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IFN- γ , but not IL-17A, is required for survival during secondary pulmonary *Francisella tularensis* Live Vaccine Strain infection

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Abstract

IL-17 and IFN- γ production by Th17 and Th1 cells, respectively, is critical for survival during primary respiratory infection with the pathogenic bacterium, *Francisella tularensis* Live Vaccine Strain (LVS). The importance, however, of these T cell subsets and their soluble mediators is not well understood during a secondary or memory response. We measured the number of CD4⁺ T cells producing IFN- γ or IL-17 in the spleen and lungs of vaccinated mice on day four of the secondary response using intracellular cytokine staining in order to identify protective T cell subsets participating in the memory response. Few bacteria were present in spleens of vaccinated mice on day four and a T cell response was not observed. In the lung, where more bacteria were present, there was a robust Th1 response in vaccinated mice but Th17 cells were not present at higher numbers in vaccinated mice compared to unvaccinated mice. These data show that the lung is the dominant site of the secondary immune response and suggest that Th17 cells are not required for survival during secondary challenge. To further investigate the importance of IFN- γ and IL-17 during the secondary response to *F. tularensis*, we neutralized either IFN- γ or IL-17 in vivo using monoclonal antibody treatment. Vaccinated mice treated with anti-IFN- γ lost more weight and had higher bacterial burdens compared to vaccinated mice treated with isotype control antibody. In contrast, treatment with anti-IL-17A antibody did not alter weight loss profiles or bacterial burdens compared to mice treated with isotype control antibody. Together, these results suggested that IFN- γ is required during both primary and secondary respiratory *F. tularensis* infection. IL-17, on the other hand, is only critical during the primary response to respiratory *F. tularensis* but dispensable during the secondary response.

Keywords

Francisella tularensis; interferon-gamma; IL-17; secondary response; intranasal; clpB

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INTRODUCTION

Tularemia is caused by the gram negative coccobacillus, *Francisella tularensis*. While the CDC reports approximately 120 cases of naturally-acquired tularemia each year in the U.S., *Francisella* has also been used as an infectious agent in bioweapons and continues to pose a realistic threat (1-3). The potential of a bioterrorism attack utilizing *F. tularensis* as the infectious agent underscores the need for a safe and effective vaccine. Vaccine development requires an understanding of what immune response components are required for protection, i.e. correlates of protection.

F. tularensis subspecies *tularensis* SchuS4 (SchuS4) is a highly virulent Type A strain. As few as 10 organisms can cause severe disease in humans with a 30-60% fatality rate if antibiotics are not administered (4). The LD₁₀₀ in mice is <10 CFU for all inoculation routes and mice succumb to infection within 5-6 days (5, 6). Because unmanipulated mice succumb to SchuS4 infection before the development of an adaptive immune response, a less virulent strain, *F. tularensis* subspecies *holarctica* LVS, is used to study components of adaptive immunity.

Both IFN- γ and IL-17A are required to mediate *F. tularensis* clearance during the primary infection. IFN- γ neutralization increases systemic bacterial burdens and mice deficient in IFN- γ succumb to an otherwise sublethal LVS infection for wild-type mice (7, 8). Treatment with recombinant IFN- γ decreases bacterial burdens further confirming the importance of IFN- γ for bacterial control (9). IL-17A is also important for bacterial control following intranasal inoculation as *IL17a*^{-/-} and *IL-17Ra*^{-/-} mice have increased LVS burden in the lung compared to wild-type B6 mice (10, 11). Furthermore, treatment with anti-IL-17A increases lung bacterial burdens compared to isotype control antibody treated mice and decreases the mean time to death (10, 12). IL-17F is a closely related homolog of IL-17A that is also produced by Th17 cells, but has not been extensively studied in the context of *Francisella* infection (13). In the case of *Staphylococcus aureus* infection, IL-17A and IL-17F seem to have redundant functions because a phenotype is only observed when mice are deficient for both isoforms (14). T cells are an important source of IFN- γ and IL-17A and are required for *F. tularensis* clearance and the development of protective immunity (15).

