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Duel immunization with SseB/flagellin provides enhanced protection against *Salmonella* infection mediated by circulating memory cells

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Abstract

The development of a sub-unit *Salmonella* vaccine has been hindered by the absence of detailed information about antigenic targets of protective *Salmonella*-specific T and B cells. Recent studies have identified SseB as a modestly protective antigen in susceptible C57BL/6 mice, but the mechanism of protective immunity remains undefined. Here, we report that simply combining *Salmonella* SseB with flagellin substantially enhances protective immunity, allowing immunized C57BL/6 mice to survive for up to 30 days following challenge with virulent bacteria. Surprisingly, the enhancing effect of flagellin did not require flagellin antigen targeting during secondary responses or recognition of flagellin by TLR5. While co-immunization with flagellin did not affect SseB-specific antibody responses, it modestly boosted CD4 responses. In addition, protective immunity was effectively transferred in circulation to parabionts of immunized mice, demonstrating that tissue resident memory is not required for vaccine-induced protection. Finally, protective immunity required host expression of IFN-γR but was independent of iNOS expression. Taken together, these data indicate that *Salmonella* flagellin has unique adjuvant properties that improve SseB-mediated protective immunity provided by circulating memory.

Introduction

Salmonella enterica serovars infect humans, pets, livestock, and poultry, causing a wide variety of clinical diseases, depending on the serovar involved and the underlying susceptibility of the host (1, 2). In the US, the economic impact of *Salmonella* infection is substantial and periodic multi-state outbreaks of gastroenteritis occur due to the ingestion of contaminated produce, meat, or processed foods (3). Public health measures have improved food handling and limited the dissemination of livestock waste, while vaccines have also been developed to reduce *Salmonella* carriage in livestock and poultry (4, 5). Despite these efforts, *Salmonella* infections cause over 1 million illnesses in the US every year (6).

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The impact of *Salmonella* infection is considerably greater in low-income countries that lack access to clean water and basic sanitation. In this environment, human-restricted serovars *Salmonella enterica* Typhi and Paratyphi can be transmitted, causing a life-threatening systemic disease known as typhoid or enteric fever (7). Recent estimates suggest that typhoid afflicts 21.65 million people and causes 433,000 deaths annually, with most cases localized to south, and southeast, Asia (8, 9). In contrast, non-typhoidal *Salmonella* serovars typically cause gastroenteritis, but are also responsible for disseminated infections of young children or adults with compromised immunity, referred to as invasive Non-Typhoidal Salmonellosis (iNTS) (10, 11). Importantly, iNTS strains are the most common bacterial isolates recovered from febrile presentations in adults and children in Sub-Saharan Africa (12). In fact, estimates suggest that iNTS is responsible for 564,000 deaths annually, meaning that typhoidal and iNTS infections together cause almost 1 million deaths annually (9). Although these systemic bacterial infections can often be successfully treated with antimicrobials, *Salmonella* serovars are increasingly resistant to multiple antibiotics and the development of an effective vaccine for vulnerable populations is required (8).

Vaccine development for typhoid has largely focused on improving the efficacy of live attenuated (Ty21a) and Vi polysaccharide (ViCPS) vaccines. These licensed vaccines are only moderately effective (50–60% over 3 years), poorly immunogenic in infants, and neither is widely used in endemic areas (8, 13–16). Ty21a is a live vaccine strain (LVS) of *S.* Typhi administered in four individual doses to patients older than 5 years of age. While attempts have been made to improve the safety and immunogenicity of LVS of *S.* Typhi, this has proved unexpectedly difficult to achieve (13). Furthermore, there are natural impediments to administering live *Salmonella* vaccines to infants, the elderly, HIV-positive individuals, and immune-suppressed populations (17, 18). ViCPS, is a purified capsule polysaccharide (CPS) that can effectively curtail a typhoid outbreak and provides protection to travelers visiting endemic areas (17, 18). The major limitation of this vaccine is that it induces short-term T cell-independent antibody responses and does not induce long term immunological memory (19, 20). Next-generation ViCPS-conjugate vaccines are being studied in clinical trials and one has already been licensed in India (16). While these new Vi-conjugate vaccines should extend the duration of immunity provided by ViCPS, they will be unable to protect against iNTS or Paratyphoid serovars since these bacteria lack expression of the Vi CPS (21). Thus, in order to develop new vaccines for typhoid and iNTS, viable alternatives to LVS and ViCPS vaccines need to be explored.

Rational vaccine development can be informed by detailed knowledge of antigen targeting in immune individuals (22). Indeed, understanding of antigen targeting in human and murine *Salmonella* infection has recently improved and sub-unit vaccine development for *Salmonella* has received greater attention (23–28). It has long been known that common bacterial products like LPS and flagellin are targeted by host antibody responses during *Salmonella* infection, making these serological responses incredibly useful for diagnostic purposes (29). Studies in a mouse model of *Salmonella* infection have confirmed that flagellin is a major target antigen of antibody and CD4 T cell responses, and that flagellin can be modestly protective when used to immunize susceptible mice (30–32). Many outer membrane proteins (OMP) of *Salmonella* have also been identified as targets of the adaptive immune response and several of these proteins have similar modest protective efficacy to

flagellin (33–35). Large proteomic studies have provided a more comprehensive understanding of *Salmonella* protein targeting by the adaptive immune system and several antigen targets show limited protective efficacy when examined in mice (25, 26, 28). One surprise to emerge from these studies is that components of the *Salmonella* Type-III-secretion System (T3SS) are common targets of the adaptive immune response and can also provide limited protective immunity (23, 25, 36–39). Thus, many experiments using individual antigens have shown modest protective efficacy as part of a sub-unit vaccine formulation. However, it is not yet clear whether a combination of multiple *Salmonella* antigens could match the much greater protective efficacy provided by a LVS *Salmonella* immunization.

Here, we report that immunizing mice with two *Salmonella* proteins, SseB and flagellin, vastly improves the overall protection against bacterial challenge. This enhanced protection required an adjuvant effect of flagellin but did not require host TLR5 expression. The enhanced protection mediated by SseB/flagellin immunization also correlated with modestly increased CD4 T cell response to SseB and was transferrable to naïve mice via circulation. Finally, this protective response required host expression of IFN-γR, but did not require iNOS expression.

Materials and Methods

Mouse strains

C57BL/6 mice were purchased from the National Cancer Institute or the Jackson Laboratory and used at 8–16 weeks of age. IFNγR-deficient (IFNγR-KO) and iNOS-deficient (iNOS-KO) mice were purchased from the Jackson Laboratory. TLR5-deficient mice (TLR5-KO) were bred at the University of California Davis from a line developed in the Akira laboratory (40) and these mice do not suffer from basal inflammatory or metabolic defects (41). All mice were cared for in accordance with University of California Davis Animal guidelines.

