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Respiratory *Francisella tularensis* Live Vaccine Strain Infection Induces Th17 Cells and Prostaglandin E$_2$, Which Inhibits Generation of Gamma Interferon-Positive T Cells[▽]

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Two key routes of *Francisella tularensis* infection are through the skin and airway. We wished to understand how the route of inoculation influenced the primary acute adaptive immune response. We show that an intranasal inoculation of the *F. tularensis* live vaccine strain (LVS) with a 1,000-fold-smaller dose than an intradermal dose results in similar growth kinetics and peak bacterial burdens. In spite of similar bacterial burdens, we demonstrate a difference in the quality, magnitude, and kinetics of the primary acute T-cell response depending on the route of inoculation. Further, we show that prostaglandin E$_2$ secretion in the lung is responsible for the difference in the gamma interferon (IFN-γ) response. Intradermal inoculation led to a large number of IFN-γ$^+$ T cells 7 days after infection in both the spleen and the lung. In contrast, intranasal inoculation induced a lower number of IFN-γ$^+$ T cells in the spleen and lung but an increased number of Th17 cells in the lung. Intranasal infection also led to a significant increase of prostaglandin E$_2$ (PGE$_2$) in the bronchoalveolar lavage fluid. Inhibition of PGE$_2$ production with indomethacin treatment resulted in increased numbers of IFN-γ$^+$ T cells and decreased bacteremia in the lungs of intranasally inoculated mice. This research illuminates critical differences in acute adaptive immune responses between inhalational and dermal infection with *F. tularensis* LVS mediated by the innate immune system and PGE$_2$.

Francisella tularensis is a facultative intracellular bacterium and the causative agent of tularemia. *F. tularensis* has a low infective dose, high morbidity, and persistence in the environment (8). The two key routes of transmission are skin contact and inhalation (6, 43, 44). There is a significant increase in morbidity and mortality associated with inhalation infections compared to skin contact infections in humans (43). The reason for this difference is not clear. An *F. tularensis* live vaccine strain (LVS) has been developed from an *F. tularensis* type B strain as a vaccine and causes a less-severe disease in humans (31), though infection in mice causes a fulminant disease that is similar to human tularemia, which also varies in severity by inoculation route (12). (All references to *F. tularensis* are to LVS unless otherwise noted.) Genomic analysis demonstrates that there is a high level of conservation on the genetic level between *F. tularensis* LVS and both type A and type B strains, though there are differences (34). Previous studies suggest a difference in the innate immune response to *F. tularensis* between the intradermal and intranasal inoculation routes (2). A careful characterization of acute adaptive immune responses, comparing intradermal and intranasal infections with similar tissue bacterial burdens, has not been performed.

T-cell-mediated responses are important in clearing primary *F. tularensis* infection and conferring long-term immunity (4, 5, 10). Mice that lack an adaptive immune response are able to partially control bacterial growth, but they fail to resolve in-

fection and eventually die (10). Secretion of gamma interferon (IFN-γ) by T cells is important for clearance of *F. tularensis* (11). Studies with IFN-γ receptor$^{-/-}$ bone marrow macrophages suggest other cytokines are also able to aid in killing intracellular *F. tularensis*. Interestingly, interleukin 12p35 knockout (IL-12p35$^{-/-}$) mice are able to clear *F. tularensis* with little to no IFN-γ response (9). IL-12p35$^{-/-}$ mice still generate Th17 T-cell responses following other bacterial infections (3, 20). Th17 T cells are a third effector CD4$^+$ T-cell subset that is important in mucosal immunity and autoimmunity (3, 20, 26, 35, 50). These T cells release IL-17, which through action on epithelial cells recruits neutrophils to the site of infection (24). These data suggest that IFN-γ-mediated responses are important in controlling *F. tularensis* infections, although IL-17 and other T-cell-mediated responses may play a part and become critical in the absence of IFN-γ.

We have demonstrated that *F. tularensis* infection of bone marrow-derived macrophages leads to the production of prostaglandin E$_2$ (PGE$_2$) and alters subsequent T-cell activation in vitro (49). A role for PGE$_2$ during *F. tularensis* in vivo infection has not yet been reported. In both humans and mice, PGE$_2$ can control dendritic-cell (DC) migration to the lymph node, block IL-12 production, and promote IL-23 production (17, 18, 25, 28, 36–38, 51). Increased IL-23 would enhance the development of Th17 cells (15). PGE$_2$ also directly down modulates human and mouse T-cell activity by decreasing calcium flux (an early stage of T-cell activation) and blocking downstream production of IL-2 and IFN-γ (1, 14, 22, 29, 42). Thus, bacterial infections that induce PGE$_2$ may alter T-cell function and gain an advantage in vivo.

