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Direct visualization of endogenous *Salmonella*-specific B cells reveals a marked delay in clonal expansion and germinal center development

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Abstract

CD4$^+$ T cells and B cells are both essential for acquired immunity to *Salmonella* infection. It is well established that *Salmonella* inhibit host CD4$^+$ T-cell responses, but a corresponding inhibitory effect on B cells is less well defined. Here, we utilize an antigen tetramer and pull-down enrichment strategy to directly visualize OVA-specific B cells in mice, as they respond to infection with *Salmonella*-OVA. Surprisingly, OVA-specific B-cell expansion and germinal center formation was not detected until bacteria were cleared from the host. Furthermore, *Salmonella* infection also actively inhibited both B- and T-cell responses to the same coinjected antigen but this did not require the presence of iNOS. The SPI2 locus has been shown to be responsible for inhibition of *Salmonella*-specific CD4$^+$ T-cell responses, and an examination of SPI2-deficient bacteria demonstrated a recovery in B-cell expansion in infected mice. Together, these data suggest that *Salmonella* can simultaneously inhibit host B- and T-cell responses using SPI2-dependent mechanisms.

Keywords

Bacterial infection; Immunity; B cells; Germinal centers; clonal expansion

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Introduction

Human infection with *Salmonella* serovars causes typhoid and non-typhoidal Salmonellosis (NTS) and both these diseases are major causes of morbidity and mortality worldwide [1, 2]. The heaviest burden of *Salmonella* infections falls upon children under 5 years of age in south and southeastern Asia and sub-Saharan Africa. Invasive NTS infections are also an emerging problem in HIV-infected adults, malaria-infected children and immune-compromised individuals, primarily in sub-Saharan Africa [3–5]. Although there are two licensed Typhoid vaccines, these provide only limited protection to the most vulnerable populations [6, 7]. The first of these, Ty21a (Vivotif®), is a safe, live attenuated vaccine that requires four doses for efficacy against typhoid. The second, virulence capsular polysaccharide (ViCPS, marketed under the name Typhim Vi®), is a purified capsule polysaccharide that is able to curtail typhoid outbreaks and provide short-term protection to travelers. However, neither of these vaccines is licensed for children younger than 2 years of age or is routinely utilized in typhoid endemic areas.

The protection elicited by vaccination with ViCPS is attributed to the induction of a T-independent antibody response of limited duration [8]. Current research is focused on developing an improved Vi capsular vaccine that uses a carrier protein to generate a T-cell-dependent antibody response and B cell memory [9]. In contrast to the ViCPS vaccine, the protection mediated by the live attenuated Ty21a vaccine is thought to require the induction of T-cell-mediated immunity [10]. In agreement with this hypothesis, *Salmonella*-specific T-cell responses are detected in Ty21a-immunized patients [11, 12]. Antibody responses are also induced by immunization with live attenuated *Salmonella* vaccines, but the role of these antibodies in protective immunity is less clear [11, 13]. The generation of improved vaccines for typhoid and NTS will require a deeper understanding of adaptive immunity to *Salmonella* infection and greater knowledge of how this pathogen is able to subvert protective responses.

Infection of susceptible and resistant mouse strains with *Salmonella* Typhimurium has provided a well-established model of typhoid and invasive salmonellosis [14]. Susceptible inbred strains, such as C57BL/6 or BALB/c mice are unable to survive primary infection with virulent *Salmonella* [15]. However, these susceptible strains resolve primary infection with attenuated bacterial strains and acquire robust protective immunity to secondary challenge with virulent bacteria [16, 17]. The resolution of a primary infection with attenuated bacteria requires a functioning immune system and specifically has been shown to require $CD4^+$ Th1 cells, IL-12, and IFN-γ [18–20]. Acquired immunity to secondary infection also relies on Th1 cells, but surprisingly demonstrates an additional requirement for B cells [21–23]. Resistant mouse strains, such as 129/SvJ, are able to resolve primary infection with virulent *S*. Typhimurium, and secondary protective immunity is largely mediated by the generation of protective antibodies [24, 25]. Thus, a central theme in both susceptible and resistant models is that B cells or antibody play an essential role in the resolution of *Salmonella* infection. Furthermore, these observations in the research laboratory largely concur with studies examining human salmonellosis. Individuals with a primary genetic deficiency in IL-12 or IFN-γ signaling are susceptible to NTS [26, 27], demonstrating the importance of Th1 cells for bacterial clearance. However, the absence of

Salmonella-specific antibodies in young children also correlates with increased susceptibility to NTS [28], suggesting that B cell responses are also critical for protection.

As with many successful pathogens, *Salmonella* have evolved sophisticated mechanisms to evade and subvert protective host immune responses [29]. For example, *Salmonella* are able to subvert macrophage phagocytosis and can survive and proliferate within *Salmonella*-containing vacuoles (SCVs), which are adapted to suit bacterial growth [30]. Genes within the *Salmonella* Pathogenicity Island 2 (SPI2) encode a Type III Secretion System (T3SS) that allows the injection of bacterial effector proteins into the cytosol of infected cells [30]. These effector proteins maintain the SCV structure by modifying filament formation and actin polymerization surrounding the vacuole [31]. In addition, some of these same effector proteins have been shown to influence the induction and maintenance of *Salmonella*-specific immune responses [14]. For example, multiple studies have demonstrated that SPI2 effector proteins can inhibit the migration and antigen presentation capability of infected dendritic cells [32–34]. Importantly, SPI2 effectors have also been implicated in eliminating *Salmonella*-specific $CD4^+$ T-cells following initial clonal expansion, in a process referred to as “culling” [35, 36]. *Salmonella* can also inhibit T-cell responses using a non-SPI2-encoded asparaginase [37, 38], highlighting the importance of T cell inhibition for bacterial survival in vivo. However, it is unclear whether similar bacterial inhibitory mechanisms are used to modulate *Salmonella*-specific B cell responses, and whether SPI2-encoded effector proteins might also be involved. A recent report demonstrated that antibody responses to *Salmonella* LPS, flagellin and outer membrane proteins can be detected early after infection, but that the antibody response to other *Salmonella* antigens is significantly delayed [39]. Importantly, this study showed that the overall germinal center reaction was also delayed [39], suggesting that *Salmonella* directly or indirectly inhibit the development of B cell responses.

Here, we have directly visualized the endogenous *Salmonella*-specific B cell response during primary infection of resistant mice. Using newly developed tetramer reagents and a methodology devised to study OVA-specific B cells [40], we visualized primary OVA-specific B cell expansion and germinal center formation during infection with *Salmonella*-OVA. Surprisingly, *Salmonella*-specific B cell expansion was markedly delayed until bacterial clearance was completed. Furthermore, *Salmonella* infection was simultaneously able to inhibit B cell expansion, germinal center formation, and $CD4^+$ T-cell responses to immunization. There was a marked deficiency in the ability of SPI2 T3SS mutants to inhibit B cell responses, suggesting that SPI2 effectors may be used by bacteria to prevent bacterial-specific B cell responses. In addition, this effect was independent of iNOS, which has previously been shown to be important for immune suppression during *Salmonella* infection. Thus, our data document a profound inhibition of the B cell response to *Salmonella* infection that, like $CD4^+$ T-cells, is largely dependent on the SPI2 T3SS.

Results

Visualization of Salmonella-specific B cells using B-cell tetramers

Although many studies have confirmed the importance of B-cell responses to *Salmonella* immunity [21–25, 39, 41], there are currently no reagents that can directly identify *Salmonella*-specific B cells in vivo. In order to identify and define the endogenous B-cell

response to an antigen expressed by *Salmonella*, we made use of χ4550-OVA, an attenuated Δ*asd* auxotrophic mutant strain that expresses approximately 70 μg of OVA per 10^8 *Salmonella* in the periplasmic space and has previously been used to examine OVA-specific T-cell responses in vivo [35, 42].

The endogenous B-cell response to OVA can be directly visualized by flow cytometry using a combination of OVA-specific B cell tetramers, "decoy" tetramers, and a magnetic bead enrichment strategy [43, 44]. In this staining approach, endogenous OVA-specific B cells bind OVA tetramers while B cells specific for the SA-PE core are gated out using a decoy tetramer. Using this methodology, populations of SA-PE-, OVA- and AF647-specific B cells can each be distinguished by flow cytometry (Fig. 1A). Our gating strategy for identifying OVA-specific B cells consisted of an initial lymphocyte gate, followed by a singlet gate and a dump channel containing CD11c, CD11b, F4/80, CD4, CD8, and Gr-1. By gating on B220+/Dump- cells, OVA-specific B cells that had bound the OVA-tetramer, but not the decoy tetramer, were clearly identified (Fig. 1B). We attempted to use this direct staining approach to identify OVA-specific B cells in mice that had been infected with *Salmonella*-OVA. In preliminary experiments, the expansion of OVA-specific B cells was detected in 5/7 mice infected with *Salmonella* expressing OVA (Fig. 1C, St-OVA). In contrast, there was no expansion of OVA-specific B cells in mice infected with the control strain lacking OVA expression (Fig. 1C, St-Comp). Thus, direct staining using antigen tetramers can be a useful approach to identify *Salmonella*-specific B cells in vivo.