Bacterial strains and infection

Attenuated *Salmonella typhimurium* strain BRD509 (AroA$^-$) was provided by Dr. D. Xu, (University of Glasgow, Glasgow, U.K) (42). Flagellin-deficient *S. typhimurium* strain BC490 was a gift from Dr. B. Cookson, (University of Washington, Seattle, WA) (31). LPS-deficient *Salmonella typhimurium* χ4700 was a kind gift from Dr. R Curtiss (Arizona State University, Tempe, AZ). *Salmonella* were cultured overnight in LB broth without shaking and diluted in PBS after an estimation of bacterial concentration using a spectrophotometer. For immunization experiments, 5×10^5 BRD509 was administered intravenously in the lateral tail vein. Immunized mice were challenged with 1000 wild-type *Salmonella typhimurium* SL1344 intravenously or 1×10^6 SL1344 administered orally by gavage. The administered dose of bacteria was confirmed by plating serial dilutions onto MacConkey agar plates counting colonies after overnight culture at 37°C. Infected mice were monitored daily and mice were euthanized if the developed a moribund state (unresponsive to gentle prodding). To assess bacterial colonization, spleens and livers from infected mice were homogenized in PBS and serial dilutions were plated onto MacConkey agar plates. After overnight

incubation at 37°C, bacterial plates were counted and bacterial burdens were calculated for each individual organ.

Generation of recombinant and purified proteins

Salmonella antigen, SseB, was cloned from *Salmonella* genomic DNA and inserted into the His-tag pRSET vector and overexpressed in *E.coli* BL21star DE3 cells (ThermoFisher Scientific). To generate an SseB protein expressing the 2W1S epitope (EAWGALANWAVDSA) (SseB-2W), the 2W1S sequence was cloned in-frame onto the C-terminus of SseB and expressed in the same *E. coli* expression system. Additional *Salmonella* antigens, CirA, IroN, and SlyB were similarly cloned and expressed in *E. coli*. Recombinant *E.coli* strains were cultured in Luria-Bertani (LB) broth and harvested after 14 hours of 1mM IPTG induction. The bacteria pellet was resuspended with BugBuster (EMD Millipore) and inclusion bodies resuspended with 8M Urea buffer. Recombinant proteins were purified using ProBond (ThermoFisher Scientific) or a Ni-NTA His-Bind resin (EMD Millipore) according to the manufacturer's protocol before dialysis against 1X PBS to remove traces of urea. Samples were subsequently concentrated using Amicon centrifugal instruments (EMD Millipore) and protein concentration were determined using BCA method (ThermoFisher Scientific). Since the methodology required the use of *E. coli* LPS as an adjuvant for immunization, residual LPS was not specifically removed from recombinant proteins prior to use.

Flagellin purification

LPS-deficient *Salmonella typhimurium* χ4700 was used to purify flagellin utilizing a modified acid-shock protocol (43, 44). Briefly, an overnight bacteria culture was spun down, washed, and resuspended in HCl/PBS (pH2) for 30 min at room temperature. Supernatants were collected and flagellin was harvested by ultracentrifugation and ammonium sulfate precipitation. Monomeric flagellin was prepared by depolymerizing samples at 70°C for 1hr and LPS was removed using detoxigel columns.

Immunization

LPS was purchased from Axxora, MPLA from Invivogen, Alum from ThermoFisher Scientific, and Freund's complete and incomplete adjuvants from Sigma-Aldrich. Mice were immunized via the lateral tail vein at four-week intervals with 100μg of recombinant protein (SseB or SseB-2W1S) and/or 100μg of purified flagellin mixed with 10μg of LPS or other adjuvants. In some experiments, mice were immunized sub-cutaneously with SseB, flagellin, IroN, CirA, or SlyB combined with CFA during the primary immunization and IFA during boosting, 4 weeks later.

Antibody ELISA

IgG2c responses to SseB immunization were measured using an ELISA method. Briefly, 96-well microtiter plates were coated with 10μg/ml of recombinant SseB and serum samples were added in serial dilution in 10% FBS/PBS. After incubation for 2 hours at 37°C, plates were washed four times with 0.05% Tween 20/PBS before the addition of biotin-conjugated Ab specific for the anti-mouse IgG2c (BD Bioscience). After a further incubation for 1 h at

37°C, plates were washed six times and incubated for 1 h at 37°C with HRP-conjugated streptavidin Ab (Extravidin, Sigma-Aldrich) diluted in 10% FBS/PBS. Plates were then washed eight times and an HRP substrate (O-Phenylenediamine dihydrochloride, OPD, Sigma-Aldrich) was used to develop the plates. After sufficient color change was observed, the reaction was stopped by adding 50μl of 2N H_2SO_4 and plates were analyzed using a spectrophotometer (SpectraMax M2, Molecular Devices).

Tetramer staining of 2W1S-specific CD4 T cells

PE-conjugated 2W1S::I-A^b tetramer was generated in house from 2W1S::I-A^b monomers produced in insect cells, as previously described (45, 46). Spleen and livers were harvested and stained with PE-conjugated 2W1S::I-A^b tetramer in the presence of Fc block (culture supernatant from the 2.4G2 hybridoma, 2% mouse serum, 2% rat serum) room temperature for one hour. After washing with 2% FBS/PBS, cells were stained with fluorochrome-conjugated antibodies specific for CD3, CD4, CD8, and CD44 (Affymetrix). Cells were then analyzed by flow cytometry using a BD LSR Fortessa (BD Biosciences). All data sets were analyzed using FlowJo software (Tree star).

Parabiosis experiments

Mice were immunized and boosted with SseB and flagellin mixed with MPLA, and 60 days later mice were surgically joined to generate a shared circulation. Parabionts were housed together for 28 days, allowing for the formation of an anastomosis and adequate transfer of vascularized lymphocytes between animals. Mice were then separated and allowed 2 weeks to recover from separation surgery. Post recovery, mice were challenged with 1000 virulent *Salmonella* (SL1344) intravenously and 5 days later all groups of mice were euthanized and bacterial burdens measured in tissues, as described above.

Statistical Analyses

Statistical differences between groups of normally distributed data were examined using Prism (GraphPad). Experimental groups were compared using an unpaired *t* test and were considered significantly different with a p value of <0.05.

Results

Duel SseB and flagellin immunization enhances protection against *Salmonella* infection

IgG responses to 117 *Salmonella* target antigens were previously identified by probing a proteomic array with sera from *Salmonella*-infected mouse strains and humans with iNTS (25). However, when examined individually, many of these immune-dominant antigens provided little or no protection to susceptible C57BL/6 mice after *Salmonella* challenge (data not shown). This would suggest that immunogenicity is a poor predictor of any target antigen's protective efficacy in *Salmonella* infection. Indeed, a recent study examining another large protein set for protective efficacy in BALB/c mice has reached similar conclusions (26). Indeed, it seems possible that *Salmonella* specifically direct host immune responses onto a set of largely irrelevant non-protective antigens (47), further complicating sub-unit vaccine development.

