of naive *Salmonella*-specific CD4 T cells in vivo. These cells showed signs of activation in the Peyer's patches as early as 3 hr after oral infection. The activated CD4 T cells then produced IL-2 and proliferated in the T cell areas of these tissues before migrating into the B cell-rich follicles. In contrast, *Salmonella*-specific CD4 T cells were not activated in the spleen and very few of these cells migrated to the liver, despite the presence of bacteria in both organs. These results show that the T cell response to pathogenic *Salmonella* infection is localized to the gut-associated lymphoid tissue and does not extend efficiently to the major sites of late infection.

Introduction

The recent development of technologies to visualize antigen-specific CD4 T cells in vivo has led to a more detailed understanding of T cell biology, particularly with regard to the earliest processes in naive T cell activation (Jenkins et al., 2001; McHeyzer-Williams et al., 1996).

Most of these studies have addressed CD4 T cell responses to protein or peptide antigens introduced into subcutaneous tissue, the peritoneal cavity, or blood. Some, but by no means all, pathogens actually use these routes to establish a natural infection. Many mammalian pathogens gain access to the host after interactions with the mucosal epithelium of the lung, intestine, or genito-urinary tract. This is typically followed by penetration of the mucosal barrier and replication in underlying tissues. One example of such a pathogen is the gram-negative bacterium *Salmonella*, which attaches to M cells in the Peyer's patches of the small intestine and induces uptake by these cells (Jones et al., 1995). Once inside the host, the bacteria rapidly migrate to a number of sites in the body, including the spleen and liver, where they replicate inside phagocytic cells (Carter and Collins, 1974; Jones and Falkow, 1996).

Various studies using gene-targeted mice have demonstrated that resistance to infection with *Salmonella typhimurium* depends upon activation of both CD4 $\alpha\beta$-T cells and B cells (Hess et al., 1996; Mastroeni et al., 2000; McSorley and Jenkins, 2000; Mittrucker et al., 2000). A more detailed understanding of CD4 T cell biology during *Salmonella* infection is lacking due to limitations in the tools available to study *Salmonella*-specific T cells. Attempts have been made in this laboratory and others to track CD4 T cell responses to heterologous proteins such as ovalbumin expressed by bacterial strains, but these experiments can be hindered by low protein levels or instability of in vivo expression (Chen and Jenkins, 1998, 1999; Pope et al., 2001).

Therefore, a number of basic questions related to T cell activation during infection with *Salmonella* remain unanswered. For example, it is not known exactly where naive, *Salmonella*-specific CD4 T cells first encounter *Salmonella* peptide-MHC complexes, an especially relevant question given that *Salmonella* can be detected in peripheral blood as early as 15 min after oral infection (Vazquez-Torres et al., 1999). Such rapid bacterial dissemination is thought to be mediated by dendritic cells that directly sample luminal contents by sending dendrites between intestinal epithelial cells (Maric et al., 1996; Rescigno et al., 2001). These studies suggest that models of intravenous antigen administration might be relevant when considering the induction of host immune responses to *Salmonella*. On the other hand, the natural process of bacterial infection involves bacterial replication and accumulation in gut-associated lymphoid tissue (Carter and Collins, 1974), suggesting that this may be the major site of initial antigen presentation and T cell activation. Indeed, ex vivo tests of CD4 T cell function indicate that *Salmonella* activates T cells both within the Peyer's patches and mesenteric lymph nodes and in other sites such as the spleen (George, 1996; McSorley et al., 2000; VanCott et al., 1998). It is also unclear whether or not activated *Salmonella*-specific T cells migrate to the liver, a major site of *Salmonella* infection and pathology. Oral infection with *Listeria*-expressing ovalbumin results in the migration to the liver of ovalbumin-specific CD8 T cells, which produce effector cytokines and presumably contribute to bacterial elimination (Pope et al., 2001). However, unlike *Listeria* infection, *Salmonella* infection of susceptible mice is uniformly fatal, raising the possibility that activated T cells do not reach the liver in this case.

The flagellin protein of *Salmonella* is known to be a source of peptide-MHC complexes for CD4 T cells from infected mice of both H-2^b and H-2^k haplotypes (Cookson and Bevan, 1997; McSorley et al., 2000). Here, we describe an adoptive transfer system that allows the visualization of flagellin-specific CD4 T cells in vivo. We demonstrate that *Salmonella*-specific CD4 T cells undergo very rapid activation in the Peyer's patches and mesenteric lymph nodes but remarkably do not respond in the spleen and fail to accumulate in large numbers in nonlymphoid sites of infection.

Results

Development of an Adoptive Transfer System to Track *Salmonella*-Specific CD4 T Cells In Vivo

A *Salmonella*-specific T cell antigen receptor (TCR) transgenic mouse was generated by using TCR-Vβ2 and

[1]Correspondence: mcsorley@uchc.edu
[2]Present address: University of Connecticut Health Center, Department of Medicine, Division of Immunology, 263 Farmington Avenue, Farmington, Connecticut 06030.

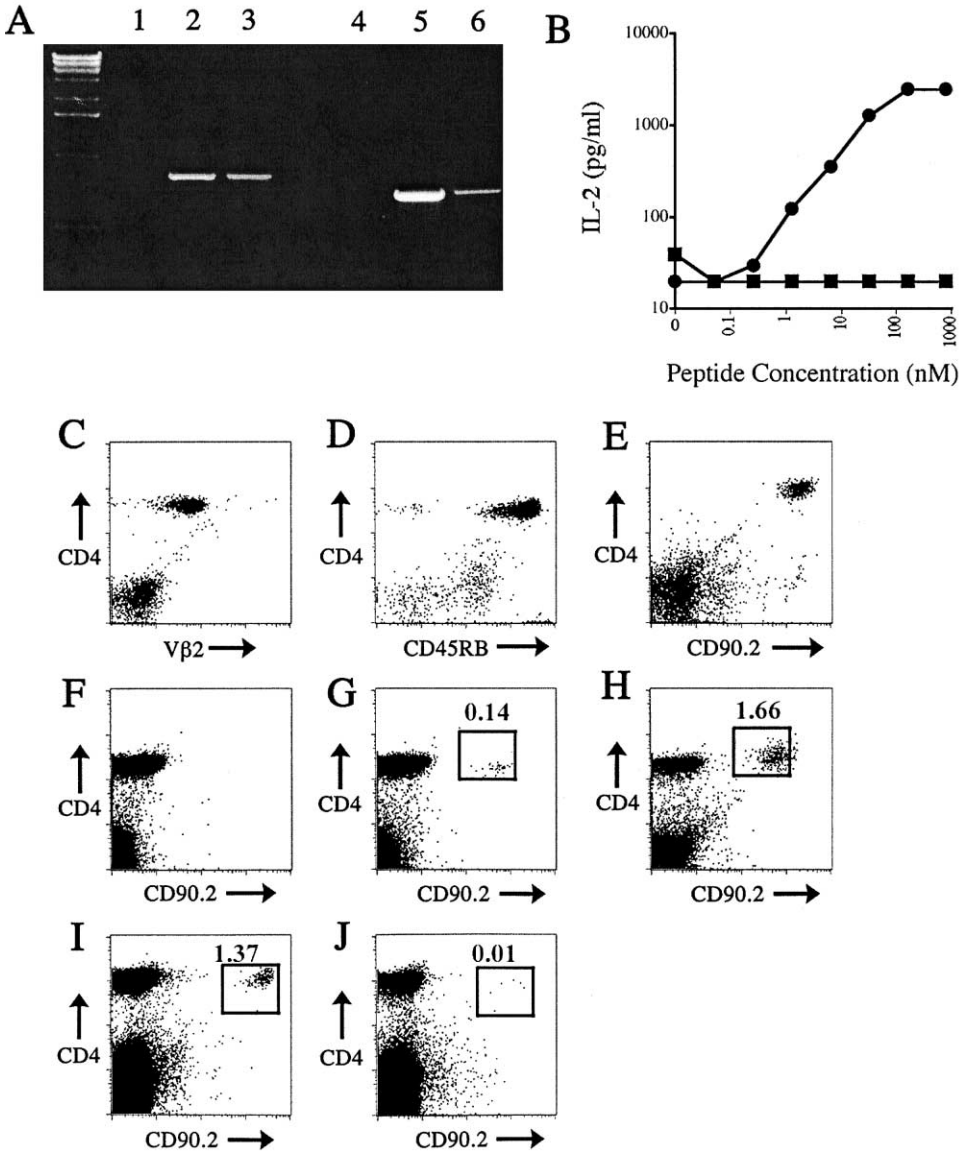

Figure 1. Development of an Adoptive Transfer System to Track *Salmonella*-Specific CD4 T Cells In Vivo

(A) Vα10-Jα10 (lanes 1–3) or Vβ2-Jβ1.2 (lanes 4–6) PCR products from genomic tail DNA of a nontransgenic littermate (lanes 1 and 4) or an SM1 TCR transgenic mouse (lanes 2–3 and 5–6).