B cell expansion and germinal center formation takes place after Salmonella infection has resolved

To visualize the kinetics of antigen-specific B cell expansion during *Salmonella* infection, resistant C57BL6x129/Sv F1 mice were infected IV with St-OVA, sacrificed at various time points later, and OVA-specific B cells identified by tetramer staining of pooled secondary lymphoid tissues. In order to monitor bacterial growth, livers were also harvested from the same mice, homogenized, and plated onto MacConkey agar. During the first 10 days of infection, *Salmonella* expanded in the liver of infected mice, however, bacterial numbers had significantly reduced by day 20 and fell below the level of detection by day 37 (Fig. 2A). This tempo of liver colonization is consistent with previous reports of spleen colonization using this same bacterial strain in resistant mice [42]. Surprisingly, OVA-specific B cell expansion was minimal throughout the period of active infection but peaked around day 45, with expanded numbers of B cells declining back to baseline by 75 days post-infection (Fig. 2A). By direct tetramer staining, naïve mice were calculated to have approximately 600 OVA-specific B cells in secondary lymphoid tissues. At the peak of clonal expansion (day 45) after *Salmonella*-OVA infection, there was an average of 3100 OVA-specific B cells, constituting a 5-fold expansion in response to infection. The unusual delayed kinetic of OVA-specific B-cell expansion was also consistent with late development of OVA-specific IgG responses in *Salmonella*-OVA-infected mice (Fig. 2B). This later IgG response largely consisted of OVA-specific IgG2c, but OVA-specific IgG1 and IgG2b were also detected in infected mice (Fig. 3).

Next, we examined the phenotype of expanded OVA-specific B cells in St-OVA infected mice by monitoring surface expression of CD38 and GL7 (Fig. 4A). CD38+GL7- staining is a useful marker of naïve or memory B cells, whereas CD38-GL7+ expression identifies germinal center B cells [45, 46]. Recently, an intermediate CD38+GL7+ population has been described and is thought to represent a population of activated B cells that have not yet committed to a germinal center or memory cell fate [44]. In our analysis, we simply refer to this intermediate population as "activated" and our overall gating scheme is summarized in Fig. 4B. In uninfected mice, tetramer binding OVA-specific B cells were uniformly CD38+GL7-, as would be expected for naïve B cells. At 37 days post-infection, prior to the peak of B-cell clonal expansion, 18% of OVA tetramer-positive B cells expressed the CD38-GL7+ phenotype, typical of germinal center B cells (Fig. 4D). This germinal center response increased and peaked at day 45 when the percentage and absolute number of OVA tetramer binding B cells expressing a CD38-GL7+ phenotype was approximately 50% of all OVA-specific B cells (Fig. 4C–D). By day 75 post-infection, the contracted pool of tetramer-positive B cells had returned to expression of a CD38+GL7- surface phenotype, similar to naïve mice (Fig. 4C–D). This modulation of B-cell surface phenotype was antigen-specific and not simply a consequence of an inflammatory response to *Salmonella*, since an increase in the number or percentage of cells expressing a germinal center phenotype was not observed when gating on OVA tetramer-positive B cells in mice infected with the control strain St-Comp (Fig. 4E and F). Overall, these data are consistent with a delayed kinetic of B-cell clonal expansion and germinal center development in mice infected with *Salmonella*.

Salmonella inhibit OVA-specific B-cell expansion and activation after immunization

The marked delay in clonal expansion and activation of OVA-specific B cells in response to *Salmonella*-OVA infection contrasted with the previously reported rapid expansion of OVA-specific B cells in response to OVA immunization [40], and suggested that *Salmonella* may actively inhibit B-cell responses. We therefore examined whether *Salmonella* infection can actively inhibit antigen-specific B-cell expansion and activation to OVA-immunization. In mice immunized with OVA/LPS, a 100-fold expansion of OVA tetramer-positive B cells was detectable 7 days post-immunization, and the majority of these expanded B cells expressed a CD38-GL7+ surface phenotype (Fig. 5A–D). Consistent with our data above, there was no detectable expansion of OVA-specific B cells in *Salmonella*-OVA infected mice at this time point and all OVA-specific B cells were CD38+GL7- (Fig. 5A–D). However, in mice immunized with OVA and also concurrently infected with *Salmonella*-OVA, there was a marked reduction in the percentage and absolute number of OVA-specific B cells when compared to OVA immunization alone (Fig. 5A and B). Furthermore, while over 80% of OVA-specific B cells in OVA/LPS immunized mice expressed a germinal center phenotype, only 40% of OVA-specific B cells had this phenotype in the presence of *Salmonella*-OVA (Fig. 5C and D). Thus, concurrent *Salmonella* infection effectively inhibits the expansion of OVA-specific B cells responding to antigen and limits the acquisition of a germinal center phenotype. Interestingly, *Salmonella* infection caused an increase in the percentage of "activated" CD38+GL7+ B cells responding to OVA immunization (Fig. 5C and D), perhaps suggesting that these cells were effectively blocked during the transition to a germinal center CD38-GL7+ phenotype. Since previous studies have reported an

inhibitory effect of *Salmonella* infection on $CD4^+$ T-cell responses [35–38], we examined whether OVA-specific B- and $CD4^+$ T-cell responses to immunization were simultaneously inhibited. Indeed, *Salmonella* infection caused a marked reduction in the percentage and total number of OVA-specific OT-II T cells and OVA-specific B-cell responses in OVA-immunized mice (Fig. 6).

SPI2-deficient bacteria have reduced ability to inhibit B-cell responses

The ability of *Salmonella* to cause a marked reduction in B-cell expansion and germinal center formation indicated that *Salmonella* inhibit B-cell responses in vivo. Previous studies have highlighted nitric oxide as an important mediator involved in the suppression of adaptive immune responses in *Salmonella*-infected mice [47–49]. However, we did not detect any significant reduction in the inhibition of OVA-specific B cell expansion by *Salmonella* infection in iNOS-deficient mice compared to wild-type controls (Fig. 7). Other studies that have examined bacterial genes contributing to immune suppression have demonstrated that a functional SPI2 T3SS is essential for the inhibitory effects of *Salmonella* on T-cell responses [33, 35, 36]. In order to examine whether the SPI2-encoded T3SS was required for inhibition of OVA-specific B cells, we introduced a chromosomal deletion at the SPI2 locus in the parental strain χ4550 (ΔSPI2). When OVA-immunized mice were infected with ΔSPI2 mutant rather than the parent strain, there was a notable recovery of OVA-specific B-cell expansion and germinal center surface phenotype in B cells responding to immunization (Fig. 8A–D).

SPI2 effector mutants' inhibition of OVA-specific B-cell responses is unaffected by bacterial load

We next utilized a SPI2 effector mutant library to examine whether individual effector proteins inhibited the B cell response to OVA. Mice were immunized with OVA mixed with heat-killed *Salmonella* (OVA/HKST) and either co-infected with WT *Salmonella*, strains that completely lack a functional SPI2 T3SS (Δ*ssaV* and Δ*sseC*), or individual strains with lesions in single T3SS effector genes. As expected by day 7, OVA-specific B cells expanded above naïve levels in mice immunized with OVA/HKST and many of these B cells expressed a germinal center or activated surface phenotype (Fig. 8A–C, OVA/HKST). In contrast, B-cell clonal expansion and germinal center phenotype development was blocked by concurrent infection with WT *Salmonella* (Fig. 8A–C, WT). Mice infected with strains lacking a functional SPI2 T3SS lost the inhibition of B cell expansion and germinal center development observed with WT *Salmonella* (Fig. 8A–C, Δ*ssaV* and Δ*sseC*), suggesting that effector proteins of the T3SS were active in inhibiting B-cell responses. However, in a screen examining individual effector mutants, most of these bacterial strains retained the ability to inhibit OVA-specific B-cell clonal expansion and the generation of a germinal center phenotype (Fig. 8A–C and data not shown). For two strains, Δ*sifA* and Δ*sseF*, the inhibition of B-cell responses was modestly lower than observed for the WT strain (Fig. 8A–C), perhaps indicating that these effector proteins actively contributed to B-cell inhibition. However, when a control non-SPI2 $phoP^c$ mutant was examined, we detected a similar recovery of the B-cell response to antigen (Fig. 8A–C). Furthermore, when bacterial loads were examined, the loss of B-cell inhibition by these individual effector mutants largely tracked with lower bacterial burdens also observed with SPI2-deficient strains, Δ*sifA*, Δ*sseF*,

and *phoP*^{c} (Fig. 8D). Thus, the failure of SPI2 mutants to affect B-cell inhibition may largely be due to the lower bacterial burdens rather than a specific effect of SPI2. In order to examine the effect of SPI2 genes independently of bacterial load in vivo, we examined whether a much higher dose of the SPI2 mutant Δ*ssaV* would recover the ability to inhibit B-cell responses in vivo. In pilot experiments we determined that 1×10^5 was the highest dose of Δ*ssaV* that would be tolerated in infected mice after IV injection. However, even a 50-fold higher dose of this Δ*ssaV* showed no recovery in the ability to inhibit OVA-specific B-cell responses in vivo (Fig. 9).