Previous sublethal infection with LVS or the attenuated mutant, LVS *clpB* (i.e. vaccination), provides 100% protection against lethal LVS challenge for at least one year (16). *clpB* encodes a highly conserved chaperone protein involved in resisting heat stress in multiple bacterial species (17-23). Despite the attenuation of LVS *clpB*, inoculation with this strain induces a robust adaptive immune response that was required for bacterial clearance (16). While the importance of IFN- γ and IL-17A during primary pneumonic tularemia are well-understood (7-10, 12), the role of T cells, defined by IFN- γ and IL-17A production, remains poorly described during secondary pneumonic tularemia. Understanding what type(s) of T cell responses are crucial for protective memory will inform further vaccine development. It is also not clear whether the secondary T cell response to the attenuated mutant and vaccine candidate, LVS *clpB*, is similar to the response induced by wild-type LVS. In this manuscript, we examined the T cell response during a secondary intranasal infection with

LVS and determined whether IFN- γ and/or IL-17A were critical for surviving a lethal LVS challenge.

MATERIALS AND METHODS

Bacteria

The live vaccine strain of *Francisella tularensis* subspecies *holartica* (LVS) was obtained from the CDC (Atlanta, GA). The LVS *clpB* (FTL_0094) deletion strain was generated as previously described (16). Bacteria were grown at 37°C on chocolate agar supplemented with 1% IsoVitalex (Becton-Dickinson). To prepare bacterial inoculations, bacteria were removed from a lawn grown on chocolate agar and resuspended in sterile PBS at an OD₆₀₀=1 (equivalent to 1×10¹⁰ CFU/mL). The number of viable bacteria was determined by serial dilution and plating on chocolate agar.

Mice

Six to ten week old female C57Bl/6J (B6) and B6.SJL-*Ptprc^aPepc^b*/BoyJ (B6-CD45.1) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). All mice were housed in specific pathogen-free conditions at the University of Arizona in accordance with the Institutional Animal Care and Use Committee (IACUC).

Inoculation of Mice

For vaccinations, mice were anesthetized with 575 mg/kg tribromomethanol (Sigma) and intranasally inoculated with 5 × 10² CFU LVS or 5×10⁴ CFU LVS *clpB*. For lethal LVS challenge experiments, mice were anesthetized with 0.25 mL of 7.5 mg/mL ketamine and 0.5 mg/mL xylazine cocktail in PBS and intranasally inoculated with 5×10³ CFU (5 × LD₅₀) LVS 120 days after the initial sublethal infection. Mice were weighed daily. Mice were sacrificed if they lost more than 25% of their starting weight.

Determination of serum anti-LVS IgG titers

High binding enzyme-linked immunosorbent assay (ELISA) plates (Costar) were coated with 0.25 µg/well LVS lysate in 100 mM sodium carbonate/bicarbonate buffer (pH 9.6). Coated plates were washed and then blocked with 5% bovine serum albumin (BSA) (w/v) in PBS for 1 hour at room temperature. 100 µL/well of 1:2 serial dilutions of mouse serum starting at 1:100 was added and incubated at room temperature for 2 hours. Plates were washed and then 100 µL/well of anti-mouse IgG conjugated to alkaline phosphatase (1:1000) (Life Technologies) was added and incubated at room temperature for 1 hour. Plates were washed and 100 µL/well para-Nitrophenylphosphate (pNpp) (Life Technologies) was added and incubated for 30 minutes at room temperature. A₄₀₅ and A₅₇₀ were measured using a micro-plate reader (Molecular Devices). A₅₇₀ was subtracted from A₄₀₅ to normalize. Half-maximal titers were determined by dividing the maximum A₄₀₅-A₅₇₀ reading by 2 and using this absorbance value as an unknown value on a best-fit dilution curve for each individual mouse. Each serum sample was analyzed in duplicate.

Western blot detection of LVS specific antibodies

Ten, 20, and 40 µg of LVS lysate were loaded onto a 4-12% Bis-Tris SDS polyacrylamide gel (Life Technologies). Proteins were separated by electrophoresis and then transferred onto a polyvinyl difluoride (PVDF) membrane. The membrane was blocked and incubated with naïve, LVS, or LVS *clpB* mouse sera (1:2000 in 5% BSA in TBST buffer (w/v)). Goat anti-mouse IgG conjugated to IRDye 800CW (Licor) was used for detection. Bands were visualized using the Odyssey Infrared Imaging System (Licor).