(B) The amount of IL-2 detected by sandwich ELISA in supernatants from lymph node cells from SM1 RAG-2-deficient mice (circles) or nontransgenic littermates (squares) cultured in vitro (1×10^6/well) for 48 hr with different concentrations of flagellin peptide (427–441). The results are representative of three individual experiments.

(C–J) Dot plots from two-color flow cytometric analysis of: lymph node cells from SM1 RAG-2-deficient mice stained with anti-CD4 and Vβ2 (C), CD45RB (D), or CD90.2 (E) antibodies; popliteal lymph node cells from female B6.PL-thy1^a-Cy mice stained with anti-CD4 and anti-CD90.2 antibodies before (F), or 4 days after (G) adoptive transfer with 2×10^6 SM1 RAG-2-deficient T cells, or 4 days after adoptive transfer with 2×10^6 SM1 RAG-2-deficient T cells and 3 days after subcutaneous injection of 100 μg of flagellin peptide 427–441 in complete Freund's adjuvant (H); mesenteric lymph node cells from female B6.PL-thy1^a-Cy mice stained with anti-CD4 and anti-CD90.2 antibodies, 4 days after adoptive transfer with 2×10^6 SM1 RAG-2-deficient T cells and 3 days following oral infection with 5×10^9 flagellated *Salmonella typhimurium* strain SL1344 (I) or nonflagellated strain BC490 (J).

TCR-Vα10 genomic DNA sequences amplified from a *Salmonella*-specific T cell clone, CN.B1 (McSorley et al., 2000). A founder line (SM1) was detected by PCR amplification of tail DNA using the appropriate TCR-specific primers (Figure 1A) and bred to RAG-2-deficient mice. Lymph node cells from SM1 RAG-2-deficient mice responded in vitro by producing IL-2 to the relevant flagellin peptide (427–441) (Figure 1B), purified flagellin, or heat-killed *Salmonella* (data not shown). Peripheral CD4 T cells from SM1 RAG-2-deficient mice expressed CD4 and Vβ2 (Figure 1C) and had the phenotype of naive T cells: CD45RB^{high} (Figure 1D), CD62L^{high}, CD90.2$^+$ (Figure 1E), CD40L$^-$, CTLA-4$^-$, CD69$^-$, and CD25$^-$ (data not shown).

The activation of SM1 RAG-2-deficient cells was studied after adoptive transfer into normal recipient mice to simulate the physiological situation where *Salmonella*-specific T cells must compete with an excess of T cells of other specificities (Pape et al., 1997). Several million SM1 RAG-2-deficient T cells from the spleen and lymph nodes of donors that were never exposed to *Salmonella* were injected intravenously into B6.PL-thy1^a-Cy mice that express CD90.1, not CD90.2, on T cells (Figure 1F). Four days after injection, a trace population of CD90.2$^+$ SM1 cells was detected in all secondary lymphoid organs analyzed (Figure 1G and Figure 3A). Similar results were obtained by detecting CD45.2$^+$ SM1 RAG-2-deficient T cells in CD45.1$^+$ B6.SJL-PtprcaPep3b/BoyJ recipient mice (data not shown). The in vivo clonal expansion of SM1 cells was observed in the draining lymph nodes of recipient mice 3 days after subcutaneous injection of the flagellin peptide 427–441 emulsified in complete Freund's adjuvant (Figure 1H), or in the mesenteric lymph nodes after oral infection with virulent *Salmonella typhimurium*, strain SL1344 (Figure 1I). Activation was antigen specific, since SMI T cells did not undergo clonal expansion in recipient mice infected with *Salmonella* strain BC490 that does not express flagellin structural genes (Figure 1J). Indeed, the frequency of SM1 T cells in the MLN of mice infected with BC490 was lower than that of uninfected recipients due to an increase in the number of other cells within the lymph node that were responding to bacterial stimuli. The weak CD90.2 staining of the CD4 negative population (Figures 1E and 1H–1J) is likely due to nonspecific staining of macrophages, as a similar population is present in untransferred, infected mice (data not shown).

Salmonella Enter the Peyer's Patches and Migrate to Mesenteric Lymph Nodes and Spleen within Twenty-Four Hours of Oral Infection

The course of *Salmonella* infection was followed in mice inoculated orally with 5×10^9 SL1344 organisms, 1 day after transfer of SM1 T cells. Bacteria were first detected 3 hr after infection in the Peyer's patches of the small intestine but had not yet reached the mesenteric lymph nodes or the spleen (Figure 2A). Over the next 3–9 hr, the number of *Salmonella* cultured from the Peyer's patches and mesenteric lymph nodes increased dramatically, whereas the spleen was not infected until 24 hr. The number of bacteria in all of these organs increased further between 24 and 48 hr, whereas peripheral nodes such as the inguinal lymph node did not contain bacteria over this 2 day time period.

Salmonella-Specific CD4 T Cells Are Activated within Three Hours of Oral Infection

By tracking transferred SM1 T cells in the same tissues, it was possible to visualize the kinetics of naive *Salmonella*-specific CD4 T cell activation in vivo. CD69 was used as an early marker of T cell activation because it is expressed on the surface of activated T cells rapidly after TCR ligation (Cochran et al., 2000). In the Peyer's patches, a number of SM1 T cells were activated to express CD69 within 3 hr of infection, and more cells were activated over the next 6–9 hr, reaching a peak 12 hr after infection (Figure 2B). Similar activation of SM1 T cells was noted in the mesenteric lymph nodes, although the kinetics of CD69 induction were delayed by 3–6 hr compared to the Peyer's patches, correlating with the tempo of infection. The expression of CD69 on SM1 cells in the Peyer's patches and mesenteric lymph nodes declined between 24 and 48 hr after infection, just as these cells underwent blastogenesis (Figure 2C). IL-2 production by SM1 T cells in the mesenteric lymph nodes was detected directly ex vivo by intracellular staining. As with CD69 expression, the kinetics of IL-2 production peaked about 12 to 15 hr after oral infection, although only 10% of the SM1 T cells could be detected making IL-2 at any one time (Figure 2D). In addition, at the peak time point for cytokine production (12 hr), a similar percentage of SM1 T cells produced TNF-α, whereas a smaller number was detected in the mesenteric lymph nodes producing IFN-γ or IL-4 (Figure 2E). Similar results were obtained from the Peyer's patches (data not shown). Therefore, as expected for naive CD4 T cells (Dutton et al., 1998), SM1 T cells in the Peyer's patches and mesenteric lymph nodes produce mainly IL-2 and TNF-α and, to a lesser degree, effector cytokines.

Surprisingly, SM1 T cells in the spleen did not express CD69, produce IL-2, or become blasts, despite the presence of bacteria by 24 hr after infection (Figures 2B–2D). Similarly, analysis of splenic SM1 T cells at later time points (72 and 96 hr) after infection did not reveal any significant induction of CD69 or blastogenesis of SM1 T cells (data not shown). In addition, no significant induction of CD69, blastogenesis, or production of IL-2 was detected within the endogenous CD4 T cell population throughout this time course (data not shown). This probably reflects the low frequency of *Salmonella*-specific CD4 T cells in the endogenous T cell repertoire.

Salmonella-Specific CD4 T Cells Undergo Clonal Expansion Exclusively within the Peyer's Patches and Mesenteric Lymph Nodes

The number of SM1 T cells was monitored in different lymphoid organs after oral *Salmonella* infection to track the clonal expansion of *Salmonella*-specific T cells in vivo. Naive SM1 T cells were detected in uninfected recipients at similar levels in all lymphoid organs with the exception of the Peyer's patches, which had a 2- to 3-fold lower ratio of SM1 cells to endogenous CD4 cells than other secondary lymphoid organs (Figure 3A and data not shown). This could be related to the fact that SM1 T cells from the Peyer's patch were not included in the transfer inoculum because SM1 RAG-2-deficient donor mice have very small Peyer's patches.