Discussion

Despite the importance of B cells in immunity to *Salmonella* and greater understanding of the antigens targeted [17, 21–25, 39, 41, 50], very few reagents have been developed to study the response of *Salmonella*-specific B cells during infection. Using a novel methodology that allows direct visualization of endogenous antigen-specific B cells, our data clearly show that the B-cell response during *Salmonella* infection is remarkably delayed until after bacterial clearance occurs. A delay in B-cell responses is unlikely to be related to antigen availability since the peak of infection is several weeks prior to B-cell clonal expansion. Thus, the most likely interpretation of these data is that *Salmonella* actively inhibit B-cell responses to bacterial antigens. Although the GC response is markedly delayed compared to immunization, previous studies have shown that the extra-follicular B-cell response can give rise to early antibody production during *Salmonella* infection [39], and this non-GC response may drive early titers of antibody to flagellin and outer membrane proteins. Our methodological approach is obviously reductionist in that it visualizes the B-cell response to a single antigen and thus may not be truly representative of the overall response to all *Salmonella* antigens. However, the fact that overall GC formation occurs late in the response to *Salmonella* infection, would tend to suggest that the delay detected in response to OVA is likely to be typical of T-cell-dependent B-cell responses to *Salmonella*. The function of such B-cell inhibition with respect to bacterial survival is not immediately apparent since antibodies do not usually contribute significantly to bacterial clearance during primary infection [21–23]. However, it is possible that these phenomena are related, and that the inability of antibody to contribute to primary bacterial clearance may derive directly from the ability of bacteria to inhibit an effective GC response. If this turns out to be the case, the identification of bacterial genes driving GC inhibition may allow for the generation of attenuated strains with markedly improved immunogenicity.

A possible explanation for the delay in B-cell responses is the lack of $CD4^+$ T cell help during the period of active infection. Previous studies examining T-cell responses to bacterial flagellin have demonstrated that $CD4^+$ T cells are initially activated but fail to survive beyond clonal expansion [51], potentially depriving *Salmonella*-specific B cells of needed T-cell help. Indeed, our data show that $CD4^+$ T-cell and B-cell responses to the same antigen were concurrently inhibited by *Salmonella* infection. An alternative possibility is that *Salmonella* directly inhibit B-cell function in vivo. Indeed, other groups have shown that *Salmonella* can actively infect B cells [52, 53], and therefore potentially regulate function via bacterial effector proteins in infected B cells. It is unclear which *Salmonella*-encoded proteins might be responsible for effects on B-cell function, but our study suggests

that inhibition is not simply mediated by a single SPI2 effector. Future studies will be needed to determine whether these effector proteins directly affect B cells or primarily affect the ability of T cells to respond to infection. Another intriguing possibility is that *Salmonella*'s inhibitory effect on B and T cells is indirect and that the effect of *Salmonella* on macrophages may actually hinder the formation of the germinal center and thus regulate B-cell proliferation. A recent study demonstrated that in the absence of marginal zone macrophages, activated B cells that migrate to the T-B border and receive help from activated $CD4^+$ T cells fail to further migrate to the follicular perimeter, proliferate and form germinal centers in response to a T-dependent antigen [54]. Since *Salmonella* have well-described cytotoxic effects on macrophages [55, 56], it is possible that this population is depleted or functionally incapable of promoting germinal center formation during *Salmonella* infection. Further work needs to be done to determine the mechanism by which macrophages promote germinal center formation and the strategies *Salmonella* use to inhibit B-cell responses.

Previous data have suggested that T-cell inhibition can be driven by the expression of SPI2 genes [33, 35, 36], although non-SPI2 genes are also reported to have inhibitory effects on the adaptive response [37, 38]. Other studies have pointed to an important role for host-induced nitric oxide in T-cell inhibition [47–49]. Our studies show that the inhibition of B-cell responses by *Salmonella* occurs in the absence of host iNOS but requires the expression of a functional SPI2-encoded needle complex by the bacteria. Although other mutations that affect in vivo growth can influence B cell inhibition, a 50-fold excess of a SPI-2 mutant was unable to generate significant inhibition, suggesting that these genes are vitally important. Despite the requirement for SPI2 genes, we were unable to unambiguously identify specific SPI2 effector proteins that mediate this effect. This is most likely because there is significant redundancy in the function of these effectors in vivo.

The fact that B-cell clonal expansion and germinal center formation eventually occurs late after infection is intriguing since bacterial loads are below the limit of detection when this response is eventually initiated. This kinetic suggests that residual antigen is retained in secondary lymphoid tissues for a considerable period after the infection has resolved. Alternatively, such a late response could be driven by a reservoir of bacteria that persists below the limit of conventional detection methods. Indeed, using an antibiotic treatment model of infection, a reservoir of *Salmonella* has been shown to persist for long periods following apparent resolution of infection [57–59]. Intriguingly, this persistent infection is below the level of conventional detection methods and can cause relapsing disease. Similar long-term persistence has been detected in resistant mice challenged with virulent bacteria [60, 61]. It seems likely therefore that some degree of low-level persistence occurs during host elimination of attenuated bacterial strains. Such persistent bacteria could potentially provide a source of antigen driving the late B-cell response detected by our methodology. However, persistence has not yet been formally established in a vaccine model with susceptible mice, and even if it occurs, this may not provide sufficient antigen to explain B cell clonal expansion.

One of the hallmarks of live attenuated *Salmonella* vaccines is their relatively poor immunogenicity [9]. Efforts to enhance the immunogenicity of vaccine strains are ongoing

and may be assisted by greater understanding of bacterial strategies to inhibit or limit the adaptive immune response in vivo. Overall, our study indicates that *Salmonella* can inhibit B-cell responses and that utilizing direct visualization approaches enhances our understanding of host-pathogen interactions in this infection model. Greater mechanistic understanding of how *Salmonella* direct this inhibition of B-cell proliferation and germinal center formation may lead to the generation of *Salmonella* vaccine strains with improved immunogenicity.

Materials and Methods

Bacteria Strains and Plasmids

Bacterial strains and plasmids utilized in this study are listed in Table 1. The χ4550 strain and pYA3149 plasmid were a kind gift from Dr. Roy Curtiss III (Arizona State University). The χ4550/pYA3149-OVA strain was provided by Dr. Marc Jenkins (University of Minnesota). The SPI2 (T3SS-2) effector-deficient mutant library was provided by Dr. Michael Hensel (Universität Osnabrück, Germany). For new strains developed for this study, the high frequency generalized transducing mutant of bacteriophage P22 (*HT105/1 int-201*) was used to transfer mutations between strains; as strain SR-11 is naturally resistant to P22 infection, cleaning transductants of phage was not necessary. A SPI2-deficient mutant of χ4550 was generated by transducing the Δ*spiB*::KSAC deletion of SPN450 into χ4550/pYA3149-Empty and selecting for kanamycin resistant transductants, yielding SPN524/pYA3149-Empty. PCR amplification (PCR Supermix, Invitrogen) was utilized to confirm null amplification of *spiB* (Primers: 5'-TGGCTGAATGAAGGTAACC-3' and 5'-CTCAGATGGACAATTTCTCC-3') and to confirm the location of the KSAC cassette as previously described [62]. An SPN524 derivative that had titrated out pYA3149-Empty was obtained by growing SPN524/pYA3149-Empty in LB + DAP, plating dilutions on LB agar + DAP, and then screening colonies for DAP auxotrophy on LB agar. Electrocompetent cells of SPN524 were then generated, as previously described [63]. Purified pYA3149-OVA (Miniprep spin kit, Qiagen) from χ4550/pYA3149-OVA was electroporated into SPN524 and electroporants selected by plating on LB agar. Presence of pYA3149-OVA was checked by purifying the plasmid and comparing band size to pYA3149-Empty after BamHI (New England Biolabs) restriction digestion and agarose gel electrophoresis.

Mice and Infections

Susceptible C57BL/6J mice (Jackson Labs) were crossed with resistant 129Sv/J mice (Jackson Labs) and the resistant F1 progeny used in most experiments. In a minority of experiments, C57BL/6 mice were used, and this is indicated in the relevant figure legends. OT-II transgenic RAG-deficient mice were backcrossed to RAG-deficient CD90.1 congenic background in our laboratory. The iNOS-deficient mice were purchased from Jackson Labs. Mice were housed in specific pathogen free conditions at the University of California Davis and all experiments were performed following approved institutional guidelines. All *S.* Typhimurium strains were grown overnight in Luria-Bertani broth and diluted in PBS after estimating bacterial counts by spectrophotometry. Mice were immunized intravenously (IV) with 10μg ovalbumin (OVA) and 10μg LPS and/or infected IV with 5×10^5 χ4550-OVA (St-OVA), χ4550-comp (St-Comp) and ΔSPI2; or IV with 5×10^3 of the NCTC 12023 and SPI2

effector mutant strains and/or 10μg ovalbumin with 10^8 Heat-Killed *Salmonella* (HKST). Infection doses were confirmed by plating serial dilutions onto MacConkey agar plates. Any moribund infected mice were euthanized as stipulated in our IACUC protocol. Bacterial growth *in vivo* was calculated by plating serial dilutions of organs homogenized in 0.01% TritonX /1XPBS onto MacConkey agar and bacterial counts were determined after overnight incubation at 37°C.

OVA and Decoy B cell Tetramer Production

Ovalbumin and decoy tetramers were produced as previously described [44]. Briefly, ovalbumin (Sigma Aldrich) was biotinylated using an EZ-link Sulfo-NHS-LC- Biotinylation kit (Thermo Fisher Scientific) using a 7:1 molar ratio of biotin to ovalbumin. Free biotin was removed via desalting columns (GE Healthcare). The molar amount of biotinylated ovalbumin was measured via Western blot by incubating a fixed amount of biotinylated ovalbumin with differing amounts of SA-PE (Prozyme) at room temperature, followed by Native PAGE (Bio-Rad Laboratories). The resulting gel was transferred onto PVDF +membrane and probed with SA-AP to determine the point at which there was free biotin on ovalbumin to bind to SA-AP. The ratio of SA-PE to biotinylated ovalbumin was then used to calculate the molar concentration of ovalbumin that was actually biotinylated. Biotinylated ovalbumin was incubated with SA-PE in a 6:1 ratio for 30 minutes. OVA-specific B cell tetramer was concentrated and excess biotinylated ovalbumin removed via centrifugation in a 100-kDa molecular weight cutoff Amicon Ultra filter (Millipore). Antigen tetramer was stored at 4°C at a concentration of 1μM. The nonspecific “decoy” tetramer was produced by conjugation of SA-PE to AF647 (Invitrogen) for 60 minutes at room temperature. Free AF647 was removed via centrifugation in a 100-kDa molecular weight cutoff Amicon Ultra filter (Millipore). The SA-PE*AF647 was incubated with 20 molar excess of biotin for at least 30 minutes at room temperature and decoy tetramer was stored at 1μM at 4°C.