Bacterial burden determination

Spleens, livers, and lungs were homogenized in sterile PBS using a Biojector (Bioject) as previously described (24). Ten-fold serial dilutions were made and plated on chocolate agar. Resulting colonies were counted 72 hours later.

Spleen and lung cell isolation

Spleens and lungs were harvested from mice and made into single-cell suspensions of mononuclear cells as previously described (16).

Immunophenotyping and flow cytometry

Antibodies used for flow cytometry were the same as in (16). Intracellular cytokine staining was performed as previously described (16). Flow cytometry data were gated and analyzed as previously described using FlowJo v7.6 (Treestar) (16). Briefly, cells were gated through a doublet discriminator and then a lymphocyte gate. Live CD3⁺ T cells were further gated as CD4⁺ or CD8⁺. IFN-γ⁺ or IL-17A⁺ cells were gated on in the CD4⁺ and CD8⁺ gates. Cytokine⁺ gates were set using isotype and fluorescence minus one controls.

Depletion of IFN-γ or IL-17A

Anti-IFN-γ (clone XMG1.2) was a generous gift from Mary Ann Accavitti-Loper (University of Alabama-Birmingham). Rat IgG1 isotype control antibody (clone HRPN), anti-IL-17A (clone 17F3), and mouse IgG1 isotype control antibody (clone MOPC-21) were purchased from BioXCell (West Lebanon, NH). Mice received 500 µg of anti-IFN-γ or rat IgG1 isotype control antibody or 100 µg of anti-IL-17A or mouse IgG1 antibody in 200 µL PBS via intraperitoneal injection on days 0, 2, and 4 post-inoculation.

Cytokine/chemokine quantification

A multiplex luminex bead-based approach was used to quantify cytokines/chemokines in clarified tissue homogenate as previously described (16). A 20-analyte assay panel was performed according to the manufacturer's protocol (Life Technologies) using a BioPlex array reader (Bio-Rad Laboratories).

Statistical analysis

Statistical significance of immunophenotyping/flow cytometry data was determined using a Kruskal-Wallis with Dunn's post-test. Bacterial burdens were log transformed and then a one-way ANOVA with Tukey's post-test was applied. A Mann-Whitney test was used for IgG half-maximal titer data. GraphPad Prism (v5.04) was used for analysis.

RESULTS AND DISCUSSION

Previous infection with sublethal LVS or LVS *clpB* decreases bacterial burdens during lethal challenge

We quantified bacterial burdens in the spleen, liver, and lung on day 4 post-lethal LVS challenge to determine how well mice vaccinated 120 days prior with LVS or LVS *clpB* were controlling infection. We measured the response on day 4 post-challenge because the secondary response occurs more rapidly than the primary response and this time point precedes the time when unvaccinated mice succumb to infection (16). As expected, bacterial burdens were significantly lower in the spleen, liver, and lung of LVS and LVS *clpB* vaccinated mice compared to unvaccinated mice (Figure 1A-C). These data indicate that prior infection with LVS or LVS *clpB* elicits an immune response that controls bacterial growth at the site of inoculation (lung) as well as distal sites (spleen and liver).

LVS and LVS *clpB* infected mice have similar antibody responses

Previous studies have suggested a contribution of antibodies to protection in challenges following primary infection (25, 26). Consequently, differences in the antibody response may impact clearance, and therefore survival, during a second infection. We determined anti-*Francisella* IgG titers in LVS and LVS *clpB* infected mice 28 and 120 days post-inoculation as a measure of the systemic antibody response elicited during the primary infection. LVS-specific IgG titers on day 28 or day 120 post-inoculation were not significantly different in LVS and LVS *clpB* vaccinated mice (Figure 2A-D). Naïve mouse sera did not contain LVS-specific IgG antibodies (Figure 2A, C). Sera from LVS and LVS *clpB* infected mice produced similar, but not identical, banding patterns (Figure 2E) suggesting mice inoculated with LVS *clpB* raise at least an equivalently diverse antibody response. These data suggest that while there was the potential for a differential contribution of antibody responses to bacterial control or survival of LVS and LVS *clpB* vaccinated mice during lethal LVS challenge, the nearly equivalent IgG titers indicate that this is not the case.