In this study, we compared acute primary T-cell-mediated responses after intradermal and intranasal inoculations with *F.*

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tularensis. Intradermal inoculation led to an early and robust IFN-γ-mediated T-cell response, while intranasal inoculation led to a mixed Th1/Th17- mediated cell response. PGE$_2$ is at least partially responsible for this difference, since inhibition of PGE$_2$ synthesis increased the IFN-γ response and decreased the bacterial load in the lung. Studies reported in this article highlight critical differences in the adaptive immune response following different routes of inoculation with *F. tularensis*.

MATERIALS AND METHODS

Bacteria. The *F. tularensis* LVS (obtained as a vaccine preparation for the Centers for Disease Control and Prevention) (4) was used in these studies. Viable bacteria were quantified by serial dilution on chocolate agar.

Mice. C57BL/6J mice were purchased from the Jackson Laboratory (Bar Harbor, ME). All animals used in this study were maintained under specific-pathogen-free conditions in the American Association of Laboratory Animal Care-accredited University of North Carolina Department of Laboratory Animal Medicine Facilities. Female mice between 6 and 8 weeks of age were used in all studies.

Infection of mice. Mice were inoculated with *F. tularensis* LVS either intranasally or intradermally. Intradermal inoculations were performed by injecting 10^5 CFU of *F. tularensis* LVS in 50 μl of phosphate-buffered saline (PBS) at the base of the tail. For intranasal inoculations, mice were anesthetized with an intraperitoneal injection of 0.4 ml of a 20-mg/ml tribromoethanol (Avertin) solution and inoculated with 5×10^2 CFU of *F. tularensis* LVS in 50 μl of PBS. Anesthetizing mice with 0.4 ml of 20 mg/ml Avertin prior to intradermal inoculation had no effect on the subsequent immune response (data not shown). These inoculation doses were chosen because immunocompetent mice survived and resulted in similar bacterial burdens in all tissues tested, allowing us to quantitatively compare immune responses in the absence of an altered peak antigen load.

Collection of airway, lung, and spleen cells. Airway cells were collected by bronchoalveolar lavage as previously described (48). Bronchoalveolar lavage fluid (BALF) was collected and frozen at −80°C for later analysis. Cells were placed in complete RPMI 1640 (RPMI 1640 supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml of penicillin, 100 μg/ml streptomycin, and 50 μM β-mercaptoethanol). Mononuclear cells were isolated from the lung as previously described (48). Mononuclear cells were purified from cell suspension by density gradient centrifugation using Lympholyte M (Cedarlane Labs, Ontario, Canada). Spleens were aseptically harvested from mice, and a single cell suspension was generated. Red blood cells were removed by ammonium chloride-potassium carbonate lysis. Live cells from all tissues were determined using trypan blue exclusion and counting on a hemacytometer. Greater than 90% cell viability was noted in all samples.

Intracellular cytokine staining. For stimulator cells, naive C57BL/6J splenocytes were plated in 24-well flat-bottom plates, 10^6 per ml, 2 ml per well, in antibiotic-free medium and infected with *F. tularensis* LVS at an MOI of 200:1 or mock infected. After 2 h, supernatants were removed and replaced with medium containing 5 μg/ml gentamicin to kill extracellular *F. tularensis* while leaving the intracellular bacteria untouched. Splenocytes were then incubated at 37°C overnight. The next day, adherent cells were extensively washed to remove T cells, secreted cytokines, and PGE$_2$. T cells from infected spleens were undetectable after washes (data not shown). Tissue mononuclear cells from infected mice at 10^6 cells/ml were added to infected antigen-presenting cells (APCs) and cultured for 24 h. Tissue APCs were added along with T cells to allow cross-presentation of antigens in order to overcome any inhibitory effects on infected APCs. Streptomycin was incubated at this step to kill all bacteria and ablate their inhibitory effects. These parameters were chosen to maximize T-cell activation and minimize the inhibitory effects of *F. tularensis* in in vitro assays, as demonstrated in our lab (data not shown). For the last 4 h, cells were treated with 10 μg/ml brefeldin A. Cells were then stained for surface markers with fluorochrome-conjugated antibodies. Cells were then fixed and stained for intracellular cytokines, following the eBioscience protocol. Cells were analyzed on a CyAN ADP flow cytometer (Dako, Fort Collins, CO). CyAN flow cytometry data were analyzed using the Summit software program, version 4.3 (Dako). All flow data included appropriate −1 controls to allow correct compensation of multicolor analysis.