B-cell Tetramer Staining, Enrichment and Flow Cytometry

Tetramer staining and enrichment were performed as previously described [43, 44]. At the indicated times after infection or immunization, mice were sacrificed and the spleen and all lymph nodes (inguinal, popliteal, brachial, axillary, cervical, lumbar, caudal, renal, pancreatic and mesenteric) were harvested and homogenized. Cells were incubated with decoy tetramer for 10 minutes at room temperature followed by OVA-tetramer for 15 minutes on ice. After washing excess tetramer, stained samples were incubated for at least 30 minutes on ice with anti-PE microbeads (Miltenyi) and enriched by passing through a magnetized column. Both the tetramer-bound and flow-through samples were subsequently stained with CD11c-, CD11b-, F4/80-, CD4-, CD8-, Gr-1- APC-Cy7, B220- eF450, CD38-AF700, GL7- FITC, and IgM- PE-Cy7. All antibodies were purchased from eBioscience, BD Biosciences, Biolegend, or Tonbo. Tetramer-specific cells were analyzed via flow cytometry (BD Canto and Fortessa) after gating on lymphocytes, singlets and B220+Dump- cells. The number of OVA tetramer-positive cells was determined by acquiring the entire bound fraction of enriched cells. In cases where there was an excess of OVA tetramer-positive cells that spilled into the flow-through, 1/5 of the flow-through samples were also acquired on the flow cytometer and collected cell numbers multiplied accordingly and added

to the total in the bound fraction numbers. Flow cytometry data was analyzed using FlowJo Software (Tree Star, Inc).

OVA-specific Antibody Responses

Blood was collected by retro-orbital bleeding and sera prepared and stored at −20°C. OVA-specific IgG2c, IgG2b, IgG1, IgG3, or IgM antibodies in serum were measured by ELISA, as previously described [64]. Briefly, high-protein binding plates (Costar) were coated overnight with ovalbumin protein in 0.1M $NaHCO_3$. After incubation in 10% FBS/PBS for 1h at 37°C, the plates were washed twice with 0.05% Tween20/PBS, and serum samples were added in serial dilution in 10% FBS/PBS. After incubation for 2h at 37°C, plates were washed four times before the addition of biotin-conjugated Ab specific for IgG2c, IgG2b, IgG1, IgG3, or IgM (BD Bioscience, eBioscience, and Biolegend). After incubation for 1h at 37°C, plates were washed six times and incubated for 1h at 37°C with HRP-conjugated streptavidin (Sigma-Aldrich). Plates were washed eight times and an HRP substrate (*O*-Phenylenediamine dihydrochloride, Sigma-Aldrich) was used to develop the plates. After sufficient color change was observed, the reaction was stopped by adding 100μl 1N H_2SO_4 and plates were analyzed using a spectrophotometer (Spectra Max M2, Molecular Devices).

Adoptive transfer and infection

Spleen, inguinal, axillary, brachial, and mesenteric lymph nodes were harvested from OT-II TCR transgenic mice and red blood cells were lysed using ACK lysing buffer (Lonza). Approximately $1–2\times10^6$ cells were transferred intravenously into C57BL/6 mice. The following day, mice were injected intravenously with either 10μg ovalbumin (OVA) plus 10μg LPS or 5×10^5 χ4550-OVA (St-OVA) plus 10μg OVA and 10μg LPS.

Statistical Analysis

Statistical analysis was performed using unpaired t tests (Prism 5, GraphPad Software, Inc.). Statistical differences between groups are highlighted with *, $P < 0.05$; **, $P < 0.01$; or ***, $P < 0.001$.

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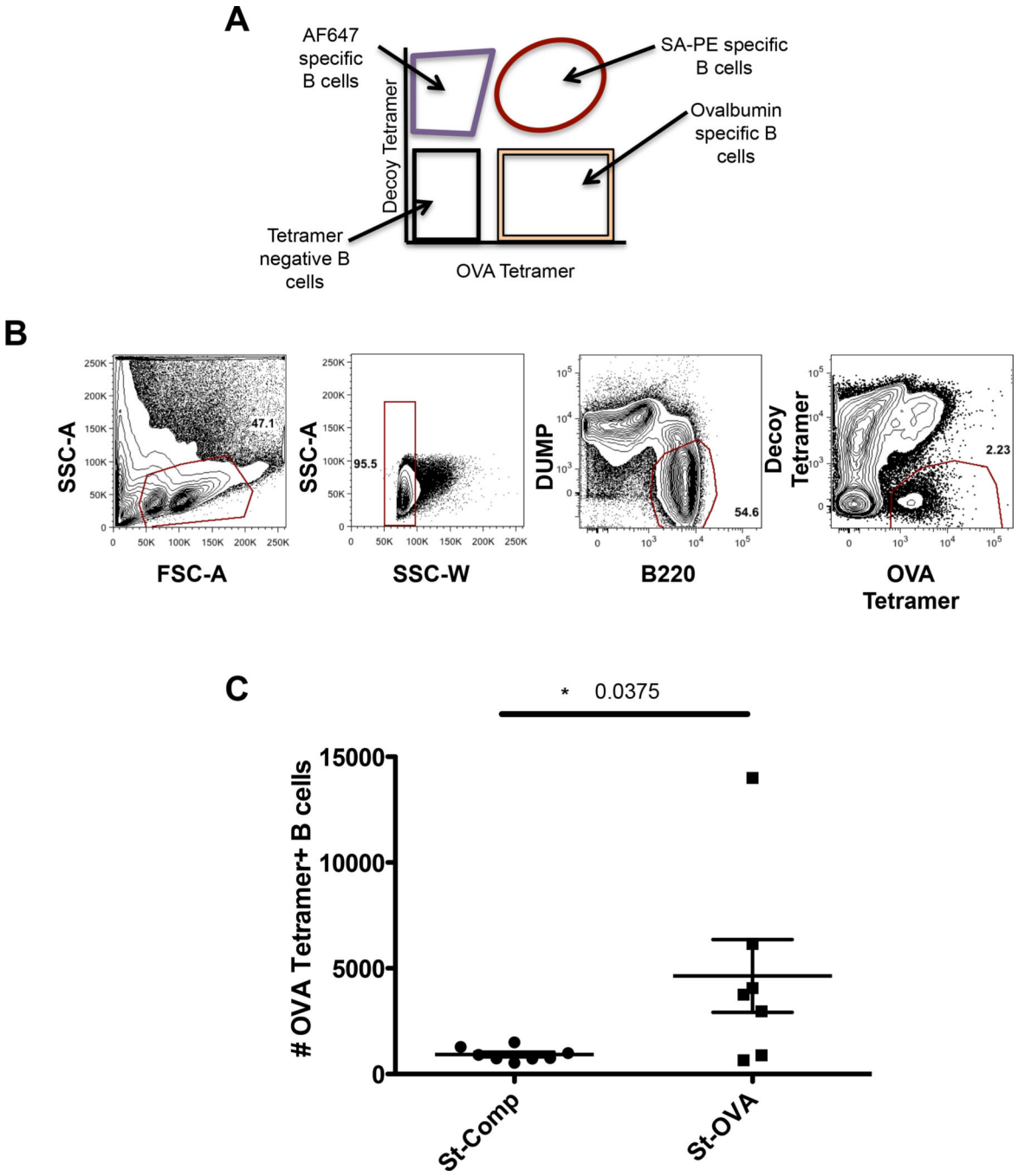

Figure 1. Ovalbumin B-cell tetramers can be used to directly visualize *Salmonella*-specific B-cell responses

(A) Description of the flow cytometry plot distinguishing OVA Tetramer$^+$ cells from SA-PE-specific and AF647-specific B cells. (B) The gating strategy for identifying OVA-specific B cells consisted of an initial lymphocyte gate, followed by a singlet gate and a dump channel containing CD11c, CD11b, F4/80, CD4, CD8, Gr-1. After gating on B220$^+$/Dump cells, OVA-specific B cells were identified by gating on OVA Tetramer$^+$Decoy Tetramer cells. (C) Mice were infected with 5×10^5 St-OVA or St-Comp and the number of OVA-specific B cells at the peak of the response was enumerated. Data are shown as mean

± SEM and were combined from two independent experiments with a total of seven to eight mice per group. Statistical significance was determined by unpaired *t* test between the indicated groups.