Although B cells contribute antibody to the immune response, they also likely serve as antigen-presenting cells since B cell deficient mice are not protected via passive transfer of sera but survive when B cells are adoptively transferred (25). *IgA*^{-/-} mice are more susceptible to a lethal LVS challenge after vaccination with inactivated LVS than wild-type mice (26). The failure of *IgA*^{-/-} mice to respond likely has both antibody and T cell involvement as there are fewer IFN- γ producing T cells in the lung compared to wild-type mice (27).

LVS and LVS *clpB* vaccinated mice have an increased lung IFN- γ -mediated T cell response compared to unvaccinated mice

We next examined the T cell response in LVS and LVS *clpB* vaccinated mice using intracellular cytokine staining (ICS) to determine what types of T cells responded in the spleen and lung during the secondary response (day 4 post-challenge). LVS and LVS *clpB* vaccinated mice had significantly more IFN- γ producing CD4⁺ and CD8⁺ T cells in the lung than unvaccinated mice (Figure 3A, B). Additionally, responding CD4⁺ and CD8⁺ T cells

from LVS and LVS *clpB* vaccinated mice produced significantly more IFN- γ per cell as measured by MFI compared to unvaccinated mice (Figure 3C, D). There was no significant difference in the number of IFN- γ producing CD4⁺ or CD8⁺ T cells or IFN- γ MFI between the lungs of LVS and LVS *clpB* vaccinated mice during re-challenge. Clearly, previous infection with either LVS or LVS *clpB* primes both CD4⁺ and CD8⁺ T cells to produce IFN- γ in the lung upon secondary infection. Additionally, these data indicated that LVS or LVS *clpB* vaccination leads to a robust IFN- γ response in the lung during re-challenge.

LVS and LVS *clpB* vaccinated mice have a similar lung IL-17A-mediated immune response compared to unvaccinated mice

Primary infection with LVS or LVS *clpB* induces Th17 expansion in the lung (16, 24). We therefore examined whether memory Th17 cells in the lung were responding during re-challenge. The number of Th17 cells in vaccinated and unvaccinated mice was not significantly different (Figure 3E). IL-17A levels did not increase significantly in vaccinated mice compared to unvaccinated mice as measured by MFI (Figure 3F). These data suggest that Th17 cells do not respond or are not present in the lung during secondary infection with LVS.

LVS and LVS *clpB* vaccinated mice lack a robust secondary response in the spleen.

Bacterial burdens were near the limit of detection (50 CFU) in the spleen on day 4 post-LVS-challenge (Figure 1A). It is possible that bacterial burdens were low in the spleen because there was a robust memory T cell response that controlled the infection. Alternatively, the robust T cell memory response in the lung could decrease bacterial dissemination to distal organs and low antigen loads will not drive a splenic response. To distinguish between these two possibilities, we examined the secondary immune response in the spleen on day 4 post-re-challenge using ICS. There were not significantly more IFN- γ producing CD4⁺ or CD8⁺ T cells in LVS and LVS *clpB* vaccinated mice compared to unvaccinated mice (Figure 4A, B). IFN- γ MFI was significantly higher in CD4⁺ T cells from vaccinated mice compared to unvaccinated mice (Figure 4C), no significant difference was observed in the IFN- γ MFI for CD8⁺ T cells (Figure 4D).

Primary intranasal infection with LVS or LVS *clpB* does not elicit expansion of Th17 cells in the spleen (16, 24). Similarly, the absolute number of Th17 cells in the spleens of LVS or LVS *clpB* vaccinated mice was not significantly different compared to unvaccinated mice (Figure 4E). IL-17A levels were not significantly increased in vaccinated mice compared to unvaccinated mice as measured by MFI (Figure 4F). These data suggest that, like a primary infection with LVS or LVS *clpB*, Th17 cells are not seen in the spleen.