Several studies report that immunization with flagellin or SseB provides susceptible mice with a modest degree of protection against *Salmonella* infection (23, 25, 31, 39). However, these two protein antigens have never been directly compared or combined together for protection studies. As a positive control, prior immunization with a live vaccine strain (LVS) of *Salmonella* reduced bacterial burdens by 4 orders of magnitude and allowed the majority of mice to survive subsequent infection with virulent *Salmonella* (Fig. 1, LVS Salmonella). In contrast, C57BL/6 mice immunized and boosted with SseB and *E. coli* LPS as an adjuvant had significantly reduced bacterial burdens in the spleen and liver (Fig. 1A and B, sseB), but displayed only minimal prolonged survival. Similarly, mice immunized with flagellin plus LPS had lower bacterial burdens than unimmunized mice, although this was only statistically significant in the liver (Fig. 1A and B, flagellin). As found with SseB alone, flagellin immunization had only a minor effect on survival following *Salmonella* infection (Fig. 1E). Thus, immunization of susceptible C57BL/6 mice with individual *Salmonella* antigens provides very limited protective immunity that compares poorly to the robust protective efficacy observed with LVS immunization.

Given the modest individual efficacy of SseB or flagellin, we explored whether immunization with both antigens would enhance protective immunity. Surprisingly, duel immunization with SseB/flagellin was highly effective, especially in the liver, where bacterial burdens in SseB/flagellin-immunized mice approached levels observed in LVS-immunized mice (Fig. 1A and B, sseB/flagellin). Importantly, duel immunization with SseB/flagellin was also effective against mucosal challenge with *Salmonella*, reducing bacterial burdens to levels close to LVS-immunized mice (Fig. 1C and D). In agreement with the much larger reduction in bacterial counts observed with duel immunization, SseB/flagellin-immunized mice survived for an extended period following challenge with virulent *Salmonella* (Fig. 1E). Indeed, SseB/flagellin-immunized mice had similar survival rates to LVS-immunized mice for one month after oral challenge (Fig. 1E). Interestingly, between 1 and 3 months post-infection many of SseB/flagellin-immunized mice slowly succumbed to *Salmonella* infection (Fig. 1E). A recent report has identified several *Salmonella* antigens that were modestly protective in BALB/c mice after subcutaneous injection with CFA (26). We confirmed that some these antigens were protective in C57BL/6 mice, but addition of these new antigens did not enhance the protective efficacy of SseB/flagellin after sub-cutaneous immunization (Supp. Fig. 1). Taken together, these data demonstrate that duel immunization with SseB/flagellin strikingly elevates protection against systemic or mucosal infection with highly virulent *Salmonella*.

It seemed likely that the increased efficacy of duel SseB/flagellin immunization was due to the initiation of two adaptive immune responses against two different target antigens. Indeed, we previously reported that flagellin and another T3SS protein (SseJ), induce anatomically and functionally distinct CD4 T cell responses (36), that could work cooperatively. The alternative explanation was that the intrinsic adjuvant properties of flagellin (48), might have simply enhanced the immune response to SseB. In order to discriminate between these two possibilities, we examined whether duel SseB/flagellin immunization could protect against flagellin-deficient *Salmonella* (BC490). Unimmunized C57BL/6 mice displayed a significantly higher bacterial burden after challenge with flagellin-deficient *Salmonella* (Fig. 2A and B), but there was no difference in survival time

after oral challenge with wild-type or flagellin-deficient bacteria (Fig. 2C). Thus, the absence of flagellin modestly enhanced bacterial virulence, agreeing with previous data where forced expression of flagellin hindered bacterial replication in C57BL/6 mice (44). Interestingly, C57BL/6 mice vaccinated with either LVS *Salmonella* or SseB/flagellin displayed robust protective immunity against challenge with wild-type (Fig 2A–C, Wild type) and flagellin-deficient bacteria (Fig. 2A–C flagellin-deficient). Thus, the protective immunity mediated by SseB/flagellin immunization did not require a flagellin-specific adaptive immune response for effective *Salmonella* clearance and suggested instead that flagellin was functioning as an adjuvant.

Bacterial flagellins are recognized by the innate immune system via the surface receptor TLR5, cytosolic sensors NAIP5/NLRC4, and a poorly defined mechanism distinct from both known pathways (49–51). We next examined whether the efficacy of duel SseB/flagellin immunization required host expression of TLR5. Immunization with LVS *Salmonella* was effective at lowering bacterial burdens in the spleen and liver of both wild-type (WT) and TLR5-deficient mice (Fig. 3A and B, LVS *Salmonella*). Furthermore, almost all WT and TLR5-deficient mice immunized with LVS *Salmonella* survived challenge infection with virulent bacteria (Fig. 3C). Similarly, duel SseB/flagellin immunization lowered bacterial burdens in the spleen and liver of both WT and TLR5-deficient mice (Fig. 3A and B). As noted above, SseB/flagellin immunization allowed C57BL/6 mice to survive for an extended period after challenge infection, and this long-term survival was comparable in immunized TLR5-deficient mice (Fig. 3C). Thus, although flagellin appears to function as an effective adjuvant for SseB, this protective effect does not require host expression of TLR5.

Many studies have shown that protective immunity against *Salmonella* infection is mediated by the contribution of both *Salmonella*-specific CD4 T cells and B cells (52). Thus, we examined whether co-administration of flagellin altered T or B cell responses to SseB. In C57BL/6 mice, the *Salmonella*-specific antibody response is dominated by the IgG2c isotype (53), thus we focused attention on SseB-specific IgG2c responses. After immunization and boosting, serum SseB-specific IgG2c responses were similar in mice immunized with SseB or SseB/flagellin (Fig 4A). Thus, modification of specific IgG2c did not correlate with the observed enhanced protection. Although epitope mapping has identified an immunodominant epitope of SseB in humans (37), peptide library screening of SseB-specific T cells in our laboratory has so far failed to uncover the immunodominant epitope in mice (Lee at al, unpublished). Therefore, to examine the effect of flagellin on CD4 T cell responses to SseB, we fused the 2W1S epitope to the carboxy-terminus of SseB and used 2W1S MHC class-II tetramers to examine SseB-2W-specific CD4 T cells. A population of tetramer-specific CD4 T cells could be detected in the spleen and liver of mice immunized with SseB-2W with and without flagellin co-administration (Fig. 4B). The addition of flagellin significantly increased expansion of 2W1S-specific CD4 T cells in SseB2W-immunized mice (Fig. 4C), indicating that flagellin has a modest enhancing effect on CD4 T cell expansion to SseB immunization.

In order to gather more information on the protective immunity mediated by duel SseB/flagellin immunization, we next examined whether the protection was mediated by resident or circulating memory responses. T central memory (TCM) and T effector memory (TEM)

cells circulate in peripheral blood while Tissue resident memory (TRM) cells remain localized to tissues (54–56). The role of circulating versus resident T memory cells has been examined in many infectious diseases but has not yet been examined in *Salmonella* infection. We used parabiosis surgery of immunized congenic mice to examine whether the protective effect of SseB/flagellin immunization could be conferred to naïve mice via circulation. Unpaired C57BL/6 mice and C57BL/6 mice that had only been surgically paired both had equivalent bacterial burdens in the spleen and liver after *Salmonella* infection (Fig. 5A and B, Naïve and Surgery Control). Thus, parabiosis surgery itself does affect *Salmonella*-specific immunity. As expected, both unpaired and paired mice that had been directly immunized with SseB/flagellin had significantly lower bacterial counts in the spleen and liver compared to naïve controls (Fig. 5A and B, SseB/flagellin). Importantly, unimmunized mice that shared a circulation with immunized mice also had significantly lower bacterial burdens in the spleen and liver (Fig. 5A and B). Thus, circulating memory mediates protective immunity conferred by SseB/flagellin immunization.