The percentage of SM1 T cells increased markedly in the Peyer's patches and mesenteric lymph nodes 2 days after infection, reaching a peak by day 3 or 4, after which the response declined (Figures 3B–3E). No clonal expansion of SM1 T cells was observed in the brachial, inguinal, periaortic, or axillary nodes (Figures 3B–3E), whereas the spleen had a small, transient increase in the percentage of SM1 T cells on day 3. Therefore, SM1 T cell clonal expansion after *Salmonella* infection was confined to mucosal lymphoid tissues and did not take place in other secondary lymphoid tissues.

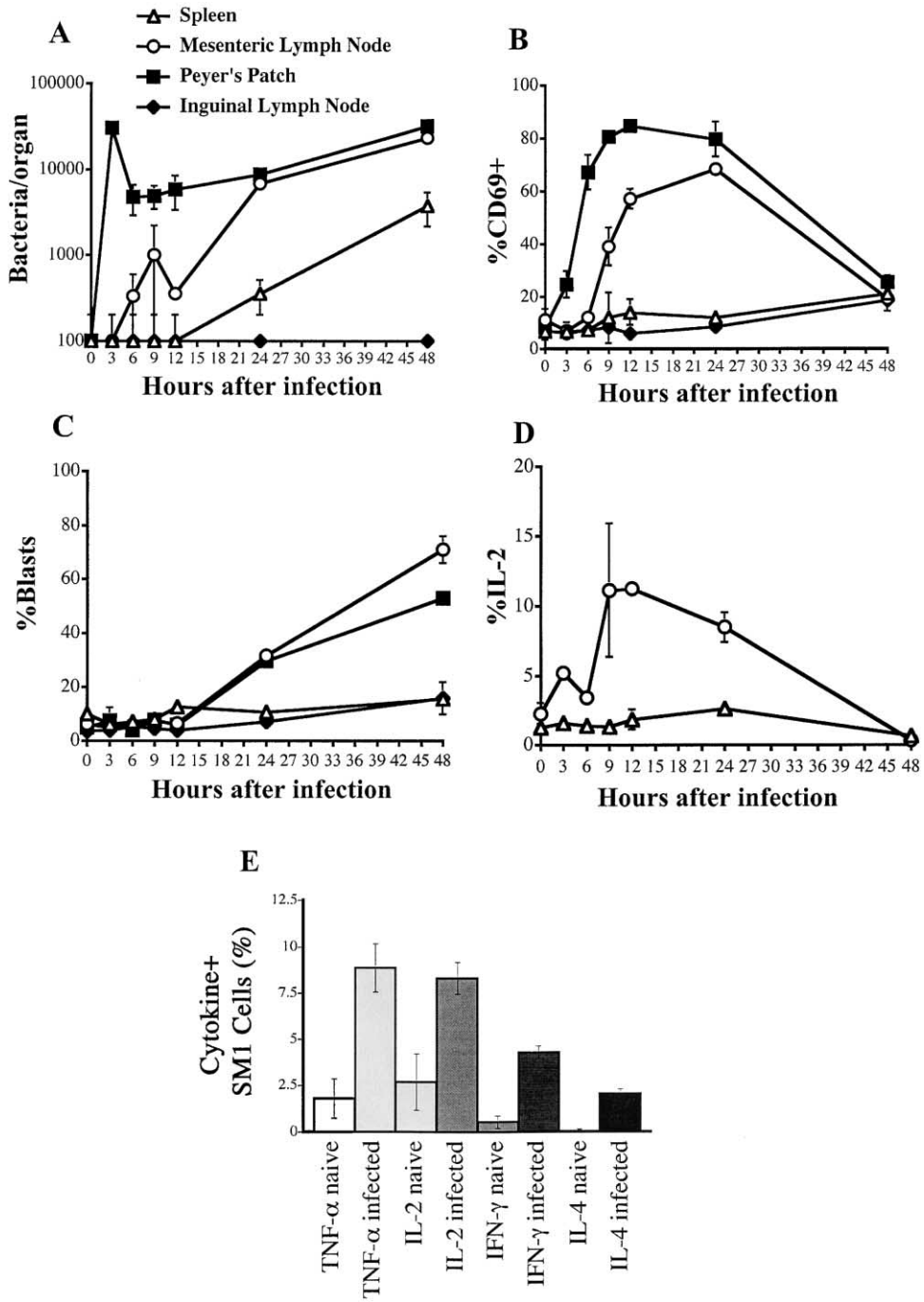

Figure 2. In Vivo CD69 Induction, Blastogenesis, and Lymphokine Production by SM1 T Cells

One day following adoptive transfer, B6.PL-thy1^a-Cy recipients of SM1 RAG-2-deficient T cells were infected orally with 5×10^9 SL1344 organisms, and the number of bacteria and the activation status of SM1 T cells in the Peyer's patches (squares), mesenteric lymph nodes (open circles), spleen (triangles), and inguinal lymph nodes (diamonds) was measured.

(A) Colony counts of viable *Salmonella* organisms.

The percentages (mean ± SD) of SM1 CD4$^+$, CD90.2$^+$ T cells expressing CD69 or blast size or containing intracellular IL-2 are shown in (B)–(D), respectively. The results represent two individual experiments. The percentage of endogenous CD4 T cells (CD4, CD90.2$^-$) from the same mice expressing these activation markers did not change over the course of this experiment (data not shown).

(E) One day following adoptive transfer, B6.PL-thy1^a-Cy recipients of SM1 RAG-2-deficient T cells were infected orally with 5×10^9 SL1344 organisms and sacrificed 12 hr later. The percentages (mean ± SD) of SM1 CD4$^+$, CD90.2$^+$ T cells containing intracellular TNF-α, IL-2, IFN-γ, or IL-4 are shown.

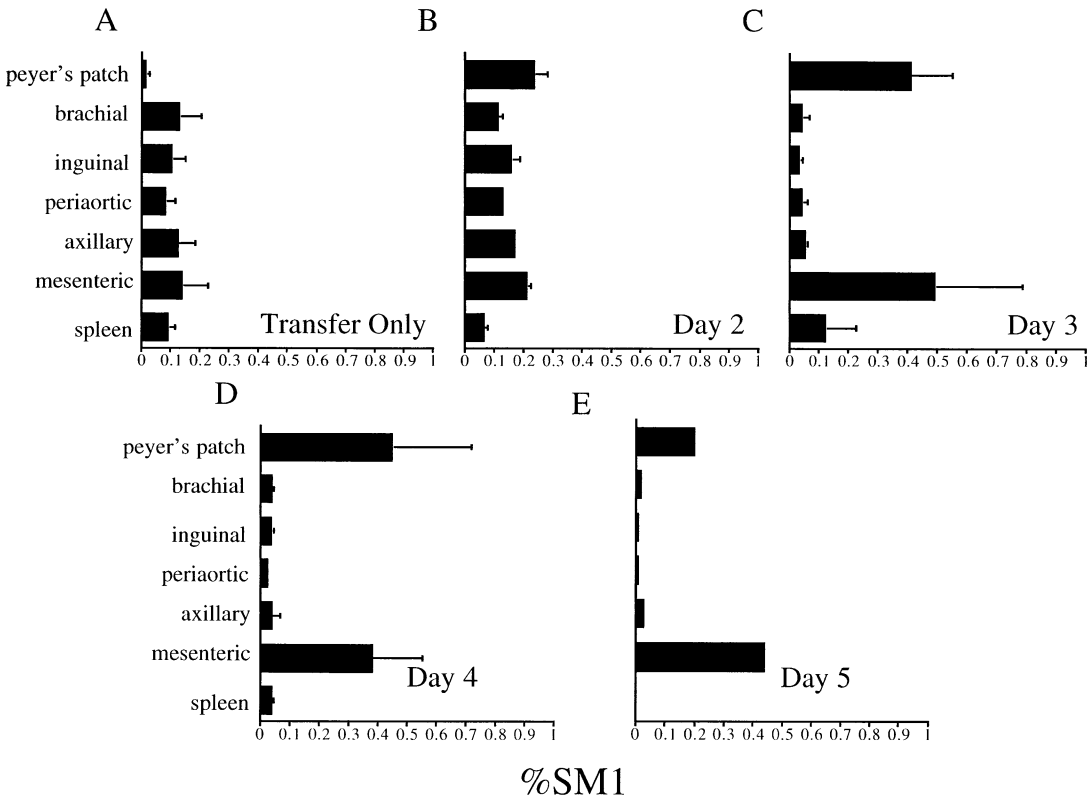

Figure 3. Clonal Expansion by SM1 T Cells in Secondary Lymphoid Organs after Oral Infection

B6.PL-thy1^a-Cy recipients of SM1 RAG-2-deficient T cells were not infected (A), or 1 day after adoptive transfer were infected orally with 5×10^9 SL1344 organisms, and groups of mice were sacrificed 2 (B), 3 (C), 4 (D), or 5 (E) days after infection. Uninfected mice were sacrificed 4 days after adoptive transfer. The percentages (mean ± SD, n = 3) of SM1 T cells in each organ are shown. Similar results were obtained in two other experiments.

