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Low-Dose *Salmonella* Infection Evades Activation of Flagellin-Specific CD4 T Cells

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Many pathogens can establish a lethal infection from relatively small inocula, yet the effect of infectious dose upon CD4 T cell activation is not clearly understood. This issue was examined by tracking *Salmonella* flagellin-specific SM1 T cells in vivo, after i.v. and oral challenge of mice with virulent *Salmonella typhimurium*. SM1 T cells rapidly expressed activation markers and expanded in response to high-dose infection but remained completely unresponsive in mice challenged with low doses of *Salmonella*. SM1 T cells, in these mice, remained unresponsive, despite massive bacterial replication in vivo. Naïve SM1 T cells in low-dose *Salmonella*-infected mice were activated rapidly after the injection of flagellin peptide, demonstrating that these T cells were fully capable of responding, ruling out the possibility of a bacterial-induced suppressive environment. The inability of flagellin-specific SM1 T cells to respond to low-dose infection was not due to Ag down-regulation, because flagellin expression was detected using a functional assay. Together, these data suggest that low-dose *Salmonella* infection can evade flagellin-specific CD4 T cell activation in vivo. *The Journal of Immunology*, 2004, 173: 4091–4099.

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nimal models of infectious disease have advanced our understanding of the pathology, genetic susceptibility, and immune response to many important human pathogens. However, by definition, the infection of animals under controlled laboratory conditions has the potential to introduce novel experimental variables that are not actually found in a natural outbreak of disease. Some important physiological variables to consider in this light are the natural route of infection, the dose of pathogen in a natural exposure, the contribution of environmental or vector-derived products to the disease, and the likely health status of the host population. Each of these has the potential to influence pathogen replication, host immune responses, or both.

Infection of mice with *Salmonella typhimurium* is the best available model to study typhoid fever, an important disease in many developing countries (1–3). This model typically involves the oral, i.v., or i.p. administration of large numbers of cultured bacteria into susceptible or resistant mouse strains (3). Intravenous infection with as few as 1–10 (or oral infection with 10⁸) virulent *Salmonella* is fatal in susceptible mouse strains (3, 4). However, when studying the immune response to *Salmonella* infection, the experimental doses used are often several orders of magnitude higher (5–8). It is unclear whether such differences in challenge dose can influence the immune response to *Salmonella*.

CD4 T cells and B cells are both important for the resolution of *Salmonella* infection (7, 9–11). Recent studies have identified *Salmonella* flagellin as a major target of *Salmonella*-specific CD4 T cells (12, 13). Furthermore, we recently reported the rapid activation of flagellin-specific TCR transgenic T cells in mucosal lymphoid tissues following oral infection with virulent *Salmonella* (8). However, it remains unclear whether, or how, bacterial challenge dose influences the activation of these *Salmonella*-specific CD4 T cells.

Recent understanding of CD8 T cell activation during infection suggests that the clonal expansion and contraction of a pathogen-specific T cell population is determined very early during infection (14–16). Therefore, one might predict that variation in the initial bacterial dose (Ag concentration) would have a profound effect upon subsequent T cell activation. Surprisingly, this appears not to be the case. Studies, using a murine model of *Listeria* infection, reported that a 25-fold dilution in bacterial challenge dose did not significantly alter the size or kinetics of the *Listeria* LL03199-specific CD8 T cell response (17).

In this study, we demonstrate that variation in the initial dose of infection with *Salmonella* has a profound effect on the response of *Salmonella* flagellin-specific CD4 T cells in vivo, and that low-dose infection can evade *Salmonella* flagellin-specific T cell activation completely.

### Materials and Methods

#### Mouse strains

SM1 RAG-deficient TCR transgenic mice expressing the CD90.1 or CD45.1 allele were produced by two backcrosses of the original C57BL/6 SM1 RAG-deficient line (8) to B6.PL-thy1a/Cy or B6.SIL-PpRcaPep3b/BoyJ mouse strains (The Jackson Laboratory, Bar Harbor, ME). C57BL/6 mice were purchased from the National Cancer Institute (Frederick, MD) and used at 8–16 wk of age. All mice were housed in specific pathogen-free conditions and cared for in accordance with University of Connecticut Health Center and National Institutes of Health guidelines.