Th1 cell production of IFN-γ causes macrophage production of nitric oxide and eventual resolution of *Salmonella* infection (1). Thus, mice that are genetically deficient in T-bet or IFN-γ display increased susceptibility to *Salmonella* infection (57, 58). In order to examine the mechanism of protection after SseB/flagellin immunization, we immunized and boosted WT, IFN-γR-, and iNOS-deficient mice before challenge with virulent *Salmonella*. As expected, unimmunized IFN-γR-deficient mice had higher bacterial burdens than unimmunized wild-type mice (Fig. 6A and B, Naive), demonstrating the critical role of IFN-γ in primary resolution of infection. Notably, in this set of experiments, protection mediated by SseB/flagellin immunization was lower than previously observed, but was still statistically significant (Fig. 6A and B, sseB/flagellin). However, bacterial burdens in the spleen and liver of SseB/flagellin-immunized IFN-γR-deficient mice were still higher than wild-type mice (Fig. 6A and B), indicating that IFN-γR is essential for protective immunity. In contrast, SseB/flagellin-immunized iNOS-deficient mice had similar bacterial loads to SseB/flagellin-immunized wild-type mice, suggesting that iNOS is dispensable for the protective immunity induced by SseB/flagellin immunization.

Discussion

A large genome-wide association study of enteric fever in Asia identified an MHC class-II association with resistance to human typhoid (59), confirming prior data in a mouse model of typhoid (60). In addition, humans and mice with natural or acquired deficiencies in CD4 T cells display increased sensitivity to invasive *Salmonella* infections (61–64). Taken together, these data point to the importance of CD4 T cells in protective immunity to *Salmonella* infection, as expected for an intra-macrophage pathogen (11). However, it should also be noted, that many experiments also support a protective role for *Salmonella*-specific B cells, via antibody-dependent and antibody-independent mechanisms (53, 65–69).

Given the requirement for cellular immunity in protection against *Salmonella* infection, it is understandable that vaccine development has focused on LVS strains that induce strong mucosal T cell responses (13). Typically, studies that have explored a sub-unit vaccine approach using purified or recombinant bacterial antigens have detected protection that is

several orders of magnitude lower than LVS *Salmonella* (23, 25, 26, 28, 31–34), especially when using highly susceptible mouse strains. Our data with duel SseB/flagellin immunization demonstrate that unexpected gains in protective immunity can be achieved by simply combining two well-documented *Salmonella* antigens. Indeed, highly susceptible C57BL/6 mice immunized with SseB/flagellin survived for over a month following challenge with bacteria that normally cause death within a week to ten days. This finding provides some confidence that further modulation of sub-unit vaccine formulation or delivery strategy could result in a sub-unit *Salmonella* vaccine with efficacy approaching LVS *Salmonella*. Unfortunately, the addition of IroN, CirA, or SlyB (26), did not increase protection, but it seems likely that there are protective antigens could improve the efficacy of SseB/flagellin immunization. Indeed, several new protective antigens were identified by Ferreira et al (28), that could be explored in this regard. Although the intravenous immunization approach used in our study is impractical for human vaccine delivery, the purpose was to maximally stimulate systemic immunity to determine the limits of protection without using a live vaccine. Furthermore, it is interesting that intravenous priming with SseB/flagellin was effective against both systemic and mucosal challenge with *Salmonella*. This fits well with previous data demonstrating that IgA or pIgR are not required for immunity against *Salmonella* (25), and that a focus on initiating robust systemic immunity rather than mucosal protection is the most effective approach to limiting *Salmonella* replication.

Although duel immunization with SseB and flagellin markedly improved protective immunity compared to either antigen alone, all immunized mice eventually died. Therefore, while it is encouraging that there was a strong protective effect with this sub-unit approach, it is clearly less effective than LVS *Salmonella*. However, since there are many populations that cannot be administered a live attenuated vaccine strain, it would still be of interest to develop a a safe sub-unit vaccine that could provide partial protection. In addition, it might be possible to enhance the moderate efficacy of either LVS-*Salmonella* or Vi-CPS vaccines by complementation with a partially protective sub-unit vaccine. It is also interesting that SseB/flagellin immunized mice appear healthy for several weeks and only succumb at late time points, with some mice becoming moribund around 90 days after challenge. High bacterial CFUs were cultured from the spleen at these late time points, confirming that death is due to relapsing *Salmonella* infection. It is perplexing that apparently healthy mice can maintain bacteria for several months without developing effective immunity. This pattern of delayed bacterial outgrowth is reminiscent of an enrofloxacin treatment model where primary infection can be temporarily curtailed but slowly relapses (70). In this relapse model, this correlates with incomplete Th1 immunity due to inappropriate T cell priming (70, 71). Both models suggest that if small numbers of *Salmonella* can evade CD4 T cell killing for a prolonged period, the adaptive immune response is eventually exhausted and overcome. Future studies will be needed to examine why the immune response fails at late time points in the SseB/flagellin immunization model.

A surprising feature of our data is the efficacy of flagellin as an adjuvant in combination with SseB. It has been known for several years that flagellin increases dendritic cell expression of CD80/86, induces inflammatory cytokines, and thus enhances B cell and T cell responses to co-administered antigens (49, 72). These inflammatory processes can be

initiated when flagellin is detected at the cell surface or in the cytosol by membrane and cytosol sensors, TLR5 and NAIP5/NLRC4. Interestingly, there is also a third mechanism for flagellin recognition based on the activity of flagellin in TLR5-, and inflammasome-deficient mice (51). It has been noted that flagellin functions as a relatively weak adjuvant when compared to LPS or other TLR ligands (44, 49, 73, 74). However, our experiments demonstrate that flagellin has adjuvant capabilities distinct from LPS or other adjuvants. Single immunization with SseB is markedly less effective that SseB/flagellin, despite the fact that LPS is added to both formulations. Greater understanding of the role of flagellin as a vaccine adjuvant is therefore required, particularly with respect to TLR5- and inflammasome-independent activity in vivo.

It is broadly accepted that *Salmonella*-specific CD4 T cells are required for protective immunity (64, 65, 76), but the relative role of circulating TCM/TEM and tissue-resident TRM cells has not yet been examined. Our data show that the protection mediated by SseB/flagellin immunization can be transferred effectively in circulation, suggesting that circulating memory populations are likely critical for *Salmonella* immunity. Given the large bacterial burden in highly vascularized tissues, this may not be entirely unexpected. However, it is possible that the superior protective immunity elicited by LVS Salmonella is due to the capacity of a live vaccine to elicit TRM CD4 T cells. Parabiosis experiments to examine this possibility are currently underway in our laboratory. While flagellin modestly enhanced the CD4 T cell response to SseB-2W1S, this enhancement was less striking than the combined effect on protective immunity. Therefore it remains possible that other non-CD4 cells contribute to the heightened protection offered by duel SseB/flagellin immunization.