Analysis of Cell Division by SM1 T Cells after Oral Infection

The small increase in splenic SM1 T cells 3 days after infection was perplexing because no CD69 upregulation, IL-2 production, or blastogenesis was observed in the spleen over the first 48 hr of infection (Figure 2) or at any other time (data not shown). This small increase could have been caused by proliferation of a small number of SM1 cells in the spleen or by migration of SM1 cells that proliferated in the Peyer's patches and mesenteric lymph nodes. The carboxyfluorescein diacetate succinimidyl ester (CFSE) dilution method (Parish, 1999) was used to distinguish between these possibilities. CFSE-labeled SM1 T cells retained high levels of CFSE after transfer into recipient mice that were not infected, indicating that these cells had not divided (Figure 4). SM1 T cells in the Peyer's patches and mesenteric lymph nodes showed progressive dilution of CFSE beginning on day 2 after infection, consistent with the possibility that the cells were dividing in these organs. In contrast, SM1 cells containing lower levels of CFSE did not appear in the spleen until day 3, and these cells showed evidence of many cell divisions (Figure 4). This, together with the lack of CD69 upregulation and IL-2 production in splenic SM1 T cells at any time, suggests that the small increase in the number of SM1 cells in the spleen is likely due to the migration of cells that proliferated extensively elsewhere, presumably the Peyer's patches and mesenteric lymph nodes.

SM1 T Cells Migrate into the B Cell-Rich Follicles of the Peyer's Patches and Mesenteric Lymph Nodes but Not the Spleen

The location of the SM1 T cells within the secondary lymphoid organs was assessed by immunohistology. After adoptive transfer, naive SM1 T cells were found within the T cell-rich areas of the Peyer's patches, mesenteric lymph nodes, and spleen (Figures 5A, 5D, and 5G). This is the expected location for naive CD4 T cells recirculating through secondary lymphoid organs (Mackay et al., 1996). SM1 T cell expansion 3 days after oral infection was confined primarily to the T cell-rich areas of the Peyer's patches and mesenteric lymph nodes, although some cells were in the edges of the B cell-rich follicles (Figures 5B and 5E). Follicular migration of SM1 T cells increased markedly over the next 24 hr (Figures 5C and 5F). The kinetics of SM1 clonal expansion and follicular migration within mucosal lymphoid tissues in response to *Salmonella* infection was similar to that described for ovalbumin-specific CD4 T cells in the peripheral lymph nodes of mice injected with ovalbumin (Kearney et al., 1994) and to SM1 cells responding to subcutaneous injection of flagellin peptide in complete Freund's adjuvant (data not shown). In contrast, splenic

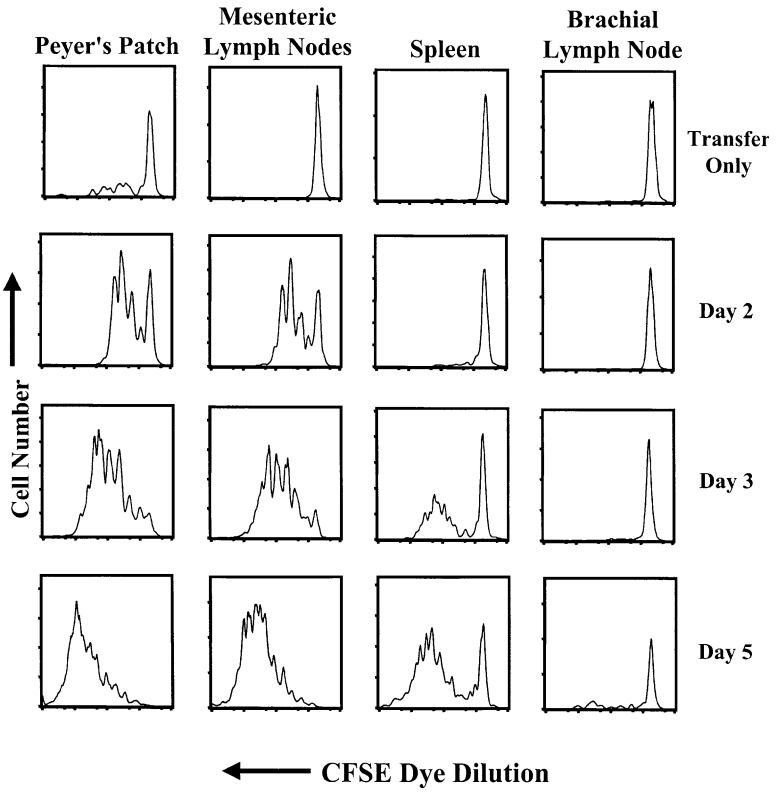

Figure 4. Cell Division History of SM1 T Cells

One day following adoptive transfer, B6.PL-thy1^a-Cy recipients of CFSE-labeled SM1 RAG-2-deficient T cells were infected orally with 5×10^9 SL1344 organisms, and groups of mice were sacrificed at the indicated times. Histograms of the CFSE content in SM1 CD4$^+$, CD90.2$^+$ T cells from the indicated secondary lymphoid organs are shown. Similar results were obtained in a separate experiment.

SM1 T cells remained in the T cell areas at all times after infection (Figures 5H and 5I), further indicating a lack of responsiveness in the spleen.

The Number of SM1 T Cells Contracts Despite Increasing Bacterial Infection

Adoptive transfer experiments using model antigens demonstrated that after the peak of clonal expansion, the number of antigen-specific CD4 T cells falls in secondary lymphoid organs and rises in nonlymphoid tissue (Kearney et al., 1994; Reinhardt et al., 2001). To observe these processes during *Salmonella* infection, the number of SM1 T cells in both lymphoid and nonlymphoid tissue after infection of recipient mice was analyzed by flow cytometry. As shown in Figures 6A and 6B, the absolute number of SM1 T cells in the Peyer's patches and mesenteric lymph nodes decreased between days 3 and 4 after infection as the number of viable bacteria in these organs increased markedly. Therefore, the reduction in the number of *Salmonella*-specific CD4 T cells cannot be explained by a loss of antigen from these sites. As described above, only a small increase in the absolute number of splenic SM1 T cells was noted despite an increasing bacterial burden at this site (Figure 6C) and the ability of antigen-presenting cells (APC) isolated from the spleens of infected mice to stimulate a flagellin-specific T cell clone in vitro (Figure 6D).

Migration of SM1 T Cells to Nonlymphoid Organs

It is well known that oral *Salmonella* infection spreads from the mucosal lymphoid tissues to the liver (Jones and Falkow, 1996; Schaible et al., 1999). Indeed, in our experiments, *Salmonella* was detected in the liver after oral infection (data not shown) with kinetics similar to those shown for the spleen (Figure 6C). It was therefore of interest to determine whether the loss of SM1 T cells from the secondary lymphoid organs correlated with the migration of SM1 cells to the liver. As expected, naive SM1 T cells were detected in the mesenteric lymph nodes of uninfected recipient mice, but very few were found in the liver or lamina propria of the small intestine (Figure 6E). Intravenous injection of flagellin peptide 427–441 with lipopolysaccharide resulted in both the expansion of SM1 T cells in secondary lymphoid organs and the migration of these cells to the liver and lamina propria (Figure 6E). Although oral *Salmonella* infection resulted in the expansion of SM1 T cells in the mesenteric lymph nodes, very few SM1 cells could be detected in the liver or lamina propria 3 or 5 days after infection (Figure 6E).