Antibodies. The following directly conjugated antibodies were used in these studies: fluorescein isothiocyanate-labeled anti-T-cell receptor beta (TCRβ) clone H57 (eBioscience, San Diego, CA) used at 1.25 μg/10^6 cells, phycoerythrin (PE)-labeled anti-IFN-γ clone XMG1.2 (BD, Franklin Lakes, NJ) used at 1

μg/10^6 cells, PE-Texas Red-labeled anti-CD11c clone N418 (Invitrogen, Carlsbad, CA) used at 0.25 μg/10^6 cells, peridinin chlorophyll protein-labeled anti-CD8 clone Ly-2 (BD) used at 1 μg/10^6 cells, peridinin chlorophyll protein-labeled anti-GR-1 clone RB6-8C5 (BD) used at 0.33 μg/10^6 cells, PE-Cy7-labeled anti-CD19 clone 1D3 (BD) used at 0.2 μg/10^6 cells, PacBlue-labeled anti-NK1.1 clone PK136 (Biolegend, San Diego, CA) used at 1.25 μg/10^6 cells, PacOrange-labeled anti-CD4 clone RM4-5 (Invitrogen) used at 0.25 μg/10^6 cells, allophycocyanin-labeled anti-IL-17a clone TC11-18H10.1 (eBioscience) used at 2.5 μg/10^6 cells, allophycocyanin-labeled anti-CD8 clone Ly-2 (BD) used at 0.5 μg/10^6 cells, and APC-Alexa 750-labeled anti-CD11b clone M1/70 (eBioscience) used at 0.5 μg/10^6 cells. All antibodies were titrated and analyzed by multicolor flow cytometry with a variety of cells before use.

T-cell analysis. After stimulation, cells were stained simultaneously with anti-TCRβ, IFN-γ, CD11c, CD8, CD19, CD4, IL-17, and CD11b. A typical gating scheme used for T-cell cytokine analysis is shown in Fig. 1A. Cells displayed in Fig. 1A are from spleens harvested 7 days after intradermal inoculation. Cells were selected by forward and side scatter; doublets were excluded by forward linear scatter versus forward area scatter. Cells were then selected as CD11c, CD11b, and CD19 negative, to exclude DCs, macrophages, and B cells. αβ T cells were then identified by TCRβ expression, followed by CD4 or CD8 expression. These TCRβ$^+$, CD4$^+$ or CD8$^+$, CD11c$^-$, CD11b$^-$, and CD19$^-$ cells were analyzed for IFN-γ and IL-17 expression. All gating is based on isotype controls.

Bronchoalveolar lavage flow analysis. Cells isolated from bronchoalveolar lavage were stained with anti-TCRβ, -CD4, -CD8, -CD19, -CD11c, -CD11b, -GR-1, and NK1.1. Cells were selected by forward and side scatter; doublets were excluded by forward linear scatter versus forward area scatter. The bronchoalveolar lavage cell gating scheme is shown in Fig. 1B. T cells were defined as TCRβ$^+$, CD4$^+$, or CD8$^+$ and negative for all other stains. B cells were defined as CD19$^+$, NK cells as NK1.1$^+$, neutrophils as CD11b^{hi} GR1^{hi}, DCs as CD11c$^+$, alveolar macrophages as CD11c$^+$ CD11b^{low} GR1^{mid}, and macrophages as CD11b$^+$ CD11c$^-$. We are aware that this marker set fails to identify either B-cell or DC subsets, but since neither of these populations contributes significantly to either the IFN-γ or IL-17 responses, these markers are sufficient for our purposes.

Enumeration of bacteria in organs. Lungs and spleens were collected and homogenized in sterile PBS. Tenfold serial dilutions were plated on chocolate agar and incubated at 37°C. Colonies were counted after 72 h.