**Adoptive transfer of SM1 T cells**

Spleen and lymph node cells (inguinal, axillary, brachial, cervical, mesenteric, and periaortic) were harvested from SM1 RAG-deficient, CD90.1 congenic (or CD45.1 congenic), TCR transgenic mice, and a single-cell suspension was generated. An aliquot of cells was stained using Abs to CD4, CD90.1 (or CD45.1), and Vβ2 (BD Pharmingen, San Diego, CA; eBioscience, San Diego, CA), and the percentage of SM1 cells was determined by flow cytometry using a FACSCalibur (BD Biosciences). SM1 T cells were defined as CD4⁺ Vβ2⁺, and these cells were the only lymphocytes present in harvested tissues from TCR transgenic mice. The total number of SM1 T cells was calculated, and the concentration was adjusted so that 2.5 × 10⁶ SM1 T cells were injected i.v. into recipient C57BL/6
Salmonella infection

Salmonella enterica serovar Typhimurium (SL1344) or the isogenic flagellin-deficient strain (BC490) were grown overnight in Luria-Bertani broth without shaking and diluted in PBS after an estimation of bacterial concentration using a spectrophotometer. BC490 was a generous gift from Dr. B. Cookson (University of Washington, Seattle, WA). Oral infections were conducted as previously described (19). Immediately before administration of bacteria by gavage, mice were given 0.1 ml of a 5% sodium bicarbonate solution to neutralize the pH of the stomach. For i.v. infections, bacteria were injected into the lateral tail vein. In all infection experiments, the dose of bacteria administered was confirmed by plating serial dilutions on MacConkey agar plates.

Flagellin immunization and in vivo cytokine stimulation

For peptide immunization and in vivo cytokine experiments, mice were injected i.v. with 200 μg of flagellin peptide 427–441 (VQNRFNFA/TL-NLGN(T) (13). To examine cytokine production directly ex vivo, spleens from injected mice were harvested at 6 h following peptide injection. These cells were immediately surface stained, fixed with formaldehyde, permeabilized using saponin (Sigma-Aldrich, St. Louis, MO), and stained intracellularly. Cytokine production therefore occurred in vivo and involved no in vitro stimulation of the cells, as previously described (20).

Flow cytometric analysis

Spleen or lymph node cells were incubated on ice for 20–45 min in Fc block (spent culture supernatant of the 24G2 hybridoma, 2% rat serum, 2% mouse serum, and 0.01% sodium azide) in the presence of the relevant primary Abs. FITC-, PE-, CyChrome-, PE-Cy5-, allophycocyanin-, or biotin-conjugated Abs specific for CD4, CD11a, CD44, CD45.1, CD69, CD90.1, IL-2, and TNF-α, were purchased from BD Biosciences or eBioscience. Streptavidin-PerCP was sometimes used to detect biotin-conjugated Abs in secondary staining. After staining, cells were analyzed by flow cytometry using a FACSCalibur. Data were analyzed using FlowJo software (Tree Star, San Carlos, CA).

Tracking SM1 T cells and Salmonella

At various times after infection, spleen or lymph node cells were harvested in Eagle’s Hanks Amino Acids medium (Biofluids, Rockville, MD) containing 2% FBS and 5 mM EDTA. Serial dilutions of each sample were first plated onto MacConkey agar plates (Difco, Detroit, MI) at 37°C to determine the extent of bacterial colonization in vivo. Following this, samples were washed in Eagle’s Hanks Amino Acids medium, and live cell counts were determined using a hemocytometer. Cells (5 × 10^6/tube) were then processed and stained as described above.

Results

In vivo tracking of Salmonella-specific CD4 T cells

We previously reported the generation of a RAG-deficient, CD90.2 TCR transgenic mouse strain (SM1) that allowed the visualization of Salmonella flagellin-specific CD4 T cells in vivo (8). This transgenic line was independently backcrossed to both B6.PL-thy1a/Cy and B6.SJL-PtprcaPep3b/BoyJ congenic mice, thus generating RAG-deficient congenic SM1 lines that express either CD90.1 or CD45.1. All peripheral CD4 T cells from RAG-deficient, CD90.1, SM1 TCR transgenic mice (hereafter referred to as SM1) expressed CD90.1 (Fig. 1A). Following adoptive transfer to C57BL/6 mice, SM1 T cells (CD4^+ , CD90.1^− ) could be detected in all lymphoid tissues, and this population expanded in response to injection of flagellin peptide and LPS (Fig. 1A). SM1 T cells responded to flagellin peptide and LPS with similar kinetics in all lymphoid tissues analyzed (Fig. 1B).