Because the extraction of T cells from nonlymphoid tissues is likely to be inefficient, it was possible that small numbers of SM1 T cells migrated to nonlymphoid organs, but these fell below the limit of detection of flow cytometry. It was also possible that SM1 T cells were migrating to nonlymphoid organs other than the small intestine and liver. We therefore used whole body immunohistology to track SM1 cells in mice that received four times as many SM1 cells as mice in previous experiments to further increase sensitivity of detection.

Only a few background events were detected on a whole body section through a mouse that did not receive SM1 T cells (Figure 7A). Naive SM1 T cells were detected at greater than background levels only in the secondary lymphoid tissues that were present on a midline section (spleen and Peyer's patches, Figures 7B and 7F) through

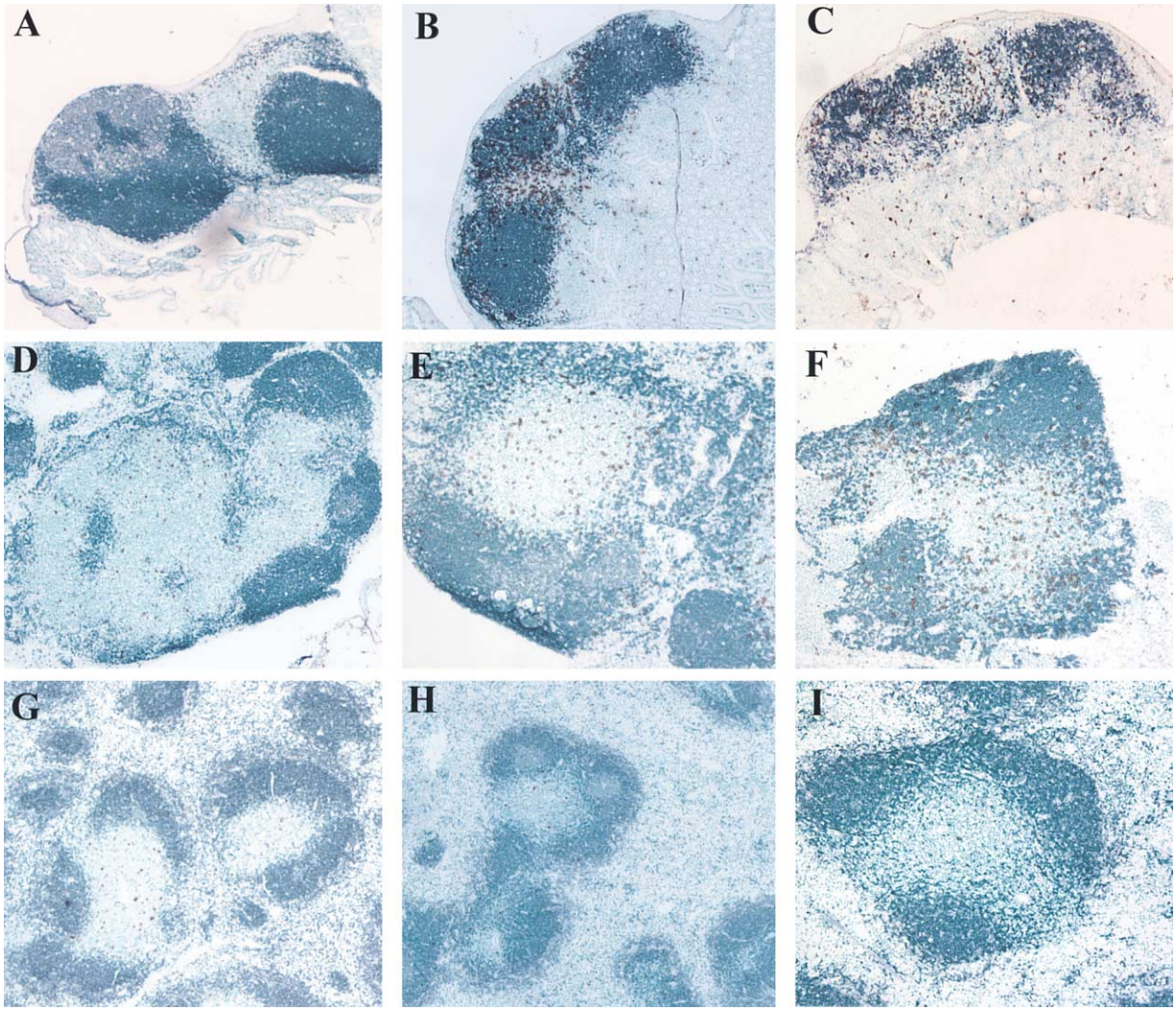

Figure 5. In Situ Detection of SM1 T Cells in Secondary Lymphoid Organs

One day following adoptive transfer, B6.PL-thy1^{a}-Cy recipients of SM1 RAG-2-deficient T cells were infected orally with 5×10^{9} SL1344 organisms, and groups of mice were sacrificed at various times. Frozen tissue sections from secondary lymphoid organs were stained with anti-CD90.2 (detected with a brown chromagen) and anti-B220 (detected with a blue chromagen) antibodies. Peyer's patches are shown in (A)–(C), mesenteric lymph nodes in (D)–(F), and spleens in (G)–(I) from uninfected mice (A, D, G), or mice infected 3 (B, E, H) or 4 (C, F, I) days earlier. The sections shown are representative of many others from two separate experiments. The blue staining can be used to identify the location of the B cell-rich follicles.

an uninfected mouse that received SM1 cells. Whole body sections from mice orally infected with *Salmonella* 3 or 5 days previously demonstrated expansion of SM1 T cells in the Peyer's patches (Figures 7C, 7D, 7G, and 7H) but not the spleen as observed by flow cytometry (Figure 3). However, using whole body immunohistology, a small increase in SM1 T cells was noted in the lungs and liver 3 and 5 days after infection (Figures 7C and 7D). No other nonlymphoid organs contained SM1 T cells at either day 3 or 5 after infection, including the lamina propria of the small intestine. Notably, the amount of SM1 migration into the lungs and liver after oral Salmonella infection was much less than that which occurred in mice injected intravenously with flagellin peptide 427–441 and lipopolysaccharide (Figure 7E). Therefore, SM1 T cells are capable of redistributing to the lungs and liver after oral *Salmonella* infection but do so inefficiently.

Discussion

We report here the development of a new adoptive transfer system that allows the direct visualization of *Salmonella*-specific CD4 T cells in vivo. A number of novel findings concerning immune responses to mucosal pathogens arise from this study.

First, the response of *Salmonella*-specific CD4 T cells in the Peyer's patches is very rapid. The induction of CD69 on *Salmonella*-specific CD4 T cells within 3 hr of oral infection indicates that *Salmonella* peptide-MHC complexes are generated within this short time period. This places constraints on the processes of *Salmonella*