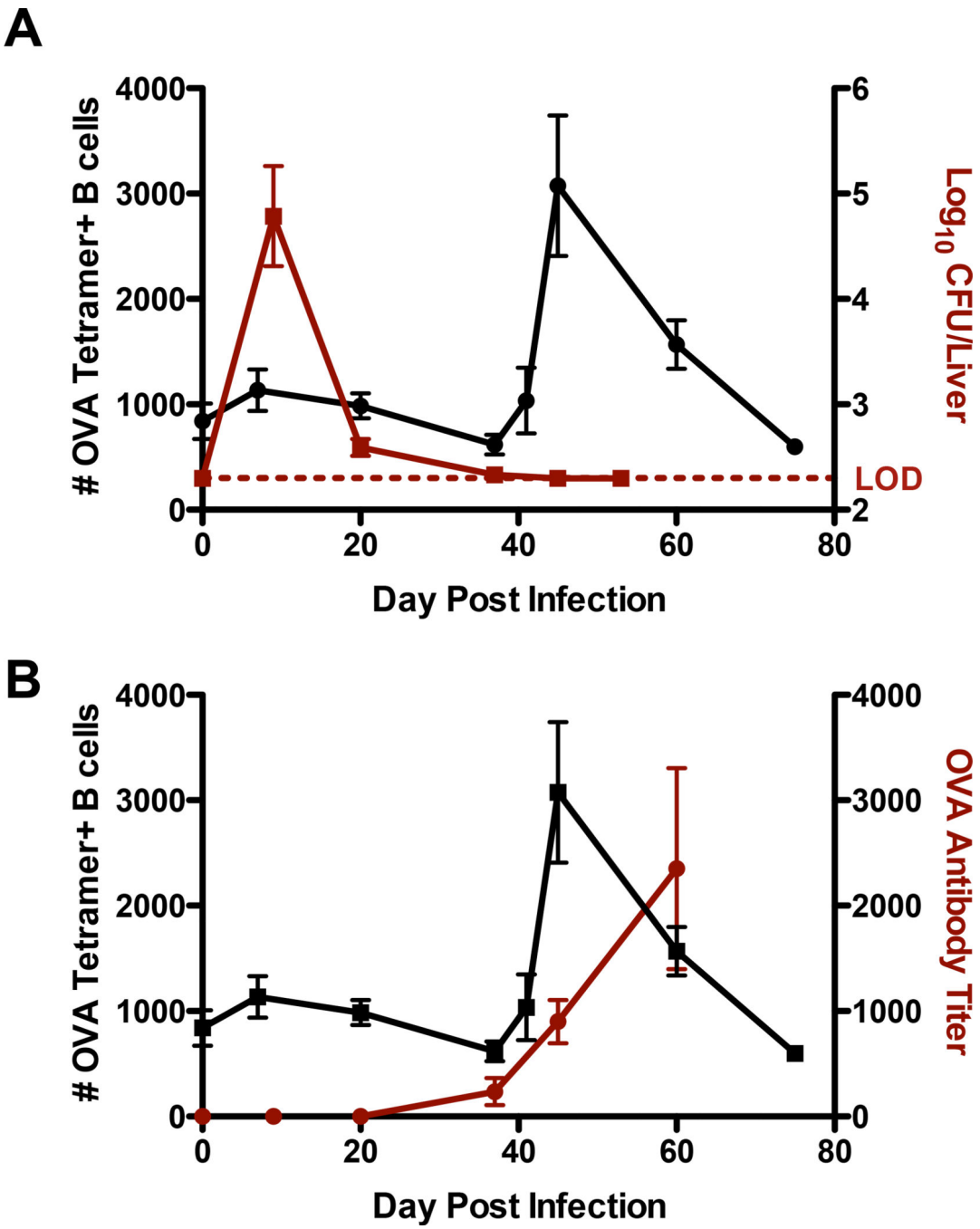

Figure 2. The peak of OVA-specific B-cell expansion occurs after *Salmonella*-OVA infection has resolved

Mice were infected IV with 5×10^5 St-OVA and at the indicated times after infection the spleen and lymph nodes were harvested and OVA Tetramer staining and flow cytometry was performed as described in Figure 1A and B. (A) Numbers of OVA-specific B cells are enumerated (black line). Livers of infected mice were collected, homogenized and plated on MacConkey agar to determine bacterial load at the indicated times post-infection. Data were pooled from one to three independent experiments with a total of two to nine mice at each time point. (B) At the indicated times post IV infection with 5×10^5 St-OVA, serum was

collected and OVA-specific IgG antibody titers (mixed antibodies: IgG1, IgG2c, IgG2b, IgG3) determined via ELISA. Data represent the mean ± SEM of two to nine mice at each time point.

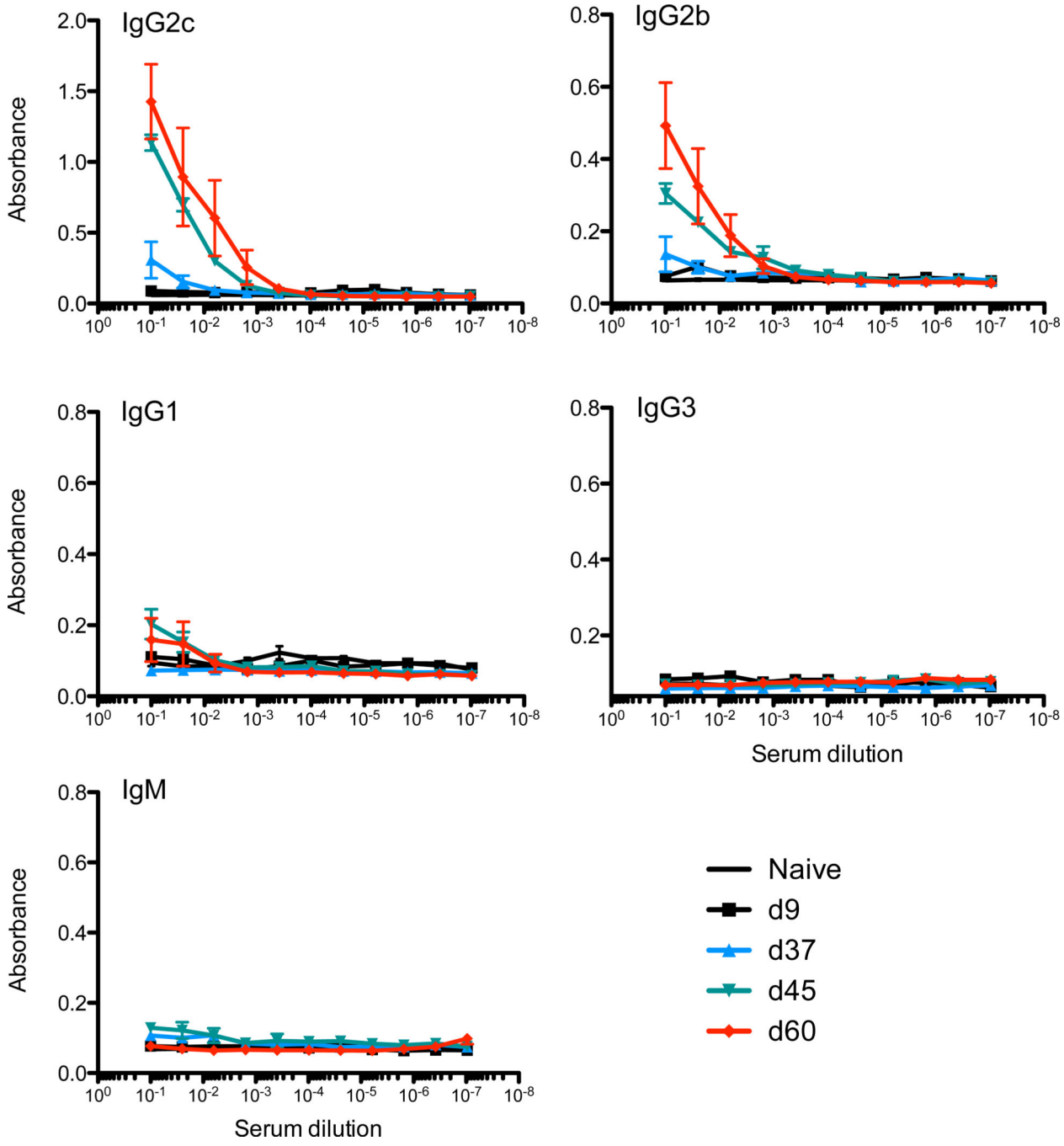

Figure 3. OVA-specific antibody responses

Mice were infected with 5×10^5 St-OVA and sera were collected at day 9, 37, 45, and 60 post-infection. The presence of OVA-specific IgG2c, IgG2b, IgG1, IgG3, or IgM was determined by ELISA. The absorbance at OD490 of respective OVA-specific antibodies is shown. Data represent the mean ± SEM of four mice per group, and are representative of two independent experiments.