FIGURE 1. SM1 T cells expand in the spleen following i.v. infection with Salmonella. A, SM1 CD90.1.CD4 T cells were incubated with CFSE at 37°C for 10 min with periodic shaking, before being washed twice in cold HBSS. SM1 T cells were incubated with CFSE at 37°C for 10 min with periodic shaking, before being washed twice in cold HBSS. In all infection experiments, the dose of bacteria administered was confirmed by plating serial dilutions on MacConkey agar plates. C57BL/6 (Transfer Only) or adoptively transferred and immunized (200 μg flagellin peptide and LPS) C57BL/6 (Peptide/LPS) were infected i.v. the following day with 200 μg of flagellin peptide and LPS. At various times after infection, spleen or lymph node cells were harvested in Eagle’s Hanks Amino Acids medium (Biofluids, Rockville, MD) containing 2% FBS and 5 mM EDTA. Serial dilutions of each sample were first plated onto MacConkey agar plates (Difco, Detroit, MI) at 37°C to determine the extent of bacterial colonization in vivo. Following this, samples were washed in Eagle’s Hanks Amino Acids medium, and live cell counts were determined using a hemocytometer. Cells (5 × 10^6/tube) were then processed and stained as described above.

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SM1 T cell activation is localized to the spleen after i.v. infection

We previously reported a restricted pattern of SM1 T cell activation following oral *Salmonella* infection; expansion was observed in mucosal lymphoid tissues but not in the spleen (8). This activation pattern did not correlate with Ag load, because the spleen is the major lymphoid site of *Salmonella* replication. To understand this phenomenon in more detail, we examined SM1 activation after i.v. infection with *Salmonella*. In contrast to oral infection, we detected *Salmonella*-specific SM1 T cell expansion in the spleen but not the mesenteric lymph nodes (MLN) (Fig. 1C). Cell division of SM1 T cells in these tissues was examined by staining with CFSE before adoptive transfer. These data demonstrated that SM1 T cells in the spleen of infected mice had undergone several rounds of cell division, whereas those in the MLN remained undivided (Fig. 1D). Together with our previous observations using oral infection (8), these data suggest that SM1 T cell expansion is typically restricted to the initial lymphoid site colonized by *Salmonella*, irrespective of the route of administration.

Low-dose infection evades initial SM1 T cell activation in vivo

This restricted pattern of SM1 T cell activation, observed using two different routes of administration, could possibly indicate some sort of bacterial evasion of T cell activation as the infection progresses in vivo. For example, it is possible that *Salmonella* rapidly reduce flagellin expression or interfere with presentation of the flagellin 427–441 epitope, or the bacteria themselves may simply be physically sequestered away from dendritic cells, thus preventing SM1 T cell activation. If either of these processes occur, SM1 activation might be restricted to the initial infected lymphoid tissue, because the bacterial load initially express flagellin, are extracellular for a short period of time at this site, and would not have had opportunity to modify Ag presentation. Thus, SM1 T cells might have a short window of time to respond to *Salmonella* before bacterial factors hinder this process. One prediction of such an evasion model is that reducing the initial *Salmonella* challenge dose should generate an immunologically silent infection with respect to SM1 T cells, because this would limit the initial numbers of extracellular *Salmonella* and the local concentration of associated Ags during the early stage of infection.

We examined this possibility by adoptively transferring SM1 T cells to C57BL/6 mice and then infecting these mice i.v. with virulent *Salmonella* over a 10,000-fold dose range. Infection with high doses of bacteria (10^5–10^6) resulted in pronounced clonal expansion of SM1 T cells in the spleen 3 days after infection (Fig. 2, bottom panels). This coincided with marked CFSE dye dilution and increased expression of CD11a and CD44 on almost all SM1 T cells (Fig. 2, bottom panels). However, infection with low doses of *Salmonella* (10^2–10^3) did not cause any clonal expansion, CFSE dye dilution, or the increased expression of CD11a or CD44 on

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3 Abbreviation used in this paper: MLN, mesenteric lymph node.
SM1 T cells in the spleen (Fig. 2, top panels). An intermediate dose (10^6) resulted in limited SM1 expansion and CFSE dye dilution. Interestingly, it appeared that almost all SM1 cells (including undivided cells) had encountered Ag at this dose, as evidenced by increased expression of CD11a, but not all of these cells divided (Fig. 2). Together, these data demonstrate that activation of SM1 T cells in vivo is critically dependent upon the challenge dose administered.