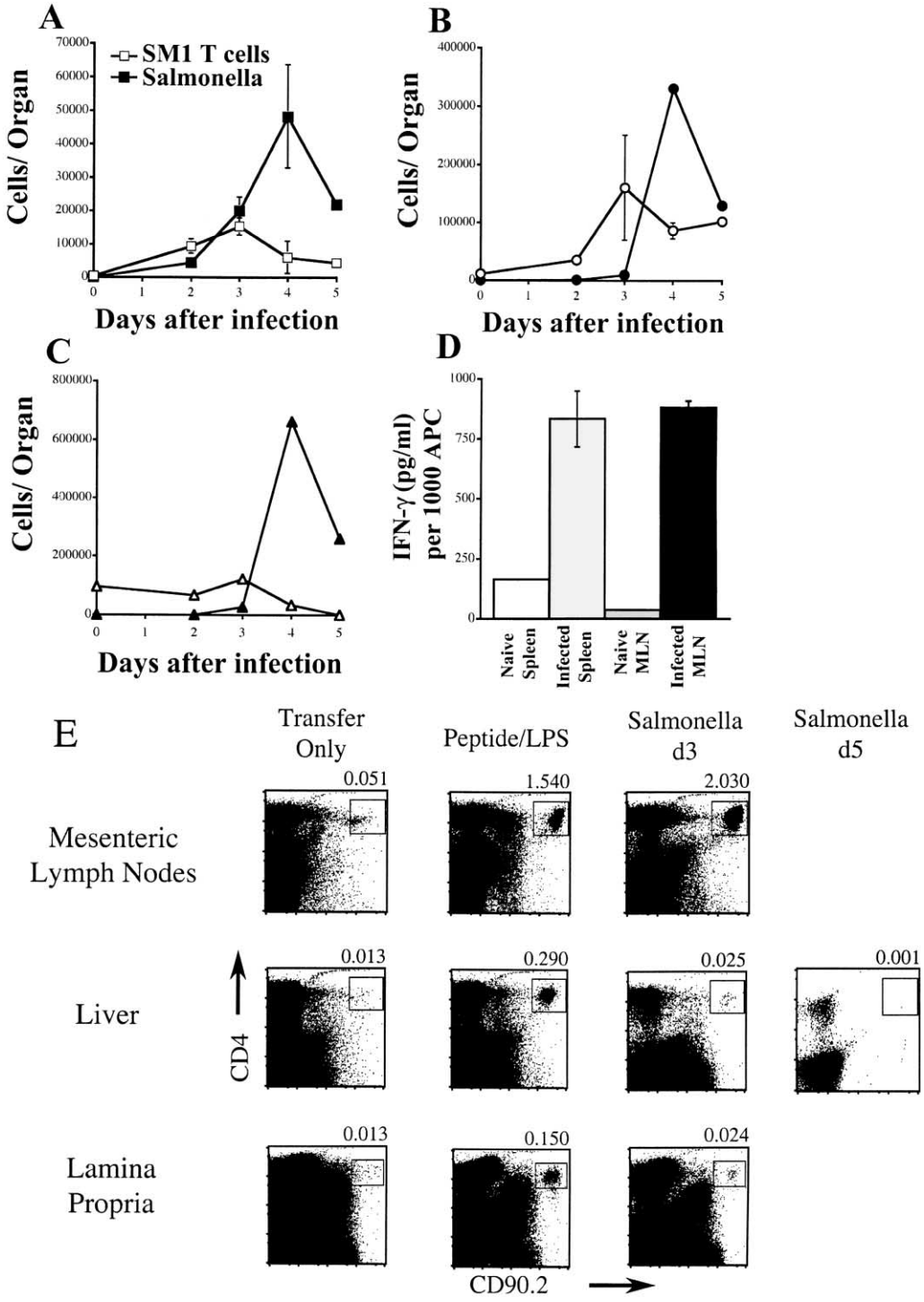

Figure 6. Clonal Contraction and Nonlymphoid Tissue Distribution of SM1 T Cells after Infection

One day following adoptive transfer, B6.PL-thy1^a-Cy recipients of SM1 RAG-2-deficient T cells were infected orally with 5×10^9 SL1344 organisms, and groups of mice were sacrificed at the indicated times. Single-cell suspensions of lymphoid and nonlymphoid tissues were prepared as described in Experimental Procedures. The absolute number of SM1 T cells was calculated by multiplying the cell counts for individual organs by the percentage of SM1 CD4$^+$, CD90.2$^+$ T cells detected by flow cytometry in the same organ. Total numbers of SM1 T cells and viable *Salmonella* recovered from Peyer's patches (A), mesenteric lymph nodes (B), and spleens (C) are shown. Each point represents mean values ± SD for three individual mice and is representative of three separate experiments.

(D) Low-density APC were prepared from the spleens and mesenteric lymph nodes of uninfected mice or mice infected orally with SL1344 organisms 4 days earlier, and cultured with the flagellin-specific T cell clone, CN.B1. IFN-γ (mean pg/ml per 1000 APC ± SD) in the culture supernatants at 48 hr is shown.

(E) Two-color dot plots of cells from the indicated organs from uninfected recipients of SM1 RAG-2-deficient T cells 4 days following adoptive transfer (transfer only), recipients of SM1 RAG-2-deficient T cells after adoptive transfer and 3 days after intravenous injection with flagellin peptide and LPS, or recipients of SM1 RAG-2-deficient T cells after adoptive transfer and 3 or 5 days after oral infection with 5×10^9 SL1344 organisms. Mice were infected or immunized 1 day following adoptive transfer.

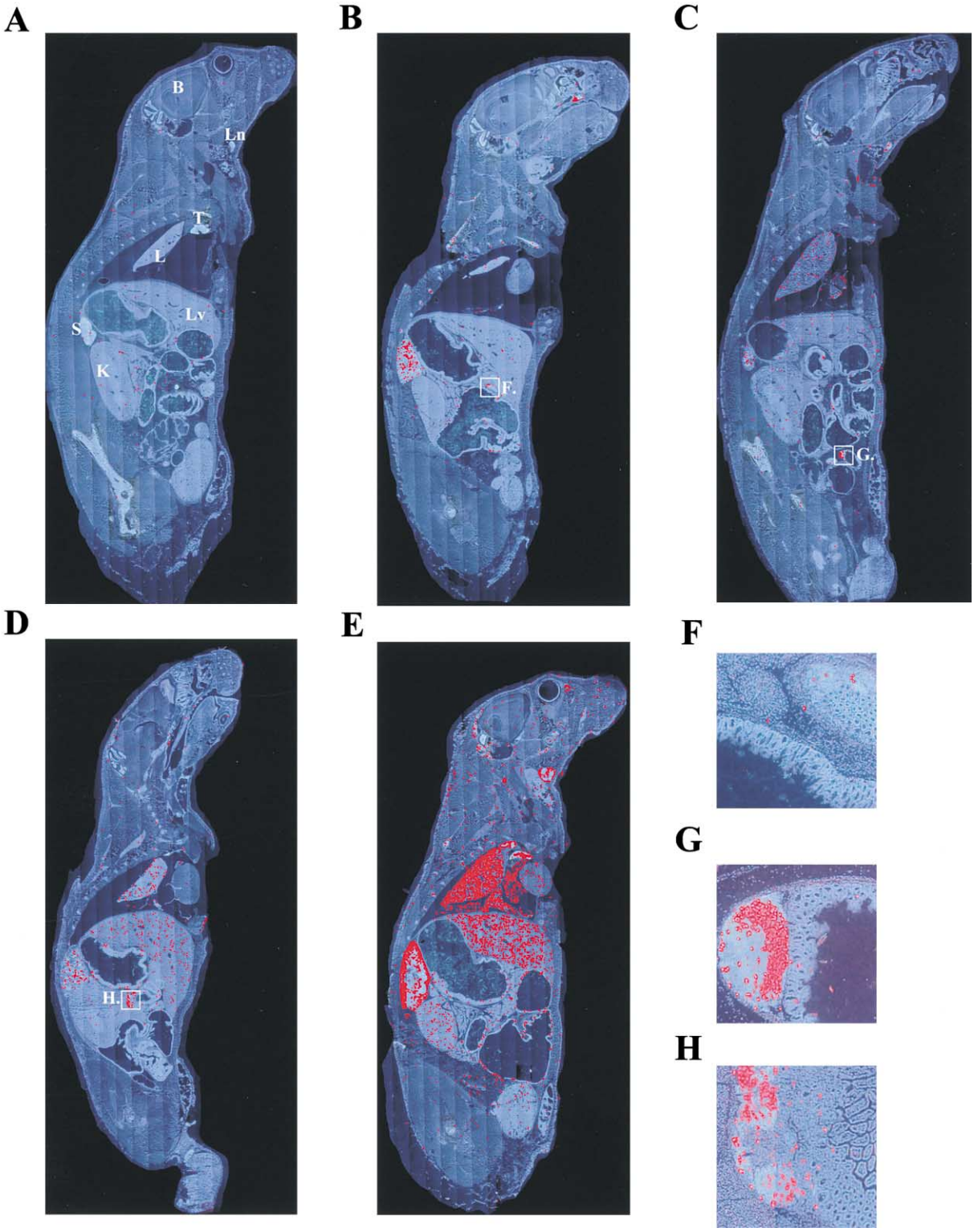

Figure 7. Tracking SM1 T Cells by Whole Body Immunohistology

Images from DAPI (blue)- and anti-CD45.2 (red)-stained sections through the following mice are shown: an untransferred B6.SJL-PtprcaPep3b/BoyJ mouse (A), an uninfected B6.SJL-PtprcaPep3b/BoyJ recipient of SM1 RAG-2-deficient T cells, 4 days after adoptive transfer (B), a B6.SJL-PtprcaPep3b/BoyJ mouse 4 days after receiving SM1 RAG-2-deficient T cells and 3 days after oral infection with 5×10^9 SL1344 organisms (C), a B6.SJL-PtprcaPep3b/BoyJ mouse 6 days after receiving SM1 RAG-2-deficient T cells and 5 days after oral infection with 5×10^9 SL1344 organisms (D), or a B6.SJL-PtprcaPep3b/BoyJ mouse 4 days after receiving SM1 RAG-2-deficient T cells and 3 days after intravenous injection of 200 μg of flagellin peptide and LPS (E). Higher power images of Peyer's patches from the whole mouse sections (B)–(D) are shown in (F)–(H), respectively. In (A), the letters refer to the following organs: B, brain; LN, lymph node; T, thymus; L, lung; Lv, liver; S, spleen; K, kidney.