Because we observed numbers of CD4⁺ IFN- γ ⁺, CD8⁺ IFN- γ ⁺, and Th17 cells in the spleens of vaccinated and unvaccinated mice on day 4 post-re-challenge that were not significantly different, these data demonstrated that the observed low bacterial burdens in the spleen were not a consequence of a robust memory T cell response. Instead, these data suggested that low bacterial burdens in the spleen did not drive splenic memory T cell expansion and that a systemic immune response does not occur by day 4 post-inoculation following intranasal secondary challenge of immune mice. Our observation that a prior intranasal inoculation

with LVS yields a secondary immune response that occurs primarily in the lung is different from what is observed with a secondary infection with highly virulent SchuS4, where a robust secondary response is observed in the spleen and not the lung (28). The most likely reason for the difference in the location of the secondary response is the route of LVS vaccination and not the infecting strain during the secondary challenge. We vaccinated mice intranasally with LVS which primes *Francisella*-specific T cells in the lung and spleen whereas Anderson, et al. vaccinated subcutaneously, a route that may not prime many lung resident memory T cells (16, 24, 28). Another possible explanation for the difference is the use of different mouse strains (B6 versus BALB/c).

IFN- γ production is required for protection from LVS secondary challenge

The secondary response in LVS or LVS *clpB* vaccinated mice induced IFN- γ producing CD4⁺ and CD8⁺ T cells in the lungs. To determine whether IFN- γ was required during the secondary response, we treated mice with anti-IFN- γ or rat IgG1 isotype control antibodies on days 0, 2, and 4 post-re-challenge. This IFN- γ neutralization regimen similar to what others have used, with more frequent administration (8). Mice were weighed daily and weight loss as a percentage of starting weight on day 6 post-re-challenge is reported (Figure 5A). While vaccinated mice receiving anti-IFN- γ had lost >20% of their initial weight, vaccinated mice receiving rat IgG1 lost less weight and their weight loss profile (data not shown) suggested they would survive the secondary infection.

We next examined bacterial burdens in the spleen, liver, and lung on day 6 post-re-challenge. We selected day 6 because unvaccinated mice begin to succumb at this time point and it allowed for maximal accumulation of bacterial growth. LVS and LVS *clpB* vaccinated mice receiving anti-IFN- γ had significantly higher bacterial burdens than vaccinated mice receiving isotype control antibody in the spleen, liver, and lung (Figure 5B-D). IFN- γ neutralization was confirmed in lung and spleen homogenates using Luminex (data not shown). These data indicate that IFN- γ production is critical during an intranasal secondary infection just as has previously been shown for primary infections with LVS (7, 8). The requirement of IFN- γ during secondary infection has also been shown using several routes of LVS vaccination and then lethal challenge with LVS, SchuS4, or another Type A strain (29-31).

IL-17A is dispensable during the secondary response to LVS

Secondary infection in LVS or LVS *clpB* vaccinated mice did not elicit a Th17 response that was greater in magnitude compared to unvaccinated mice. We therefore hypothesized that IL-17A is not required for protection during secondary infection. To test this hypothesis, we treated unvaccinated, LVS vaccinated, or LVS *clpB* vaccinated mice with anti-IL-17A or mouse IgG1 isotype control on day 0, 2, and 4 post-re-challenge. The IL-17A neutralization regimen is similar to what has been used by other groups, but with more frequent antibody administration (12). Mice were weighed daily and weight loss as a percentage of starting weight on day 6 post-re-challenge is reported (Figure 6A). IL-17A neutralization did not affect weight loss profiles of either vaccinated or unvaccinated mice (data not shown).

We then determined bacterial burdens in the spleen, liver, and lung on day 6 post-re-challenge. LVS and LVS *clpB* vaccinated mice had spleen and liver bacterial burdens near the lower limit of detection for both treatment groups (Figure 6B-C). Lung bacterial burdens were not significantly different in LVS and LVS *clpB* vaccinated mice receiving anti-IL-17A compared to isotype control (Figure 6D). Bacterial burdens in the spleen, liver, and lung of unvaccinated mice were not significantly different irrespective of antibody depletion (Figure 6B-D). Luminex analysis of clarified tissue homogenates determined IL-17A levels are below the detection limit in the spleen and lungs of mice receiving anti-IL-17A and isotype control antibody, irrespective of vaccination status (data not shown). These data indicate IL-17A is produced at low levels in during the secondary infection, further supporting our conclusion that IL-17A is dispensable during a recall response.