We next sought to determine whether this sensitivity to challenge dose was a unique property of i.v. infection. Therefore, we examined SM1 T cell activation in the MLN after infection with different oral doses of Salmonella. Again, a 10,000-fold range in bacterial numbers was examined, although these doses were higher overall than those used for i.v. infection, because the LD50 using oral or i.v. routes differs by ~4 logs (4). Oral infection with a high dose (10^10) of Salmonella resulted in significant SM1 T cell expansion within the MLN (Fig. 3, bottom panels). Furthermore, almost all SM1 cells in these mice had undergone multiple rounds of cell division and expressed high levels of CD11a (Fig. 3, bottom panels). In contrast, at the lowest oral dose used (10^4), SM1 T cells did not expand at all, display any evidence of CFSE dye dilution, or increase the expression of CD11a (Fig. 3, top panels). Intermediate doses (from 10^2 to 10^6) all caused increasing degrees of clonal expansion, CFSE dye dilution, and increased expression of CD11a (Fig. 3). Therefore, SM1 T cell activation is sensitive to differences in the initial infectious dose of Salmonella administered, irrespective of the challenge route used. These data, together with the restricted location of SM1 T cell activation in vivo, are consistent with a model in which SM1 T cell activation is determined by the initial concentration of bacterial Ags in primary infected lymphoid tissues.

**SM1 T cells remain unactivated in mice infected with low doses of Salmonella**

The lack of SM1 activation at low challenge doses was surprising, because Salmonella is a rapidly replicating pathogen and would thus be expected to rapidly increase Ag concentration over time. We thought it plausible that Salmonella evade SM1 T cell activation following low-dose infection. However, an alternative possibility was that SM1 T cell activation is merely delayed following low-dose infection until sufficient bacterial replication has occurred in vivo. We examined this issue by analyzing SM1 T cell activation and bacterial counts in the spleen at days 3 and 5 following i.v. infection with varying doses of Salmonella.

Examining Salmonella growth curves, after administration of different doses, demonstrated that bacteria replicated at a similar rate between days 0 and 3, irrespective of the initial challenge dose (Fig. 4A). Challenge with low Salmonella doses eventually resulted in similar or higher bacterial loads in the spleen than were initially administered to high-dose challenge mice. For example, 3 days after infection with 10^8 Salmonella, >10^9 bacteria were detected in the spleen (Fig. 4A), a dose that resulted in moderate SM1 T cell activation (Fig. 2). Similarly, by day 5, the number of Salmonella in the spleens of mice administered 10^2 or 10^5 bacteria had increased to >10^7, 10-fold higher than the highest initial bacterial dose used in these experiments (Fig. 4A). Mice administered high doses (10^4–10^6) of Salmonella succumbed to infection between days 3 and 5 (Fig. 4A). These data clearly show that, after low-dose infection, bacterial replication generates sufficient numbers of Salmonella in the spleen to activate SM1 T cells.

However, our analysis of SM1 T cell activation in the spleen of these same mice revealed that SM1 T cells do not expand at any time point after low-dose infection, whereas SM1 T cells expand in mice administered higher doses (Fig. 4B). Furthermore, SM1 T cells recovered from the spleen of low-dose-infected mice at day 5 had not diluted CFSE or increased the surface expression of CD11a or CD44 (Fig. 4C). Therefore, we conclude that SM1 T cell activation correlates only with the initial bacterial challenge dose rather than with the absolute bacterial burden in vivo.

**Peptide activation of SM1 T cells in Salmonella-infected mice**

These data are compatible with a model where Salmonella actively suppress T cell activation in vivo. Alternatively, replicating Salmonella may reduce flagellin expression in vivo, or be sequestered away from APCs, thereby limiting the amount of available Ag to stimulate SM1 T cells. To discriminate between these possibilities, we injected low-dose Salmonella-infected mice with flagellin peptide 427–441 to see whether it was possible to activate SM1 T cells in the presence of a Salmonella infection.