migration from the stomach to the Peyer's patches and entry into the body, as well as antigen acquisition, processing, and presentation by APC. An APC thought to be involved in this process is the myeloid dendritic cell found in the subepithelial dome of Peyer's patches. This cell is thought to acquire antigen from the basolateral surface of the M cell and migrate to the T cell area where it can engage a naive T cell (Iwasaki and Kelsall, 1999, 2000). However, the rapid T cell activation reported here suggests that capture of soluble flagellin by dendritic cells within the T cell area itself, followed by rapid presentation to nearby T cells, may also be involved. Such a mechanism of antigen presentation may be facilitated by the fact that flagellin is an abundant surface protein that is also secreted from the bacteria (Gewirtz et al., 2001). The intrinsic proinflammatory properties of flagellin after ligation of TLR-5 (Eaves-Pyles et al., 2001; Gewirtz et al., 2001; Hayashi et al., 2001) may serve to both increase migration of dendritic cells from the dome and enhance the uptake of soluble flagellin by dendritic cells already in the T cell areas. In either case, the fact that the CD4 T cell response begins in the Peyer's patches and not the mesenteric lymph nodes argues against the suggestion that the major mechanism of antigen presentation involves APC that migrate from the lamina propria via afferent lymph (Maric et al., 1996; Rescigno et al., 2001) after directly sampling lumenal contents.

Second, we noted that the kinetics of SM1 T cell expansion and contraction in the Peyer's patches and mesenteric lymph nodes are unrelated to the bacterial load within these organs. Indeed, the activation of SM1 T cells in response to *Salmonella* infection showed very similar kinetics to that noted after subcutaneous injection of flagellin peptide 427–441 (S.J.M., unpublished data). Therefore, as noted for CD8 T cells (Kaech and Ahmed, 2001; Mercado et al., 2000), CD4 T cells appear to undergo a stereotypic early activation program that is independent of antigen load.

Third, these experiments suggest that the capacity of *Salmonella*-specific T cells to respond is restricted by their anatomic location. The absence of splenic SM1 T cell activation was striking, considering that bacteria colonize this organ and SM1 T cells within the same host respond in the Peyer's patches and mesenteric lymph nodes. These data contrast with recent experiments indicating that the spleen is a major anatomical site of CD8 T cell activation following oral infection with Listeria (Huleatt et al., 2001; Pope et al., 2001). The lack of splenic responsiveness in our study is not likely to be due to an absence of antigen presentation in the spleen, because APC from the spleen and mesenteric lymph nodes of infected mice activated a flagellin-specific T cell clone in vitro. Our current hypothesis is that the APC and SM1 T cells are physically separated from one another in the spleen, thus creating an anatomical barrier to T cell activation. Indeed, preliminary studies show that *Salmonella* organisms in the spleen are in the red pulp, whereas naive SM1 T cells are in the white pulp during the first few days of infection (S.J.M., unpublished data).

Finally, our data indicate that *Salmonella*-specific CD4 T cells migrate inefficiently to the liver and not at all to the lamina propria of the small intestine after oral *Salmonella* infection. The failure of SM1 T cells to migrate into the lamina propria is in contrast to a recent study by Vezys et al. (2000), which showed that CD8 T cells specific for an antigen expressed in the small intestine expanded in the mucosal lymphoid tissues and then migrated into the lamina propria. This difference could be explained by the fact that antigen-specific T cells may migrate preferentially to sites of antigen deposition. The antigen in the Vezys study was produced in the lamina propria, whereas the antigen in our study was not because *Salmonella* does not colonize this tissue.

Lack of antigen, however, can not explain the minimal migration of SM1 T cells to the liver, since the liver is the major nonlymphoid organ infected by *Salmonella*. The minimal migration of SM1 T cells to this site could be related to a defect in T cell migration brought about by the *Salmonella* infection, or it could be a secondary effect of a small seeding pool of activated T cells perpetuated by the failure of SM1 expansion in the spleen. In either case, the net result of minimal movement of *Salmonella*-specific CD4 T cells to the major nonlymphoid site of bacterial replication could contribute to the lethal outcome of this infection.

Experimental Procedures

Mice

Female C57BL/6 (H-2^b) were purchased from the National Cancer Institute (Frederick, MD) and used at 8 to 16 weeks of age. Male and female B6.PL-thy1^a-Cy (CD90.1 congenic) and B6.SJL-PtprcaPep3b/BoyJ (CD45.1 congenic) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Female C57BL/6 RAG-2-deficient mice were purchased from Taconic (Germantown, NY) and bred under specific pathogen-free conditions in our facility.

Generation of SM1 TCR Transgenic Mice

T cell clone CN.B1, specific for flagellin peptide (427–441)-I-A^b, expresses a TCR composed of Vα10Jα10 and Vβ2Dβ1Jβ1.2 (McSorley et al., 2000). Flanking primers were designed to amplify the rearranged receptor sequences from this clone based on genomic sequences from these TCR loci (GenBank accession numbers AE000663, AE000665, AE005402, and M64239). The primers used were as follows: Vα10 5′-TTCTGTCCCGGGCAGCCACACAAGCAC CATGAAGAGGCTGCT-3′; Jα10 5′-AGCAGCGGCCGCACAAATCT CAGAGGACTCTCCCTCATTCCTT-3′; Vβ2 5′-GAGAGAACTCGAGG TCTCAGAGATGTGGCA-3′; and Jβ1.2 5′-GCATCCTTCCGCGGAT TACCAGAACAGTA-3′.

PCR products were sequenced before insertion into the TCR vectors pTα and pTβ at the recommended restriction sites (Kouskoff et al., 1995). Prokaryotic DNA sequences were then removed from both vectors prior to injection into fertilized B6 eggs. Subsequent PCR screening of genomic DNA from offspring resulted in the identification of a founder TCR transgenic line, termed SM1 (Figure 1A). SM1 mice were backcrossed to RAG-2-deficient C57BL/6 mice to obtain SM1 RAG-2-deficient offspring that contained SM1 CD4 T cells and no other lymphocytes.

Stimulation of SM1 T Cells In Vitro

Lymph node cells from SM1 RAG-2-deficient mice or nontransgenic littermates were incubated in vitro (1×10^6/well in 96-well plates) with either flagellin peptide 427–441, purified flagellin protein, or heat-killed *Salmonella typhimurium*. IL-2 was measured in culture supernatants 48–72 hr after stimulation by sandwich ELISA.

Bacterial Strains, Infection, Immunization, and Antigen Preparation

Mice were infected with a virulent strain of *Salmonella*, SL1344, as previously described (McSorley et al., 1997). Bacteria were grown overnight in LB broth without shaking and diluted in PBS after esti-

mation of bacterial concentration using a spectrophotometer. Before oral infection by gavage, mice were given 0.1 ml of 5% sodium bicarbonate solution to neutralize stomach acids. The effective dose of bacteria administered to mice was verified by plating bacteria on MacConkey agar plates and incubating overnight at 37°C. In all experiments, the estimated bacterial concentration differed from the actual concentration by less than 3-fold (data not shown). Heat-killed *Salmonella* was prepared from an overnight culture of SL1344 incubated at 65°C for 1 hr. Bacterial strains were periodically assessed for motility in soft agar to confirm flagellin expression prior to infection, and bacteria were also checked for motility after harvest from infected mice. Flagellin was prepared from phase-fixed bacterial strains BC115 or BC116 (McSorley et al., 2000) using both shearing and differential centrifugation methods, as previously described (Kanto et al., 1991; Stocker and Campbell, 1959). Flagellin was further isolated using a G-200 sephadex column, collecting fractions that contained a single band of the relevant molecular weight assessed by Coomassie-stained SDS-PAGE gels (data not shown). These fractions were concentrated using centricon concentrators (Amicon, Beverly, MA) and pooled before being used in stimulation assays. In some experiments, mice were given a subcutaneous injection in the footpad with 100 μg of flagellin peptide (427–441) emulsified in Complete Freunds Adjuvant or an intravenous injection with 200 μg of flagellin peptide (427–441) and 25 μg of lipopolysaccaride.