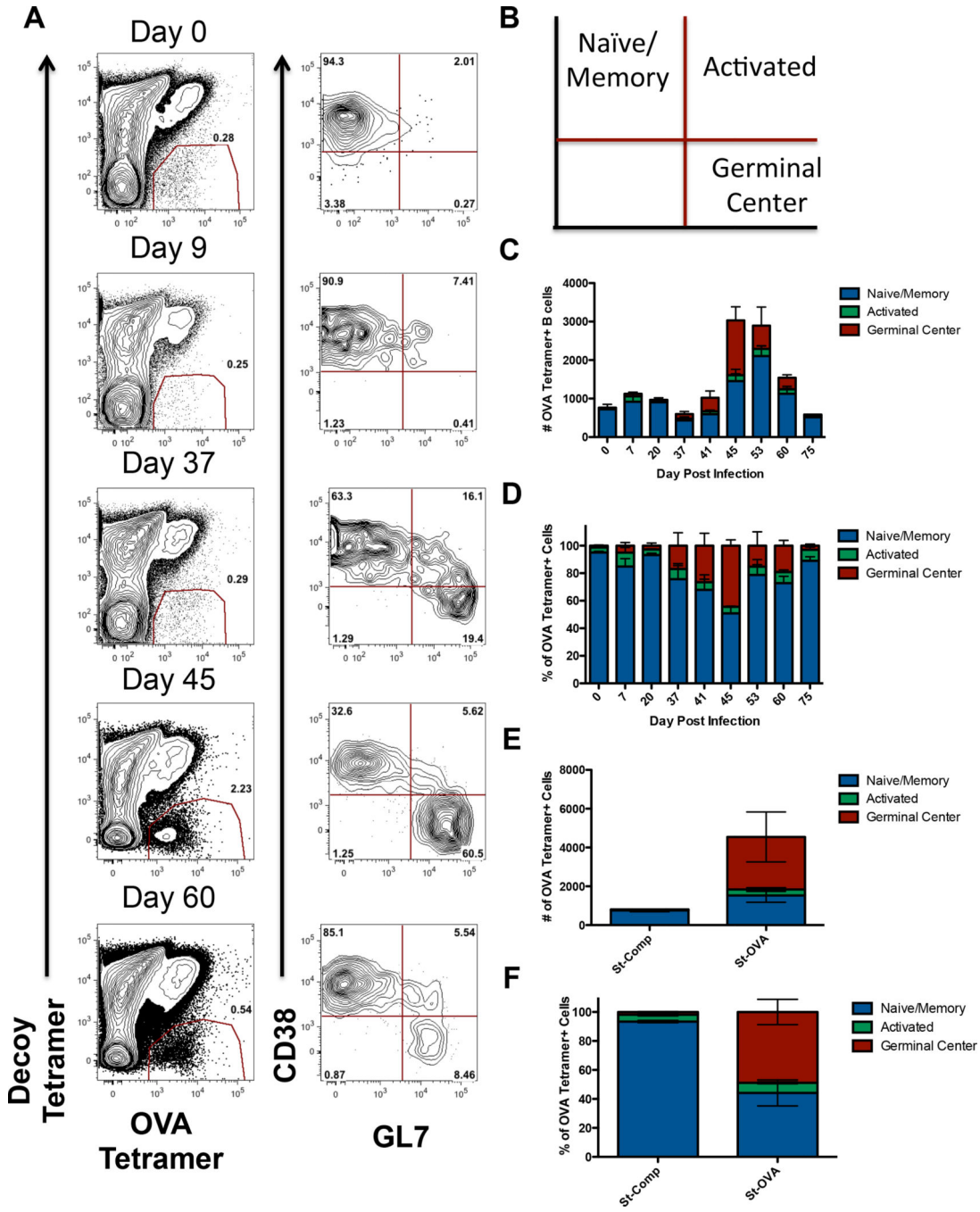

Figure 4. The peak of OVA-specific germinal center formation occurs after *Salmonella*-OVA infection has resolved

(A–D) Mice were infected with 5×10^5 St-OVA. Spleen and lymph nodes were harvested at the indicated times following infection and stained with OVA and Decoy tetramers to identify OVA-specific B cells as described in Figure 1A and B. Cells were further stained with CD38 and GL7. (A) Representative flow cytometry plots show staining of OVA-specific B cells, which were further examined with staining of GL7 and CD38 at the indicated time points. (B) Plot shows gating strategy used for examining the following populations. $CD38^+GL7^-$ cells represent the naïve and memory B cells, $CD38^+GL7^+$ cells

are activated B cells and $CD38^{lo}GL7^{+}$ cells represent B cells in a germinal center reaction. (C) The numbers of OVA-specific B cells with a naïve/memory, activated or germinal center phenotype were enumerated. (D) The percentage of OVA-specific B cells with naïve/memory, activated or germinal center phenotype are shown. (C and D) Data represent mean + SEM and were combined from one to three separate experiments with a total of two to nine mice per time point. (E and F) Mice treated as in (A) or infected with 5×10^5 St-Comp. (E) The number of OVA-specific B cells with a naïve/memory, activated or germinal center phenotype 45–49 days post-infection are shown. (F) The percentage of OVA-specific B cells with a naïve/memory, activated or germinal center phenotype 45–49 days post-infection are shown. (E and F) Data represent mean+ SEM and were pooled from two independent experiments with a total of seven to eight mice per group.

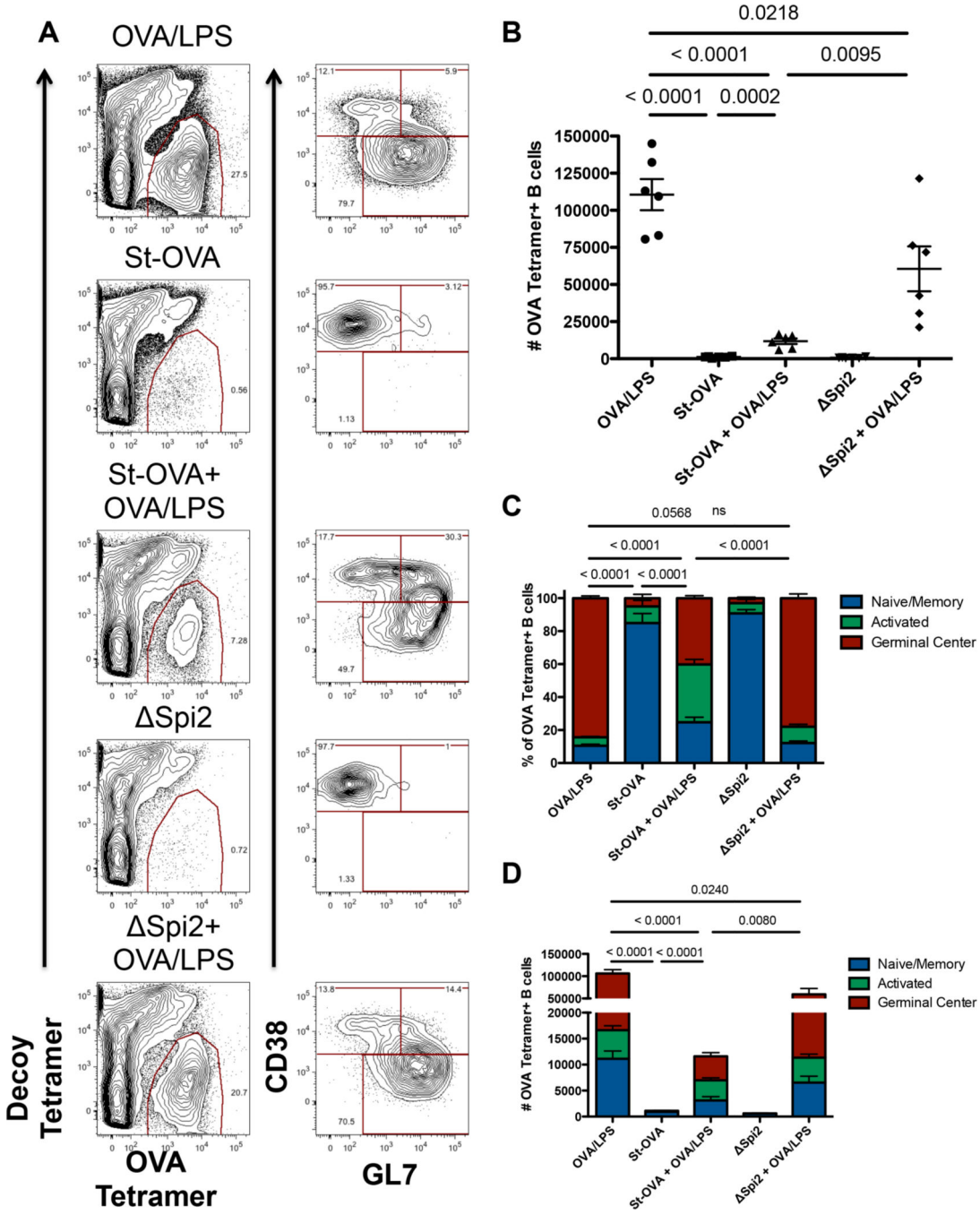

Figure 5. OVA-specific B-cell expansion and activation during immunization are inhibited by *Salmonella* and require SPI2 genes

Mice were infected IV with 5×10^5 St-OVA or ΔSPi2 and/or immunized with 10 μg OVA and 10 μg LPS. Seven days post-infection/immunization, the spleen and lymph nodes were harvested and OVA Tetramer staining and flow cytometry was performed as described in Figure 1A and B. $CD38^+GL7^-$ cells represent the naïve and memory B cells, $CD38^+GL7^+$ cells are activated B cells and $CD38^{lo}GL7^+$ cells represent B cells in a germinal center reaction. (A) Representative flow cytometry plots of the indicated infection/immunization strategies are shown. (B) The numbers of OVA-specific B cells in the indicated groups were

enumerated. (C) The percentage of OVA-specific B cells in the indicated groups with naïve/memory, activated or germinal center phenotype are shown. (D) The number of OVA-specific B cells in the indicated groups with naïve/memory, activated or germinal center phenotype is shown. (B–D) Data were combined from three independent experiments with a total of six mice per group. P values reflect statistical analysis via unpaired *t* test between numbers of germinal center phenotype cells.