C57BL/6 mice were adoptively transferred with SM1 T cells, and some were infected the following day with 10^7 Salmonella. Three days later, these mice were injected i.v. with 200 μg of flagellin peptide 427–441, and spleens were harvested 6 h later to examine in vivo activation. Preliminary experiments indicated that
6 h is the peak time point for naive SM1 cells to produce IL-2 in vivo (data not shown). SM1 T cells in uninfected (Fig. 5A, Transfer Only) or infected (Salmonella-infected) mice that did not receive peptide injection were uniformly CD69\textsuperscript{low} and produced very little IL-2 or TNF-\textalpha. In contrast, peptide injection increased CD69 expression and induced the production of IL-2 and TNF-\textalpha by SM1 T cells in both uninfected and Salmonella-infected mice (Fig. 5A, \textalpha\textsuperscript{peptide}). These data demonstrate that SM1 T cells remain fully capable of responding to specific peptide even in the presence of a Salmonella infection.

We examined this issue further by analyzing SM1 expansion and cell division, 2 days after peptide stimulation in low-dose-infected mice. SM1 T cells proliferated and increased expression of CD11\textalpha in response to flagellin peptide injection in both infected and uninfected mice (Fig. 5B, +peptide). Furthermore, SM1 T cells in Salmonella-infected mice appeared to undergo slightly more clonal expansion in response to peptide than SM1 cells in naive mice (Fig. 5B). These data argue strongly against the possibility that low-dose Salmonella infection actively suppresses the activation of SM1 T cells in vivo.

Adoptive transfer of infected spleen cells activates SM1 T cells

Therefore, our data are consistent with a model whereby Salmonella rapidly down-regulate flagellin expression in vivo, or a model in which replicating Salmonella are sequestered away from APCs, thus limiting Ag presentation during low infection. In either of these models, T cell activation would only occur in lymphoid tissues that contain a sufficient amount of extracellular bacteria or free Ag that came directly from the initial challenge inoculum. Any other site of bacterial replication would presumably have insufficient available flagellin to activate SM1 T cells.

We attempted to discriminate between these models by directly staining infected spleen sections with a flagellin-specific mouse mAb or anti-flagellin polyclonal antiserum. However, these experiments were unsuccessful due to poor sensitivity of flagellin staining and cross-reactivity of the antiserum with non-flagellin bacterial proteins. Therefore, we decided to develop a functional assay to examine flagellin expression in vivo, using flagellin-specific SM1 T cells as our detection reagent.

First, we adoptively transferred SM1 T cells into C57BL/6 mice that already had an established Salmonella infection and examined whether these T cells became activated. We presumed that this would give the bacteria sufficient time to modulate flagellin expression in vivo. Increased CD69 expression was detected on SM1 T cells that had been transferred into Salmonella-infected mice, compared with uninfected mice (Fig. 6). However, this staining was very weak and only 2-fold greater than the staining observed after transfer into mice infected with flagellin-deficient Salmonella.
To examine the possibility that *Salmonella*, and therefore flagellin, is sequestered away from APCs, we decided to transfer *Salmonella*-infected splenocytes from an infected mouse into a naive, SM1-transferred recipient. If flagellin expression is compartmentalized away from circulating SM1 T cells in the spleen, then disrupting the splenic architecture, and transferring a single-cell suspension of infected splenocytes into another mouse, should increase the chance of detecting SM1 T cell activation. Conversely, if *Salmonella* do not express any flagellin after several days of infection, SM1 T cells should not become activated by the

**FIGURE 5.** SM1 T cells in infected mice respond to flagellin peptide in vivo. C57BL/6 mice were adoptively transferred with 2 × 10⁶ SM1 T cells, and groups of mice were infected i.v. with 10⁵ *Salmonella* (SL1344). Three days later, some mice were immunized i.v. with 200 µg of flagellin peptide (+peptide), and others were left untreated. A, Six hours later, spleen cells were harvested and surface stained using mAbs against CD4, CD90.1, and CD69. Cells were then fixed and stained for intracellular IL-2 and TNF-α production. Plots show only SM1 T cells after applying a box gate, as shown in Fig. 2, and are representative of two individual experiments. B, Forty-eight hours later, spleen cells were harvested and surface stained using mAbs against CD4 and CD90.1. SM1 T cells are defined by the box gate shown in the expansion plots. Numbers above the boxed gate represent the percentage of SM1 T cells in the spleen. CFSE and CD11a plots show SM1 T cells, as defined by the box gate shown in expansion plots.
FIGURE 6. Transfer of SM1 T cells to mice with established Salmonella infection causes weak CD69 activation in vivo. C57BL/6 mice were infected i.v. with 10^7 Salmonella (SL1344) or aflagellate Salmonella (BC490). Two days later, SM1 T cells were adoptively transferred into infected or uninfected (Transfer Only) mice, and 24 h later, spleens were harvested to examine SM1 T cell activation. Plots show CFSE and CD69 expression after gating on SM1 T cells, using a boxed gate, as shown in Fig. 2. The positive control for increased CD69 expression is SM1 T cells taken from mice injected with both 10^7 Salmonella and SM1 T cells on the same day and harvested 24 h later.