Overall, our data show that combining two previously known antigenic targets of *Salmonella*-specific immunity markedly improves protective efficacy against challenge infection. Surprisingly, this enhancement is largely due to an adjuvant effect of flagellin and correlates with a modestly increased frequency of vaccine-specific CD4 T cells. The protection mediated by SseB/flagellin immunization was transferrable in circulation and required expression of IFN-γR, suggesting that circulating effector Th1 cells are responsible for enhanced protection. Together, these data increase confidence in the possibility of developing a simple sub-unit vaccine to protect against systemic Salmonellosis and support continued investigation to explore alternatives to live attenuated *Salmonella* vaccines.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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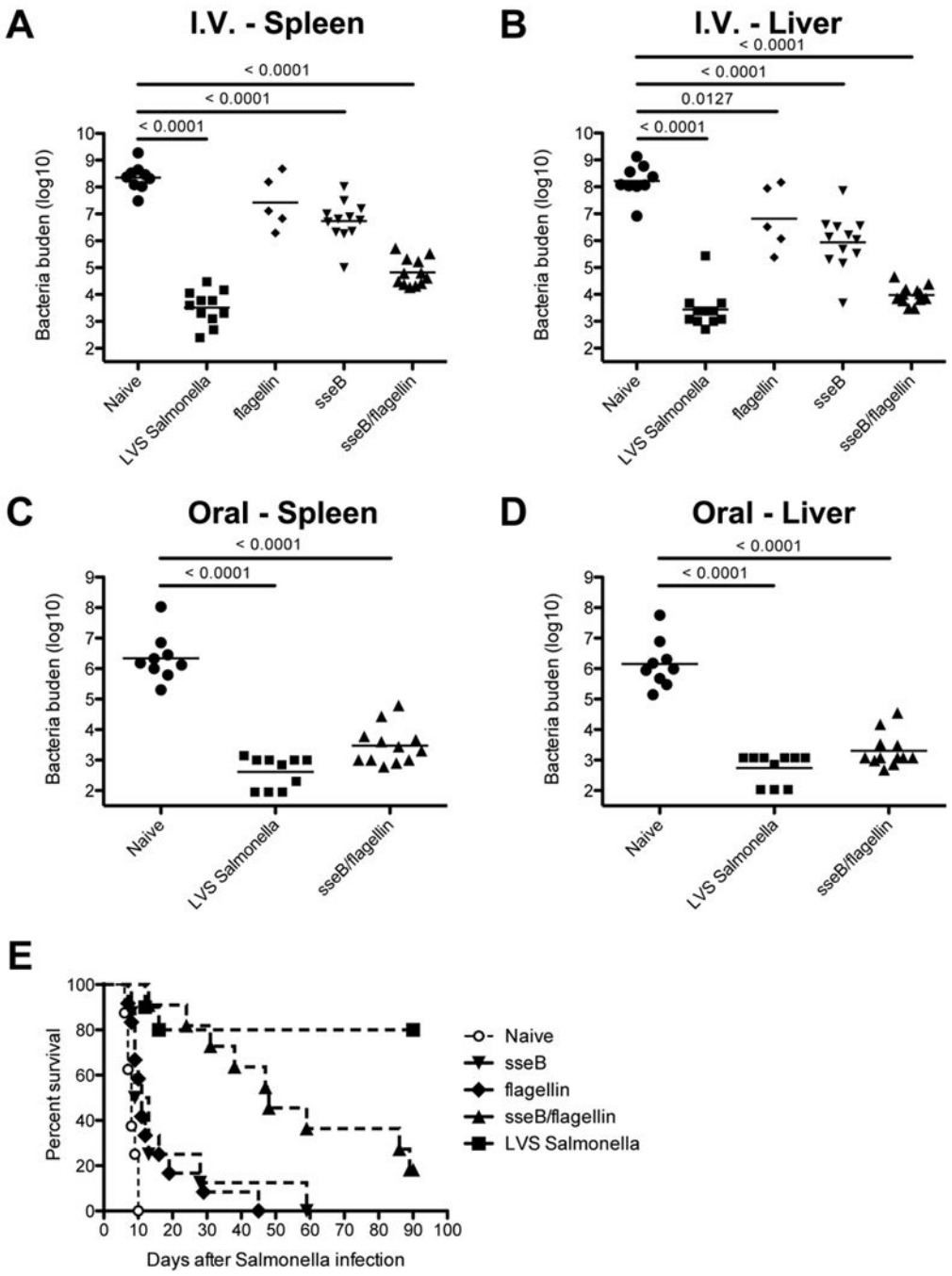

Figure 1. Duel SseB and flagellin immunization enhances protection against *Salmonella* infection

C57BL/6 mice were immunized intravenously twice at 4 week intervals with 100μg of SseB, flagellin, or a combination of SseB and flagellin (sseB/flagellin) mixed with 10μg of LPS. As a positive control, groups of mice were vaccinated intravenously with 5×10^5 of a live vaccine strain (LVS) of *S. typhimurium*. (A–D) Four weeks after the second immunization, all groups of mice were infected intravenously with 1000 virulent wild-type *Salmonella* SL1344 (A–B), or orally with 5×10^7 SL1344 (C–D). Bacterial loads were determined in spleens (A, C) or livers (B, D) at 4 days post infection. Data bars show the mean bacterial

burden per group with individual mice shown as scatter plots. Numbers indicate statistical significance between unimmunized (naïve) mice and each immunized group and list the *p* value. (E) Four weeks after boosting, mice were infected orally with 1×10^6 SL1344 and mice were monitored for the development of a moribund state. Data show the percentage of surviving mice in each group and are representative of eight to twelve mice per group.