We did not observe a significant increase in bacterial burdens or weight loss profiles for unvaccinated mice receiving anti-IL-17A compared to mice receiving isotype control antibody as other groups have shown using very similar neutralization regimens (10, 12). This could suggest that the neutralization antibody we used was not effective. We confirmed the antibody was functional and administered at the desired dose, and could therefore rule out non-neutralizing antibody. We believe the most likely explanation for our result is the time point at which bacterial burdens in the unvaccinated mice were determined. Anti-IL-17A treated mice had no difference in weight loss or survival compared to carrier-treated mice until day 8 post-inoculation (12). *IL-17a*^{-/-} mice also do not succumb to an intranasal LVS infection until after day 8 post-inoculation (10). We harvested tissue two days before others started to see a difference in weight loss or survival. Taken together, it is likely we fail to see a phenotype because the naïve mice treated with anti-IL-17A are sacrificed before bacterial burdens are significantly increased. Furthermore, because we selected a higher challenge inoculum dose, naïve mice succumb to infection by day 7 post-inoculation, making it impossible to observe differences on day 8 (10).

An important finding from these experiments was that there were no statistically significant differences between LVS and LVS *clpB* vaccinated mice for all parameters measured. These data indicated that the protective immune response elicited by previous infection with either strain is nearly identical. Because LVS *clpB* provides 100% protection from death during lethal challenge (with no culturable bacteria recovered 14 days after lethal challenge) and causes less overall disease during the primary infection, this strain is an excellent vaccine candidate.

Overall, we propose the following model: during primary intranasal infection with LVS, IL-17A produced by innate and adaptive cells in the lung initially controls the infection until IFN- γ production by CD4⁺ and CD8⁺ T cells levels increase and clear the infection. Primary infection with LVS and LVS *clpB* also induces an antibody response. During secondary intranasal infection with LVS, CD4⁺ and CD8⁺ T cells in the lung expand and produce IFN- γ . Th17 cells do not expand in the lung during secondary infection. The secondary response in the lung controls the infection and bacteria have limited spread to distal organs such as the spleen and thus the T cell response is not significantly different than unvaccinated mice. In our model, IFN- γ is absolutely required for survival during a secondary infection whereas IL-17A was dispensable.

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Highlights

- IFN- γ is required for survival during secondary pneumonic tularemia
- IL-17A is dispensable for survival during secondary pneumonic tularemia
- Vaccination with LVS *clpB* elicits the same memory T cell response as LVS

Figure 1. Previous infection with LVS or LVS *clpB* decreases bacterial burdens on day 4 post-rechallenge

B6 mice were intranasally inoculated with 5×10^2 CFU LVS or 5×10^4 CFU LVS *clpB*, or left unvaccinated (Unvx). On day 120 post-inoculation, all mice were challenged with 5×10^3 CFU LVS. On day 4 post-rechallenge, bacterial burdens were determined in the A) spleen, B) liver, and C) lung by plating serial dilutions of organ homogenate on chocolate agar. $n=3-4$ mice/group. Data are combined from 2 independent experiments. The dashed line indicates the limit of detection of 50 CFUs. Statistical significance was determined on log-transformed data using a one-way ANOVA with Tukey's post-test.

Figure 2. Antibody responses are similar in LVS and LVS *clpB* infected mice

B6 mice were intranasally inoculated with 5×10^2 CFU LVS or 5×10^4 CFU LVS *clpB*, or left uninfected. On day 28 (A, B) or 120 (C, D) post-inoculation, sera was collected and the amount of LVS-specific IgG antibodies were determined using an ELISA. Half-maximal titers were calculated for LVS and LVS *clpB* infected mice. $n=7-11$ mice/group. Data are combined from 2 independent experiments. Each sample was analyzed in duplicate. A Mann-Whitney test was used to determine statistical significance. E) 10, 20, and 40 μ g (left to right) of LVS whole cell lysate was probed with sera from either naïve, LVS, or LVS *clpB* inoculated mice collected 28 days post-inoculation. LVS-specific antibodies were detected using goat anti-mouse IgG secondary antibody.