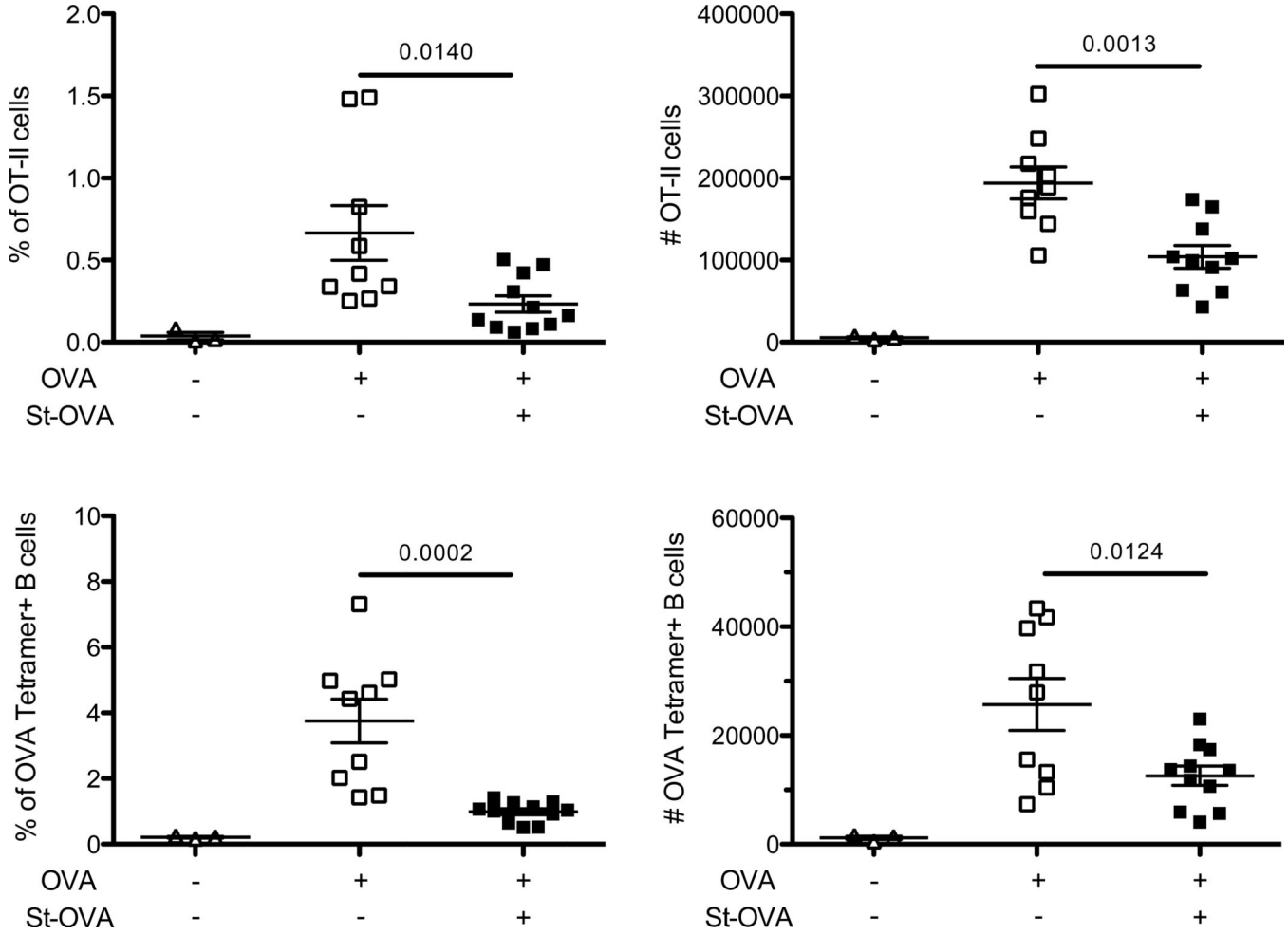

Figure 6. *Salmonella* infection inhibits OVA-specific T-cell response

C57BL/6 mice were adoptively transferred with OT-II TCR transgenic T cells. The following day, recipient mice were injected IV with either 10 μg OVA/10 μg LPS or 5×10^5 St-OVA plus 10 μg OVA/10 μg LPS. On day 7, the spleen and lymph nodes were harvested and cells were stained with antibodies specific for CD4 and CD90.1 to detect OVA-specific $CD4^+$ T cells. OVA Tetramer staining was also performed to detect OVA-specific B cells as described in Figure 1A and B. The percentage (left) and absolute number (right) ± SEM of OVA-specific $CD4^+$ T cells (top row) and OVA-specific B cells (bottom row) are shown. Data are pooled from three independent experiments with a total of nine to twelve mice in each group. Statistical significance was determined by unpaired *t* test between the indicated groups and p values are displayed.

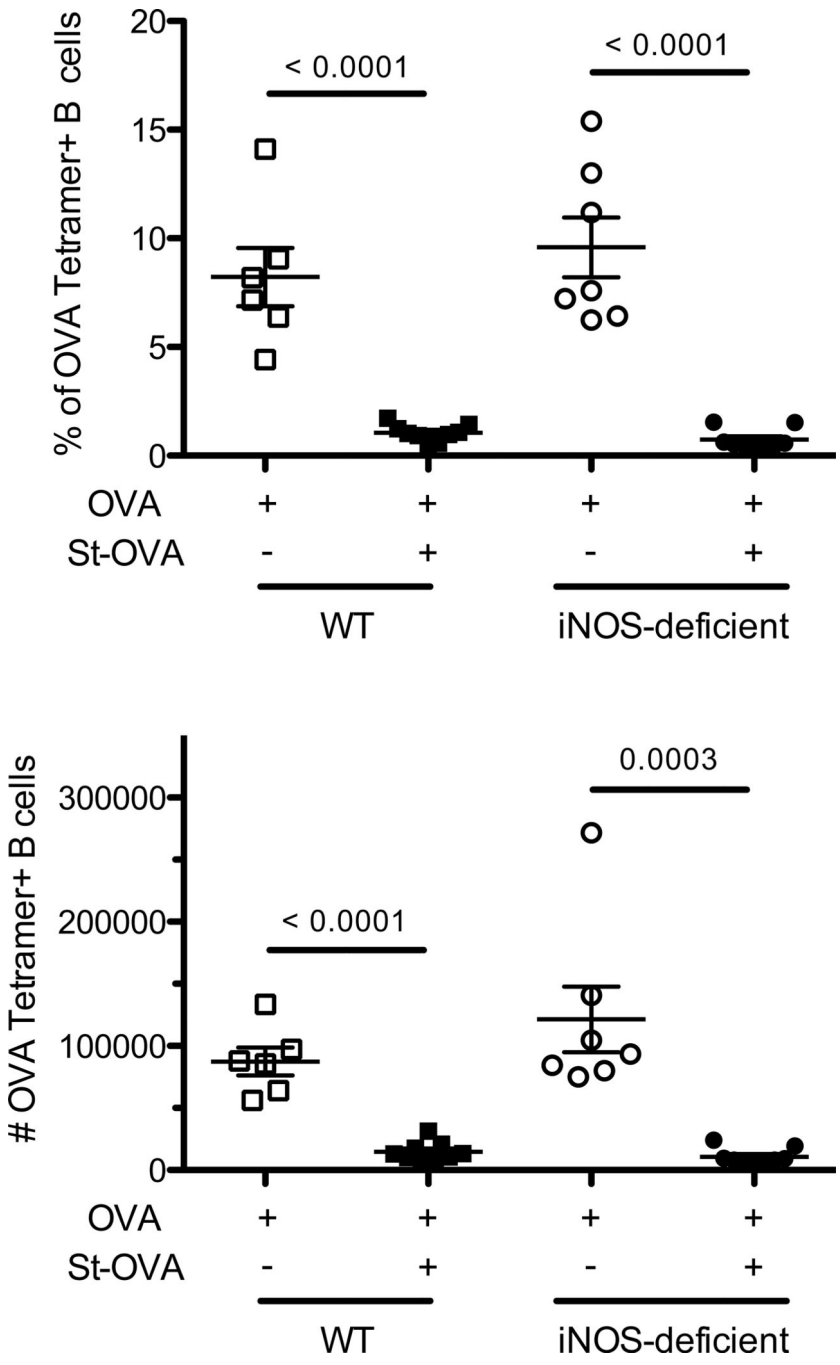

Figure 7. OVA-specific B-cell expansion occurs in *Salmonella*-infected iNOS-deficient mice

C57BL/6 (WT) and iNOS-deficient mice were injected IV with either 10 μg OVA/10 μg LPS or 5×10^5 of St-OVA plus 10 μg OVA/10 μg LPS. On day 7, the spleen and lymph nodes were collected and cells were stained with OVA Tetramer to detect OVA-specific B cells as described in Figure 1A and B. The percentage (top) and absolute number (bottom) ± SEM of OVA-specific B cells are shown. Data are combined from three independent experiments with a total of six to nine mice in each group. Statistical significance was determined by unpaired *t* test between the indicated groups and p values are displayed.