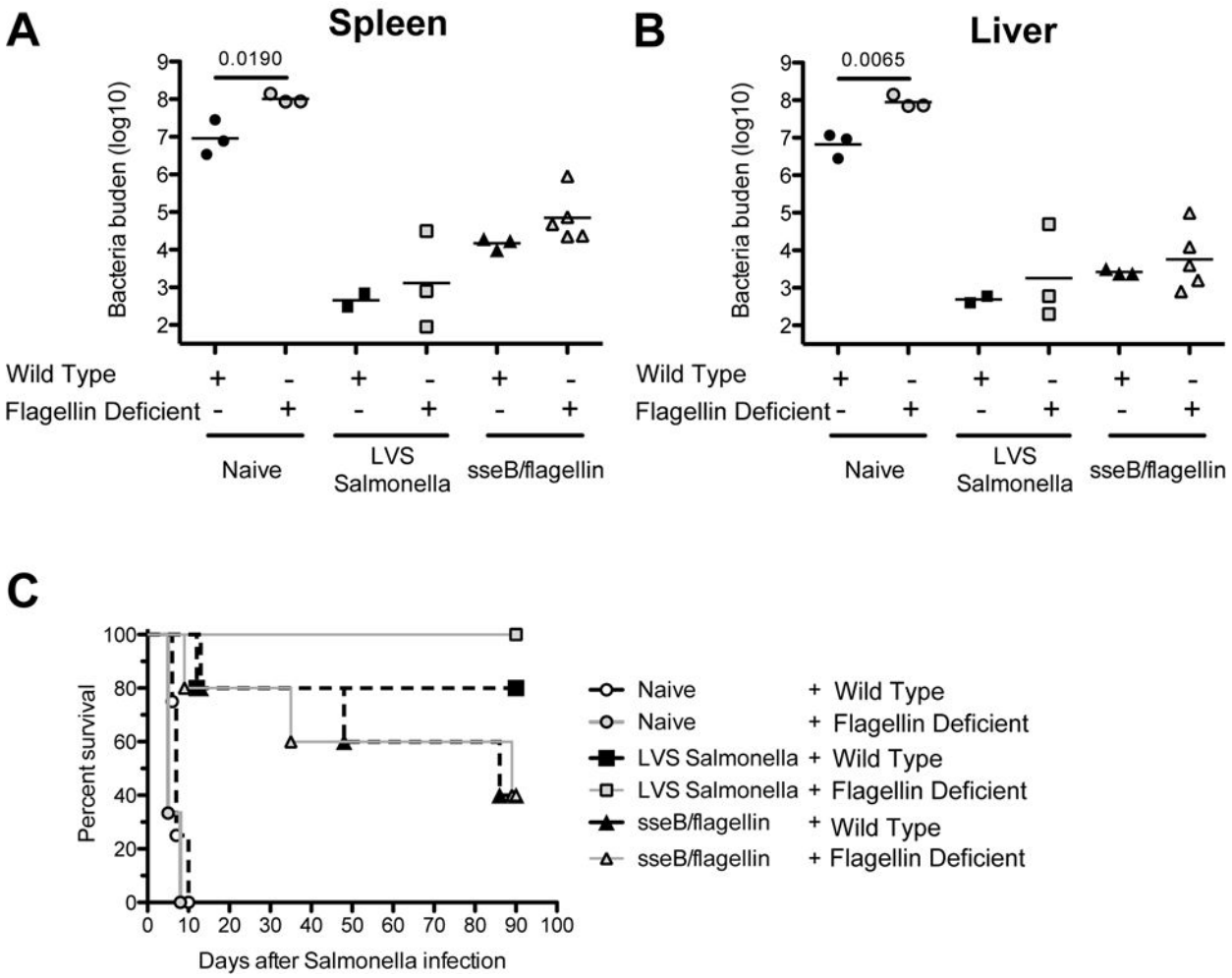

Figure 2. Protection by duel SseB/flagellin immunization does not require a flagellin-specific adaptive immune response

Groups of C57BL/6 mice were immunized intravenously twice with combination of SseB and flagellin and LPS at 4-week intervals. As a positive control, mice were vaccinated intravenously with 5×10^5 LVS Salmonella. (A–B) Four weeks after boosting, groups of mice were infected intravenously with 1000 wild-type *Salmonella* (SL1344) or flagellin-deficient *Salmonella* (BC490) and bacterial burdens were determined in (A) spleens and (B) livers, 4 days later. Data bars show the mean bacterial burden per group with individual mice shown as scatter plots. Numbers indicate statistical significance between unimmunized (naïve) mice and each immunized group and list the *p* value. (C) Four weeks after the second immunization, groups of mice were infected orally with 1×10^6 wild-type or flagellin-deficient bacteria and mice monitored for the development of a moribund state. Data show the percentage of surviving mice in each group and are representative of three to five mice per group.

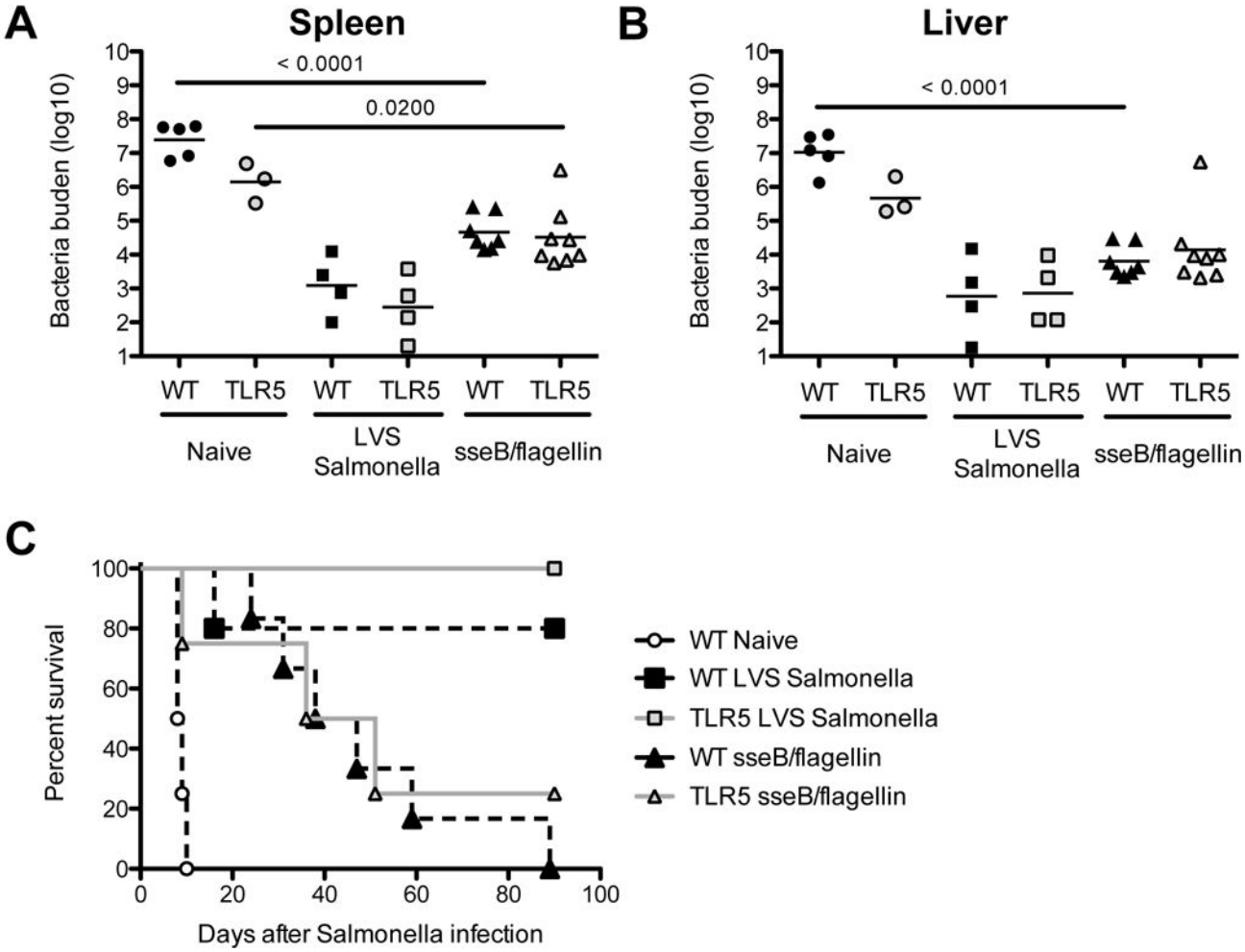

Figure 3. Protection by SseB/flagellin immunization does not require host expression of TLR5

Groups of C57BL/6 (WT) and TLR5-deficient (TLR5) mice were immunized intravenously twice at four week intervals with SseB and flagellin mixed with additional LPS. As a positive control, WT and TLR5-deficient mice were vaccinated intravenously with 5×10^5 LVS Salmonella. (A–B) Four weeks after boosting, groups of mice were infected intravenously with 1000 virulent *Salmonella* and bacterial burdens determined in the spleen (A) and liver (B) 4 days later. Data bars show the mean bacterial burden per group with individual mice shown as scatter plots. Numbers indicate statistical significance between unimmunized (naïve) mice and each immunized group and list the *p* value. (C) Immunized mice were infected orally with 1×10^6 SL1344 and examined for the development of a moribund state. Data show the percentage of surviving mice in each group and are representative of two to seven mice per group.