Figure 3. Vaccinated mice have increased numbers of lung IFN- γ producing CD4⁺ and CD8⁺ T cells compared to unvaccinated mice

B6 mice were intranasally inoculated with 5×10^2 CFU LVS or 5×10^4 CFU LVS *clpB*, or left unvaccinated (Unvx). On day 120 post-inoculation, all mice were challenged with 5×10^3 CFU LVS. On day 4 post-rechallenge, lung cells were re-stimulated with LVS infected B6-CD45.1 antigen presenting cells for 24 hours. Brefeldin A was added during the last 4 hours of culture. The number of A) CD4⁺ IFN- γ ⁺ or B) CD8⁺ IFN- γ ⁺ T cells in the lung was determined by flow cytometry. To determine change in mean fluorescent intensity (MFI) for each sample, the MFI of the IFN- γ negative population was subtracted from the MFI of the IFN- γ positive population for C) CD4⁺ or D) CD8⁺ T cells. E) Number of CD4⁺ IL-17A⁺ T cells in the lung were determined by flow cytometry. F) Change in mean fluorescent intensity (MFI) for each sample was determined by subtracting the MFI of the IL-17A negative population from the MFI of the IL-17A positive population. $n=6$ mice/group. Data are combined from 3 independent experiments. Statistical significance was determined using a Kruskal-Wallis test with Dunn's correction.

Figure 4. The secondary response is nearly absent in the spleen

B6 mice were intranasally inoculated with 5×10^2 CFU LVS or 5×10^4 CFU LVS *clpB*, or left unvaccinated (Unvx). On day 120 post-inoculation, all mice were challenged with 5×10^3 CFU LVS. On day 4 post-rechallenge, spleen cells were re-stimulated with LVS infected B6-CD45.1 antigen presenting cells for 24 hours. Brefeldin A was added during the last 4 hours of culture. The number of A) CD4⁺ IFN-γ⁺ or B) CD8⁺ IFN-γ⁺ T cells in the spleen was determined by flow cytometry. To determine change in mean fluorescent intensity (MFI) for each sample, the MFI of the IFN-γ negative population was subtracted from the MFI of the IFN-γ positive population for C) CD4⁺ or D) CD8⁺ T cells. E) Number of CD4⁺ IL-17A⁺ T cells in the spleen were determined by flow cytometry. F) MFI for each sample was determined by subtracting the MFI of the IL-17A negative population from the MFI of the IL-17A positive population. n=6 mice/group. Data are combined from 3 independent experiments. Statistical significance was determined using a Kruskal-Wallis test with Dunn's correction.

Figure 5. IFN- γ depletion increases bacterial burdens

B6 mice were intranasally inoculated with 5×10^2 CFU LVS or 5×10^4 CFU LVS *clpB*, or left unvaccinated (Unvx). On day 120 post-inoculation, all mice were challenged with 5×10^3 CFU LVS. Mice were treated with anti-IFN- γ or isotype control intraperitoneally on days 0, 2, and 4 post-inoculation. A) Percentage of starting weight on day 6 post-inoculation. Bacterial burdens were determined on day 6 post-inoculation in the B) spleen, C) liver, and D) lung by plating serial dilutions of organ homogenate on chocolate agar. $n=3-5$ mice/group. Data are combined from 2 independent experiments. The dashed line indicates the limit of detection of 50 CFUs. Statistical significance for CFUs was determined on log-transformed data using a Student's t-test comparing anti-IFN- γ to isotype control within each group.

Figure 6. IL-17A depletion has no effect on bacterial burdens

B6 mice were intranasally inoculated with 5×10^2 CFU LVS or 5×10^4 CFU LVS *clpB*, or left unvaccinated (Unvx). On day 120 post-inoculation, all mice were challenged with 5×10^3 CFU LVS. Mice were treated with anti-IL-17A or isotype control intraperitoneally on days 0, 2, and 4 post-inoculation. A) Percentage of starting weight on day 6 post-inoculation. Bacterial burdens were determined on day 6 post-inoculation in the B) spleen, C) liver, and D) lung by plating serial dilutions of organ homogenate on chocolate agar. $n=3-5$ mice/group. Data are combined from 2 independent experiments. The dashed line indicates the limit of detection of 50 CFUs. Statistical significance for CFUs was determined on log-transformed data using a Student's t-test comparing anti-IL-17A to isotype control within each group.