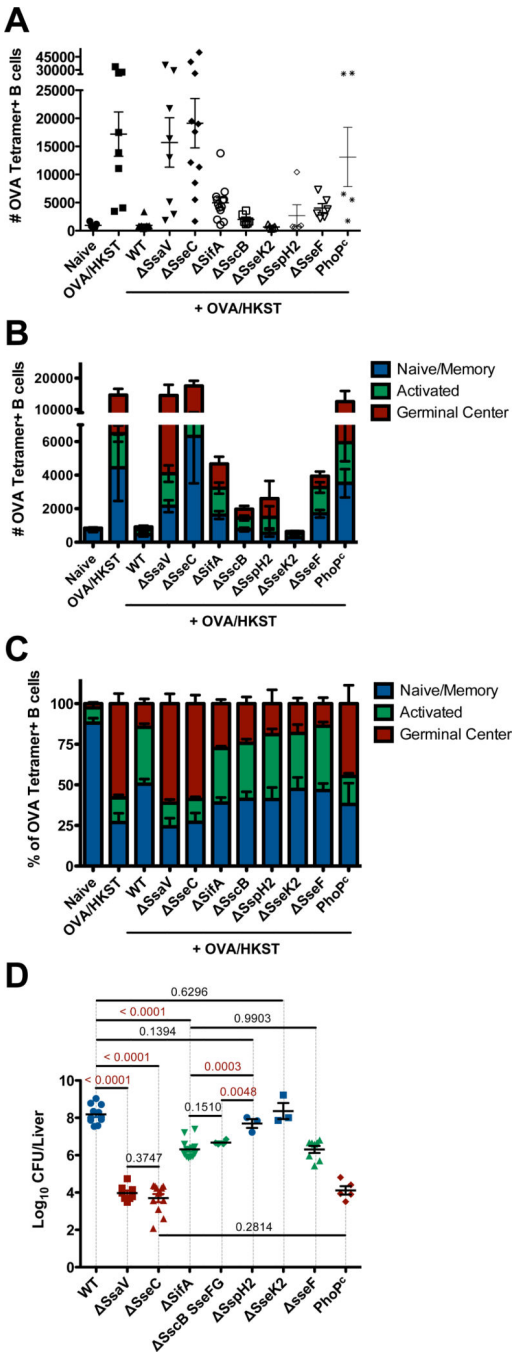

Figure 8. SPI2 effector proteins SifA and SseF inhibit expansion and germinal center formation of OVA-specific B cells

Mice were infected IV with 5×10^3 of the indicated *S*. typhimurium Wild Type or SPI2 effector mutant strains along with 10 μg OVA/10^8 HKST. Seven days post-infection/ immunization, the spleen and lymph nodes were harvested and OVA-Tetramer staining and flow cytometry were performed as described in Figure 1A and B. $CD38^+GL7^-$ cells represent the naïve and memory B cells, $CD38^+GL7^+$ cells are activated B cells and $CD38^{lo}GL7^+$ cells represent B cells in a germinal center reaction. (A) The numbers of

OVA-specific B cells in the indicated groups were enumerated. (B) The number of OVA-specific B cells in the indicated groups with naïve/memory, activated or germinal center phenotype are shown. (C) The percentage of OVA-specific B cells in the indicated groups with naïve/memory, activated or germinal center phenotype are shown. (A–C) Data represent mean + SEM and were combined from two to six independent experiments with a total of five to eleven mice in each group. (D) Seven days post-infection livers of infected mice were collected, homogenized, and plated on MacConkey agar to determine bacterial load. Data were pooled from one to four separate experiments with a total of three to fourteen mice per group. (A–D) Statistical significance was determined by unpaired *t* test between the indicated groups and p values are as displayed.

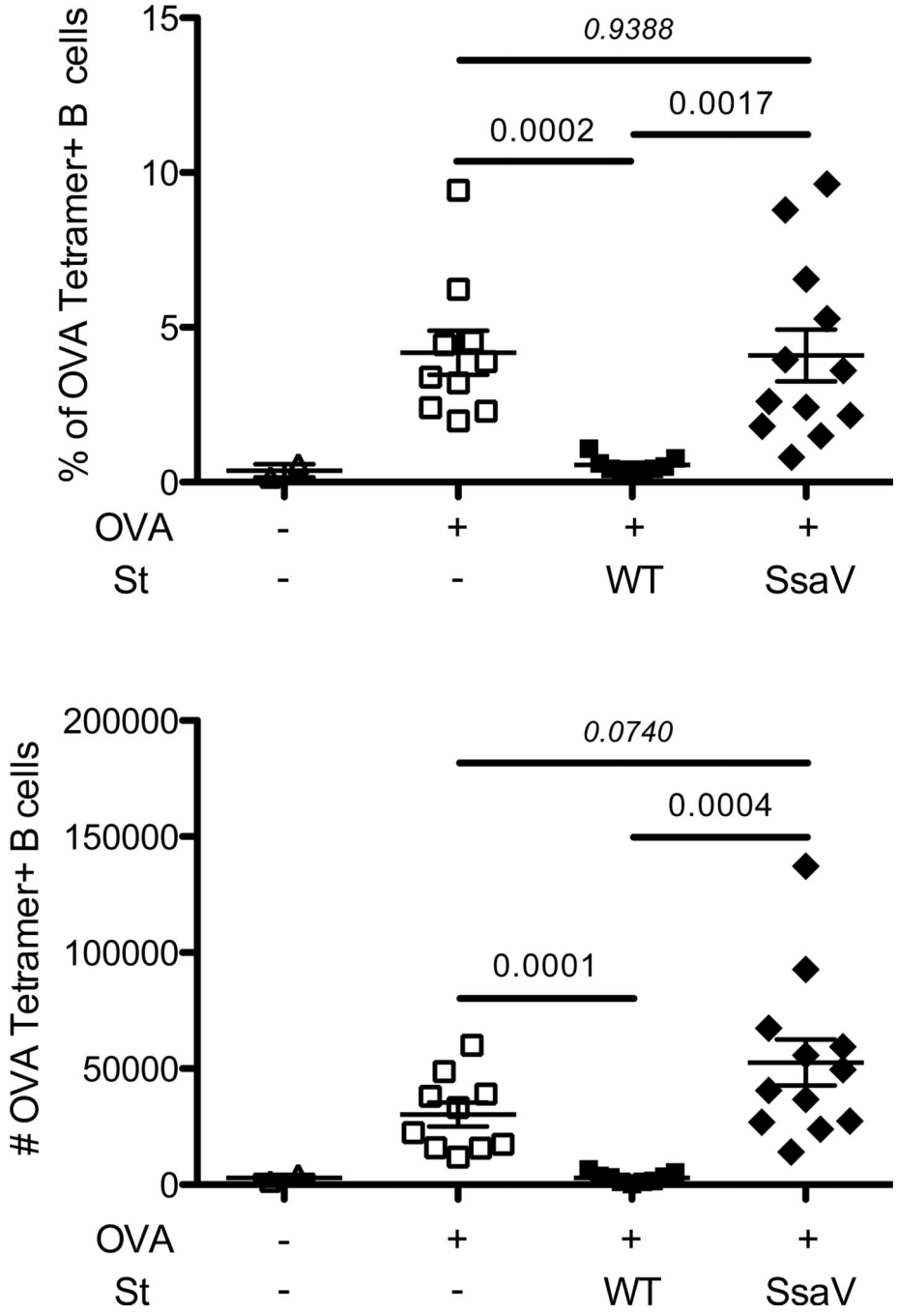

Figure 9. High dose infection with a SPI2 mutant does not inhibit expansion of OVA-specific B cells

Mice were infected IV with 2×10^3 of NCTC 12023 (WT) or 1×10^5 of SPI2 effector mutant strain Δ*ssaV* (SsaV) along with 10 μg OVA/10^8 HKST. On day 7, spleen and lymph nodes were harvested and OVA Tetramer staining was performed as described in Figure 1A and B. The percentage (top) and absolute number (bottom) ± SEM of OVA-specific B cells are shown. Data were pooled from three to four independent experiments with a total of nine

to twelve mice in each group. Statistical significance was determined by unpaired *t* test between the indicated groups and p values are displayed.

Designation	Relevant genotype	Source
S. Typhimurium strains		
χ4550	SR-11 *gyrA1816* Δ*asdA1*[*zhf-4*::Tn10] Δ*crp-1* Δ*cya-1*	{Schodel, 1994 #1836}
SPN450	IR715 Δ*spiB*(+25 to +1209)::KSAC	{Raffatellu, 2009 #1189}
SPN524 (ΔSPI2)	χ4550 Δ*spiB*(+25 to +1209)::KSAC	This study
NCTC 12023	Wild Type	
HH104	Δ*sseC::aphT*	{Hensel, 1998 #1837}
HH107	Δ*sseF::aphT*	{Hensel, 1998 #1837}
HH108	Δ*sseG::aphT*	{Hensel, 1998 #1837}
MvP373	Δ*sscBsseFG::aph*	{Kuhle, 2004 #1838}
MvP376	Δ*sspH2::aph*	{Chakravortty, 2002 #1839}
MvP389	Δ*sifB*	{Chakravortty, 2002 #1839}
MvP390	Δ*sspH1*	{Chakravortty, 2002 #1839}
MvP392	Δ*sseJ*	{Chakravortty, 2002 #1839}
MvP393	Δ*sseI*	{Chakravortty, 2002 #1839}
MvP394	Δ*slrP*	{Chakravortty, 2002 #1839}
MvP498	Δ*pipB2::aph*	{Halici, 2008 #1831}
MvP505	Δ*sopD2::aph*	{Halici, 2008 #1831}
MvP509	Δ*sifA::aph*	{Halici, 2008 #1831}
MvP570	Δ*sseK1::aph*	{Halici, 2008 #1831}
MvP571	Δ*sseK2::aph*	{Halici, 2008 #1831}
MvP873	Δ*gogB*	{Halici, 2008 #1831}
MvP874	Δ*pipB*	{Halici, 2008 #1831}
PhoP^{c}	Constitutive PhoP/Q expression	{Miller, 1990 #1840}
Plasmids		
pYA3149-Empty	*asdA*+	{Schodel, 1994 #1836}
pYA3149-OVA	*asdA*+, OVA expression	{Chen, 1999 #165}