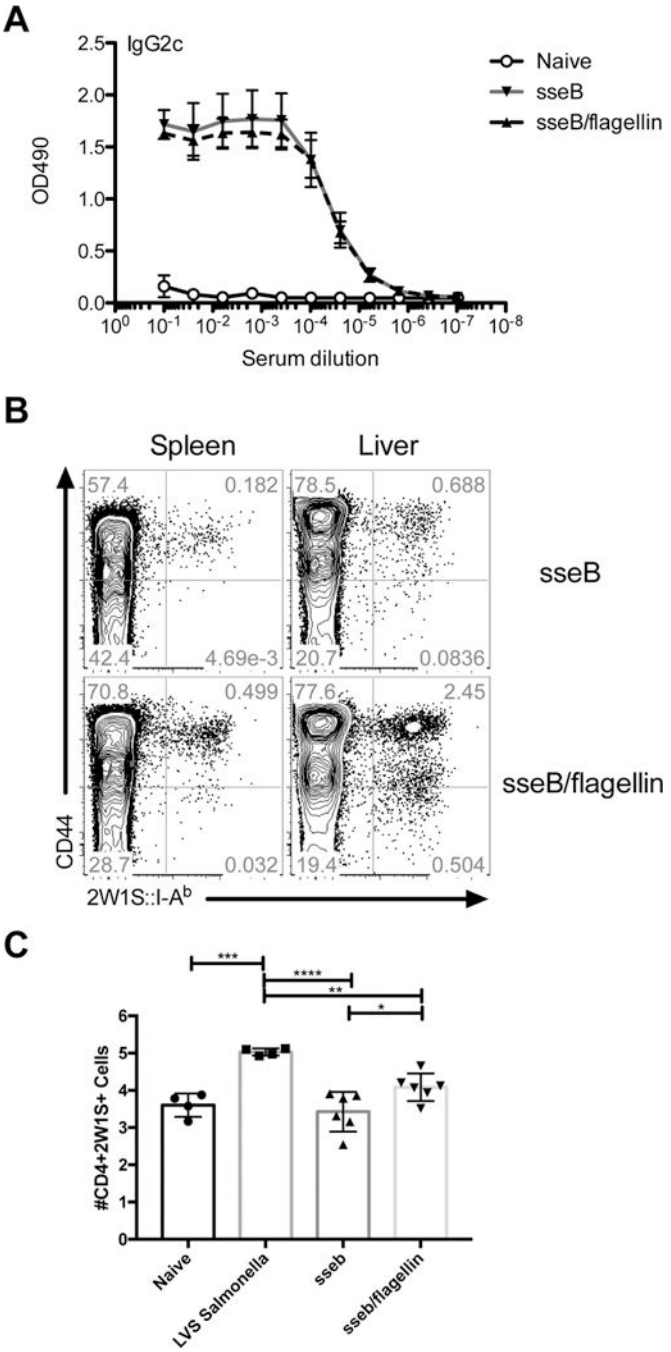

Figure 4. Duel SseB/flagellin immunization modestly enhances *Salmonella*-specific CD4 T expansion

C57BL/6 mice were immunized twice intravenously with 100μg of SseB-2W1S (sseB) or a combination of SseB-2W1S and flagellin (sseB/flagellin) mixed with 10μg of LPS. (A) Four weeks after the second immunization, sera was collected and examined for the presence of SseB-specific IgG2c by ELISA. Data show OD405 of SseB-specific IgG2c in SseB- or SseB/flagellin-immunized mice. Data represent the mean ± SEM of three to eight mice per group. (B) Four weeks after boosting, mice were re-immunized and 7 days later, spleens and livers were harvested and stained with 2W1S::I-Ab tetramer, CD4, and CD44 to detect

2W1S-specific CD4 T cells. Data show representative FACS plots showing tetramer-positive CD4 T cells. (C) Mice were immunized LVS *Salmonella*-2W, 100μg of SseB-2W1S (sseB), or 100μg of SseB-2W1S and 10μg flagellin (sseB/flagellin) and 10μg of LPS. Seven days later, spleens and livers were harvested and stained with 2W1S::I-Ab tetramer, CD4, and CD44 to detect 2W1S-specific CD4 T cells. Data show bar graphs of absolute number of 2W1S-specific CD4 T cells in the spleen of immunized mice. Numbers indicate statistical significance between unimmunized (naïve) mice and immunized mice and list the *p* value. These data combined two independent experiments each with two to three mice per group.