transfer of infected splenocytes, because there would be no specific Ag to which to respond. The transfer of infected spleen cells to uninfected mice resulted in marked up-regulation of CD69 and CD11a on SM1 T cells in the recipient (Fig. 7A). Furthermore, a small amount of SM1 cell division was noted 48 h after splenocyte transfer (Fig. 7A). Extensive cell division would not be expected at this early time point. SM1 T cell activation was completely Ag specific and did not occur after transfer of splenocytes from mice infected with aflagellate Salmonella (Fig. 7A). We conclude that some flagellin expression is maintained by Salmonella growing in vivo, but that this Ag is sequestered away from APCs. However, as infected splenocytes were taken from high-dose-infected mice in this particular experiment, it remained possible that SM1 T cell activation did not represent maintenance of flagellin expression, but rather the maintenance of APCs that had captured flagellin early after infection. Therefore, we infected a group of mice with low-dose Salmonella and transferred infected splenocytes to naive mice at various times later. The adoptive transfer of infected splenocytes from mice that had been administered low-dose Salmonella 5 days previously resulted in significant activation of SM1 T cells. These data suggest that some flagellin expression is maintained by Salmonella growing in vivo, but circulating CD4 T cells are unable to detect this Ag due to Ag sequestration.

Discussion
We have examined how altering challenge dose influences the activation of Salmonella flagellin-specific T cells, using a recently developed TCR transgenic adoptive transfer system. Surprisingly, the activation of Salmonella-specific T cells appears to be critically regulated by the size of the initial inoculum rather than the actual bacterial load in the infected tissue. Although we have not exhaustively examined all possible parameters of T cell activation, it seems reasonable to conclude that SM1 T cells are not activated by low-dose infection. SM1 T cells do not increase in number, undergo cell division, or increase expression of CD69, CD11a, or CD44, despite massive replication of bacteria in the same anatomical site. Therefore, it appears that lowering the challenge dose allows Salmonella to evade detection by Salmonella flagellin-specific CD4 T cells.

These data suggest paradoxically that Salmonella infection might be particularly pathogenic at the lowest doses, because it would be more likely that such an infection would avoid CD4 T cell activation. It is already known that natural Salmonella infection can occur with very low doses of bacteria (21). Indeed, it is possible that detection of Salmonella-specific CD4 T cell activation to high-challenge doses in the laboratory may be something of an artifact and bear little resemblance to natural Salmonella infection. At the very least, our data indicate that modulation of infectious dose can have a profound impact on T cell responsiveness and therefore more attention should be paid to this aspect of laboratory models. We have not yet examined the effect of modulating bacterial dose upon the activation of Salmonella-specific effector/memory T cells. It remains possible that low-dose Salmonella infection can also evade memory SM1 T cell activation. If so, this may represent an unforeseen impediment to vaccine development and go some way toward explaining the difficulty in generating effective Salmonella vaccines based upon the induction of Salmonella-specific memory T cells. We are currently examining this issue in our laboratory.

Very few Ags have been defined as targets of Salmonella-specific CD4 T cells, and even fewer MHC class II epitopes have been mapped (12, 13, 22). Therefore, it is not yet clear whether SM1 T cells represent an unusual CD4 T cell population, or whether they are representative of the endogenous Salmonella-specific CD4 T cell response. It is clear that CD4 T cell responses to undefined Ags occur in Salmonella-infected patients and in mice that have resolved infection with attenuated Salmonella (23–26), although the effect of challenge dose upon these responses has rarely been examined. Because flagellin represents ~8% of the protein content of Salmonella (27), the specific evasion of a T cell response to this Ag could contribute to bacterial virulence. Alternatively, the evasion we have observed may represent a general mechanism to increase virulence at low challenge doses. Future studies to define other CD4 target Ags are necessary to examine this issue in more detail.