Flow Cytometry and Antibodies

Freshly isolated spleen or lymph node cells were incubated for 15–45 min in Fc blocking solution (spent culture supernatant from the 24G2 hybridoma containing anti-Fc receptor antibody, 2% rat serum, 2% mouse serum, and 0.01% sodium azide) together with the relevant primary antibodies. Fluoroscein isothiocyanate (FITC), phycoerythrin (PE), CyChrome, or biotin-labeled antibodies specific for CD4, CD90.1, CD90.2, CD45.1, CD45.2, Vβ2, B220, CD8, CD25, CD40L, CD45RB, CD62L, CD69, CTLA-4, IL-2, TNF-α, IFN-γ, and IL-4 were purchased from PharMingen (San Diego, CA). Streptavidin-PerCp or streptavidin-PE (PharMingen) were used to detect biotin-labeled antibodies. After staining, cells were washed and analyzed using a FACscan flow cytometer (Becton-Dickinson, Mountain View, CA) and FlowJo software (TreeStar, San Carlos, CA). Before intracellular cytokine staining, cells were fixed in 2% formaldehyde after surface staining and permeablized by washing in 0.5% saponin (Sigma, St. Louis, MO) as described (Khoruts et al., 1998).

Adoptive Transfer of SM1 RAG-2-Deficient CD4 T Cells

Spleen, mesenteric, and other peripheral lymph nodes were harvested from SM1 RAG-2-deficient TCR transgenic mice and in some experiments stained with the dye CFSE (Parish, 1999). An aliquot of these cells was stained using antibodies specific for CD4 and Vβ2 and analyzed using a FACScan flow cytometer (Becton-Dickinson) to determine the frequency of SM1 cells. Recipient B6.PL-thy1^a-Cy mice were injected intravenously with 1–3 × 10^6 SM1 RAG-2-deficient CD4 T cells. For whole body histology experiments, B6.SJL-PtprcaPep3b/BoyJ were injected with 8 × 10^6 SM1 RAG-2-deficient CD4 T cells. Mice were always immunized or infected 1 day following adoptive transfer.

Tracking SM1 T Cells and Viable *Salmonella* in Secondary Lymphoid Organs

At various times after infection, a single-cell suspension of spleen, lymph nodes, and Peyer's patch cells was prepared in Eagle's Hanks Amino Acids Medium (EHAA) (Biofluids, Rockville, MD) containing 2% fetal calf serum and 5 mM EDTA. Samples were plated onto MacConkey agar plates (Difco, Detroit, MI) and incubated overnight at 37°C to assess bacterial counts. Cells from each organ (5 × 10^6/tube) were then stained as described above and analyzed by flow cytometry.

Detection of SM1 T Cells in Histological Sections

Organs were embedded in O.C.T. (Sakura Finetek, Torrance, CA) and frozen in liquid nitrogen at various times after infection. Sections (6–8 μm) from these organs were cut and dehydrated in acetone and stored at −80°C until needed. After thawing, sections were treated with 3% H_2O_2, Fc blocking solution, and an avidin/biotin blocking kit (Vector Laboratories, Burlingame, CA) before incubation with biotin-labeled antibodies specific for CD90.2 or B220 (PharMingen). Sections were then incubated with Vectastain ABC Elite (Vector Laboratories), developed with DAB or Vector SG (Vector Laboratories), stained with 5% methyl green, and mounted.

Isolation of APC from Infected Mice and In Vitro Stimulation of a Flagellin-Specific T Cell Clone

APC from spleens and mesenteric lymph nodes were isolated as previously described (Ingulli et al., 1997). In brief, organs were subjected to mild collagenase digestion at 37°C for 25 min. Low-density cells were selected by centrifugation on a 35% bovine serum albumin gradient (Sigma) and cultured at various numbers in 96-well round-bottom plates with a flagellin-specific T cell clone (CN.B1, 5 × 10^4 per well) (McSorley et al., 2000). Supernatants were harvested 48 hr later and the presence of IFN-γ detected by sandwich ELISA (PharMingen).

Releasing CD4 T Cells from Nonlymphoid Organs

Recipient mice were sacrificed and perfused with PBS, and liver and intestine were harvested in EHAA. Livers were dissociated in the presence of Collagenase VIII (Boehringer Mannheim, Indianapolis, IN) and Dnase (Sigma) and incubated at 37°C for 20 min before washing in EHAA. The resulting preparation was centrifuged on a Percoll gradient, the interface collected and washed before staining of cells as described above. Lamina propria cells were isolated as previously described (Pope et al., 2001). In brief, the small intestine was dissected free of fat and Peyer's patch tissue, washed in CMF/HEPES medium, and digested with collagenase D and VIII with Dnase. Undigested tissue was digested again with collagenase D and VIII with Dnase, and a cell suspension from these digests was isolated using a Percoll gradient.

Whole Mouse Immunohistology

Whole body sections through B6.SJL-PtprcaPep3b/BoyJ recipients of SM1 RAG-2-deficient T cells were cut and stained as previously described (Reinhardt et al., 2001). Mice were sacrificed, perfused, embedded in O.C.T., and frozen in liquid nitrogen before 10 μM whole body sections near the midline were prepared using an LKB 2250 cryomicrotome. Sections were dehydrated in acetone for 10 min and fixed in 1% formaldehyde for 15 min. Endogenous peroxidase activity was eliminated by incubating the sections in 1% H_20_2/0.1% sodium azide for 1 hr. Fc binding sites and endogenous sources of biotin were blocked with 1% mouse serum, 1% rat serum, and anti-FcR monoclonal antibody (24G2) followed by an avidin/biotin blocking step (Vector Laboratories) before incubation with biotin-labeled anti-CD45.2 antibody for 30 min. The sections were then incubated sequentially with streptavidin-peroxidase and biotinyl tyramide from the TSA-Biotin kit (NEN Life Science Products, Boston, MA) according to the manufacturer's instructions. The deposited biotin was detected with streptavidin-Cy3 (Caltag, Burlingame, CA), and the sections were counterstained with a 10 μg/ml solution of DAPI (Boehringer-Mannheim) in PBS. Each section was placed on a 7.3 × 10.2 cm glass plate, bathed in mounting medium (Vector Laboratories), and covered with a coverslip. Two sets of images covering the entire surface of each section were acquired with a CCD camera attached to an Olympus fluorescence microscope equipped with an automated stage driven by Metamorph software (Universal Imaging Corporation, Downingtown, PA), one set in the DAPI channel and another in the Cy3 channel. Photoshop 5.5 software (Adobe Systems Incorporated, San Jose, CA) was used to assemble a total of approximately 600 DAPI and 600 Cy3 images into a single image for each section. This was accomplished by first producing 16–18 rows, each comprised of 30–37 adjacent images, which were then stacked sequentially to form the final image. The red objects on the assembled Cy3 image were enlarged 5-fold so that individual T cells could be seen at the resulting low magnification. The DAPI and Cy3 images were then overlaid to give the final dual-color composite images.

Acknowledgments

We thank Jennifer Walter for expert technical assistance, Drs. Brad Cookson and Damo Xu for providing bacterial strains, Micki Dyer

and Sandra Horne from the University of Minnesota Cancer Center ES Cell/SPF Animal Facility for production of transgenic mice, and Gerald Sedgewick for image analysis expertise. This work was supported by a fellowship from the Irvington Institute for Immunological Research (to S.J.M.) and by grants from the National Institutes of Health (AI27998, AI35296, and AI39614 to M.K.J.).

Received August 16, 2001; revised January 8, 2002.

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