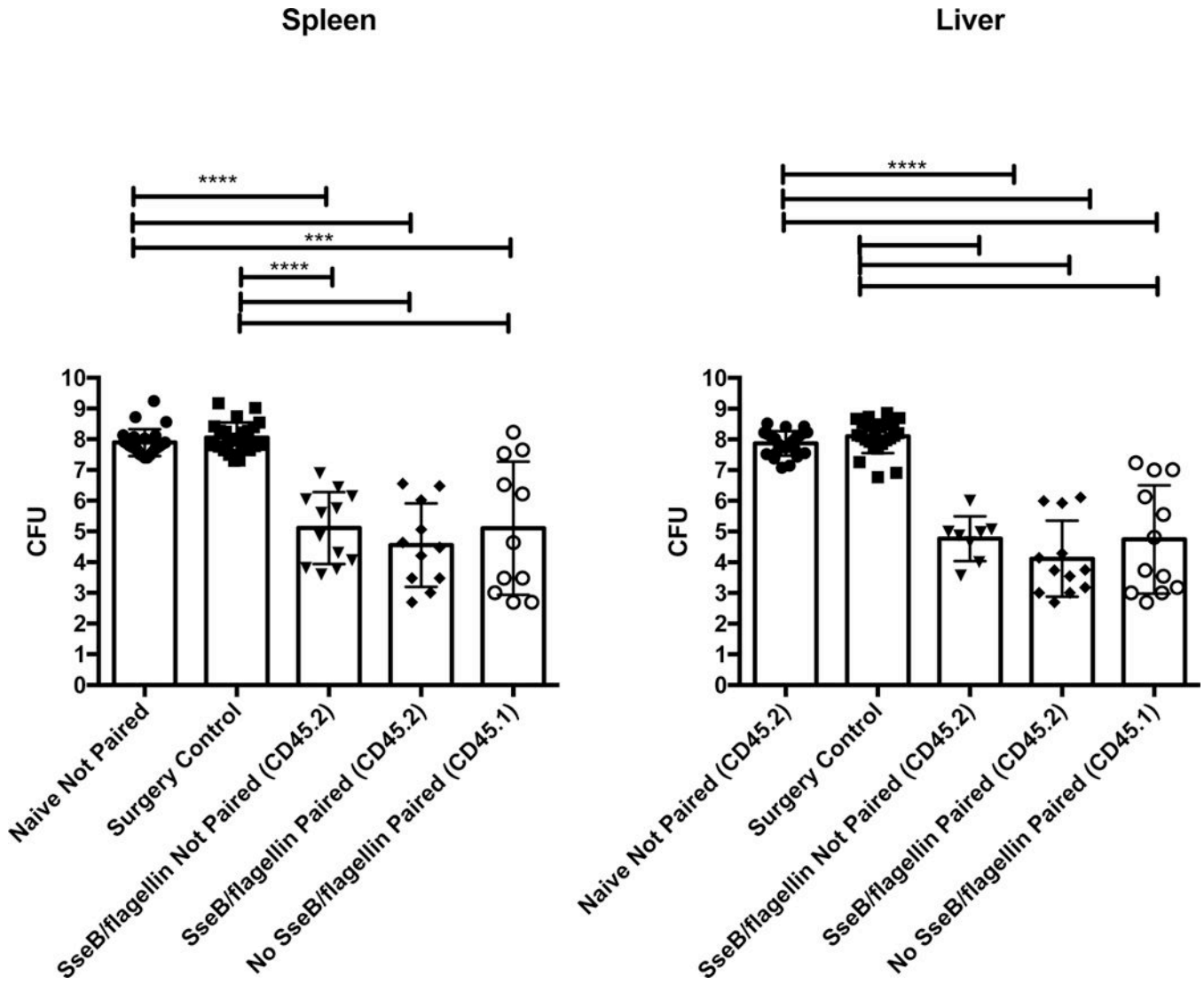

Figure 5. Protective immunity mediated by SseB/flagellin immunization is transferred in circulation

C57BL/6 mice were immunized twice intravenously with 100μg of SseB-2W1S (sseB) or a combination of SseB-2W1S and flagellin (sseB/flagellin) mixed with 10μg of MPLA. (A) Four weeks after the second immunization, mice were surgically joined for 28 days. Post separation surgery, mice were rested for 2 weeks before all groups of mice were infected intravenously with 1000 virulent wild-type *Salmonella* SL1344. Bacterial loads were determined in spleens or livers at 5 days post infection. Data bars show the mean bacterial burden per group with individual mice shown as scatter plots. Numbers indicate statistical significance between groups of mice and list the *p* value (*** indicates $p < 0.001$ and **** indicates $p < 0.0001$).

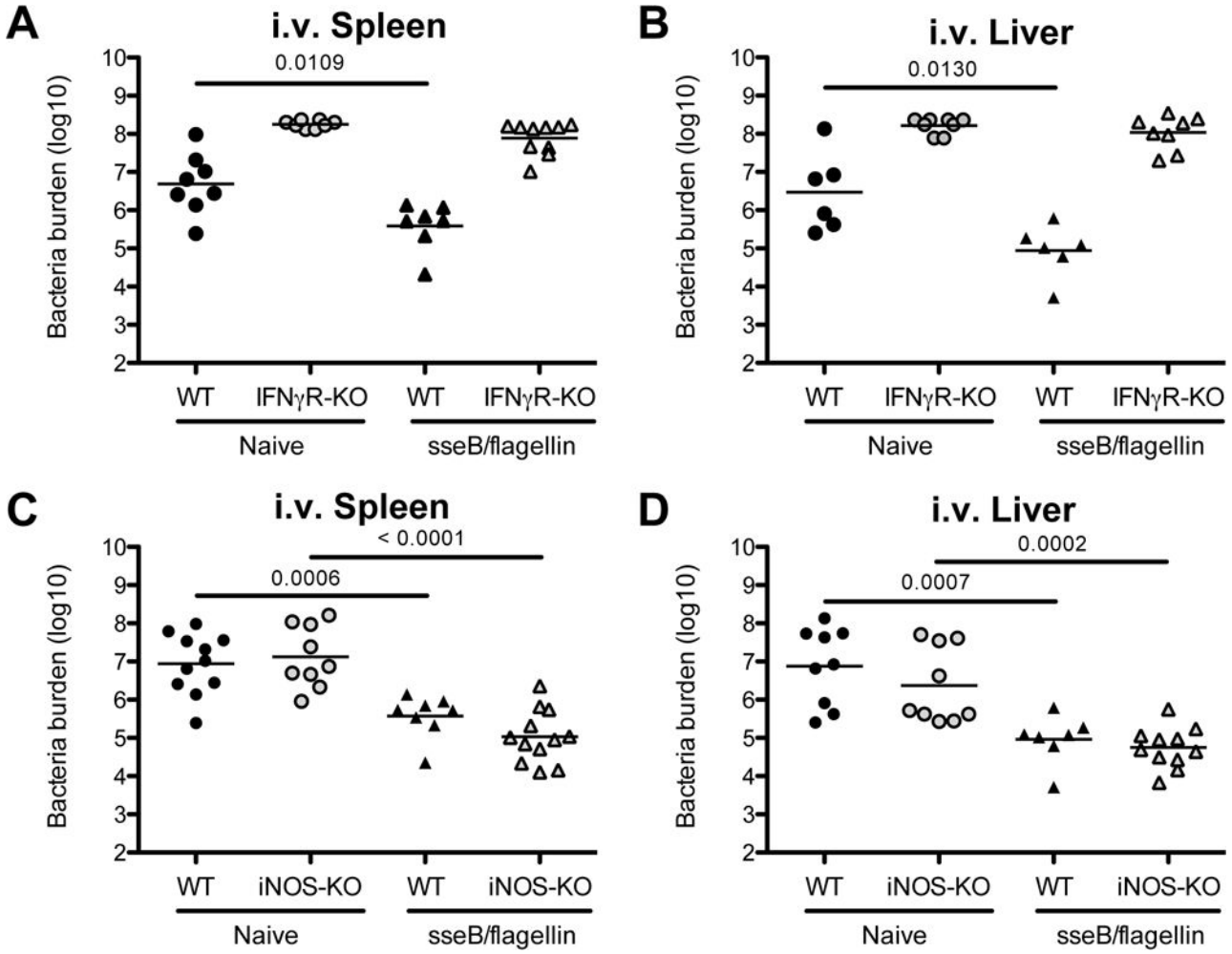

Figure 6. IFN-γ signaling is essential for protective immunity by SseB/flagellin immunization

(A–B) Groups of C57BL/6 (WT) and IFN-γR-deficient (IFN-γR-KO) mice, or (C–D) WT and iNOS-deficient (iNOS-KO) mice were immunized intravenously twice with a combination of SseB and flagellin mixed with LPS. Four weeks after the second immunization, mice were infected intravenously with 1000 virulent *Salmonella.* Bacterial burdens were determined in spleens (A, C) and livers (B, D) 4 days later. Data bars show the mean bacterial burden per group with individual mice shown as scatter plots. Numbers indicate statistical significance between groups of mice.