How might Salmonella evade detection by flagellin-specific T cells? It is possible that Salmonella rapidly deplete or modify the function of dendritic cell populations that are required for in vivo CD4 T cell activation. Indeed, dendritic cells are selectively depleted in patients with sepsis (28). However, careful analysis of dendritic cell populations during Salmonella infection has not revealed any rapid loss of these cells in vivo (29, 30). Furthermore, injection of flagellin peptide rapidly activated SM1 T cells in infected mice (Fig. 5), a process that is likely to be dependent upon the presence of dendritic cells in vivo. We think it unlikely that Salmonella infection substantially modulates Ag presentation or dendritic cell numbers, although future experiments using CD11c-DTR transgenic mice (31) should allow us to examine this issue. A more likely scenario is that Salmonella carefully regulate the expression of flagellin in vivo, as previously suggested (32, 33). It has proved difficult to directly examine this hypothesis due to the lack of flagellin-specific reagents. Therefore, it remains possible that flagellin is rapidly down-regulated by Salmonella in vivo. However, our data show that infected splenocytes can rapidly activate SM1 T cells in vivo following adoptive transfer (Fig. 7). Although we cannot rule out the possibility that Salmonella rapidly reinstate expression of flagellin during the generation of this single-cell suspension before transfer, it seems more likely that some flagellin expression is maintained in vivo, but that CD4 T cells do
not have access to the Ag. Such Ag sequestration is not entirely unexpected, because *Salmonella* reside primarily in red pulp and marginal zone macrophages (34), whereas naive CD4 T cells migrate through the spleenic white pulp (35). Future experiments are planned to examine this hypothesis directly in vivo.

Our data conflict with recent studies of CD8 responses to *Listeria* where bacterial dose was shown to be somewhat irrelevant for T cell activation (17). Interestingly, T cell activation to *Listeria* does not seem to display the same pattern of restricted activation that we have observed with *Salmonella* (36, 37). We think it likely that these differences between these models are due to the pathogenesis of the different bacteria used in these studies. *Salmonella* is thought to primarily infect macrophages in vivo, whereas *Listeria* is known to infect other cell types (1, 2, 38). Therefore, Ag may be more uniquely sequestered away from dendritic cells during *Salmonella* infection.

In conclusion, our data support the idea that low-dose infection with *Salmonella* evades flagellin-specific CD4 T cell activation. Rapid intracellular infection of macrophages may serve to limit the access of dendritic cell to bacterial Ags, thus preventing *Salmonella*-specific T cell priming, even as pronounced bacterial replication occurs. Strategies to interfere with this process may lead to the generation of more effective vaccines against typhoid fever.

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**References**


**FIGURE 7.** Splenocytes from *Salmonella*-infected mice can activate flagellin-specific SM1 T cells in vivo. A, C57BL/6 mice were infected i.v. with 10⁵ *Salmonella* (SL1344) or aflagellate *Salmonella* (BC490). Two days later, splenocytes were harvested from these infected mice and injected i.v. into naive C57BL/6 mice (10% of whole infected donor spleen per recipient) that had been adaptively transferred with CFSE-labeled SM1 T cells the previous day. Two days after injection of infected splenocytes, SM1 T cells were analyzed for evidence of T cell activation in the spleen of recipient mice. The Transfer-Only group was transferred with SM1 T cells but did not receive any infected splenocytes. CFSE, CD69, CD11a, and CD44 plots show only SM1 T cells as defined by the boxed gate in the expansion plots (top row). Numbers show the percentage of cells in each gate or each quadrant. B, C57BL/6 mice were infected i.v. with 10⁴ *Salmonella* (SL1344), and splenocytes were harvested on various days later. Harvested splenocytes were injected i.v. (10% of total infected spleen per injection) into C57BL/6 mice that had been adaptively transferred with CFSE-labeled SM1 T cells the previous day. Twenty-four hours after injection of infected splenocytes, SM1 T cells in the spleen of recipient mice were analyzed for evidence of T cell activation. CFSE and CD69 plots show only SM1 T cells as defined by a boxed gate. Numbers show the percentage of cells in the boxed gate.