Determination of secreted cytokines and prostaglandins. PGE$_2$ in BALFs was measured using a standard PGE$_2$ enzyme immunoassay kit (Assay Design, Ann Arbor, MI) with slight modifications to the standard protocol. BALF samples were first treated with protein G-coupled Sepharose beads to remove murine immunoglobulin G (IgG) in the samples. Capture plates were than pretreated with PGE$_2$ capture antibody for 15 min prior to addition of samples. Samples were mixed with PGE$_2$ conjugate and then placed on plates. The manufacturer's directions were then followed. Total IgG levels in BALF were also determined at this time. There was no correlation between IgG levels and PGE$_2$ levels (r^2 = 0.3232). IL-17 was measured using an enzyme-linked immunosorbent assay (ELISA) kit (eBioscience) as per the manufacturer's instructions. Analysis of sample optical density was measured with a SpectraMax M2e microplate reader (Molecular Devices, Sunnyvale, CA).

Statistical analysis. Data were analyzed using Student's unpaired *t* test. The GraphPad Prism 4.03 software program (Graphpad Software, San Diego, CA) was used for analysis. Statistical significance was defined a *P* value of ≤0.05, ≤0.01, or ≤0.001. All data were pooled from a minimum of two experiments with four mice per time point per group in each experiment.

RESULTS

Route of infection did not determine peak bacterial burden in organs. We inoculated 6- to 8-week-old C57BL/6J female mice either intradermally with 10^5 CFU or intranasally with 5×10^2 CFU. Inoculation doses were confirmed by immediately plating the inoculums on chocolate agar. At multiple times after inoculation, the numbers of organisms in lung and spleen tissue were determined. Early and late after inoculation, a modestly increased bacterial burden was seen in the lung of intranasally inoculated mice compared to results for intradermally inoculated mice. At day 3 through day 7 postinoculation, similar numbers of organisms were present in the lung regardless of the inoculation route (Fig. 2). Similar peak bacterial
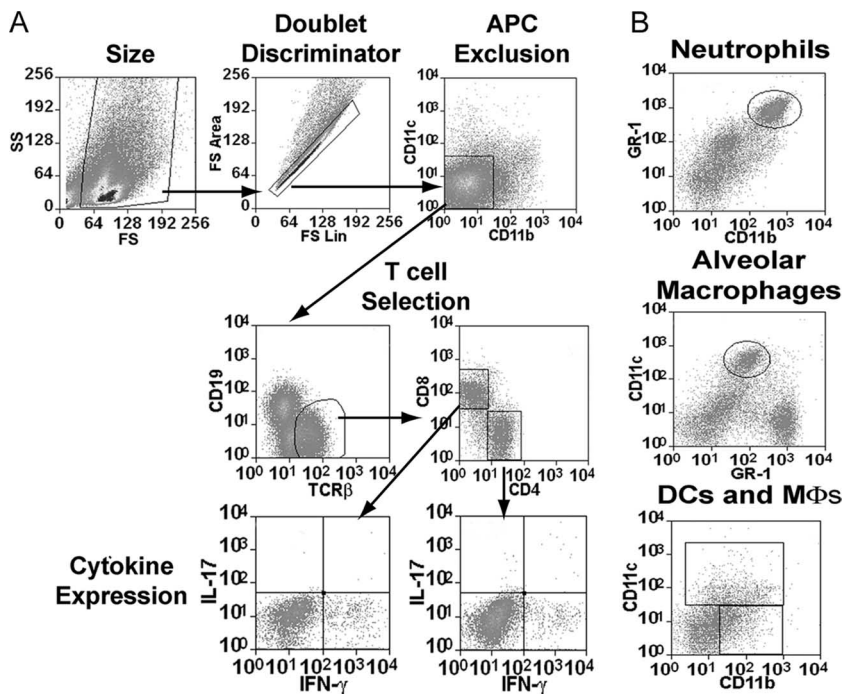
FIG. 1. Gating scheme for cell identification and intracellular cytokine staining. (A) Gating scheme used for identification of T cells and characterization of intracellular cytokine expression. Staining of spleen cells from mice 7 days after intradermal inoculation is shown. (B) Cellular characterization of neutrophils, alveolar macrophages, dendritic cells, and macrophages in the bronchoalveolar spaces. Staining of BAL cells from mice 10 days post-intranasal inoculation is shown.

loads were seen in the lungs of intranasally and intradermally inoculated mice. A delay in clearance was seen in the lungs of intranasally inoculated mice. A trend in kinetics of accumulation and clearance of bacteria similar to that in the lung was seen in the spleen. The spleen was cleared of bacteria by day 14 postinoculation regardless of the route. The bacterial burden was also similar in the liver between the two routes of inoculation and was cleared in both groups by day 14 (data not shown). These inoculation doses were used for further studies since they resulted in similar bacterial burdens that were below the lethal dose. This allowed to us to analyze differences in acute primary adaptive immune responses. We have chosen to
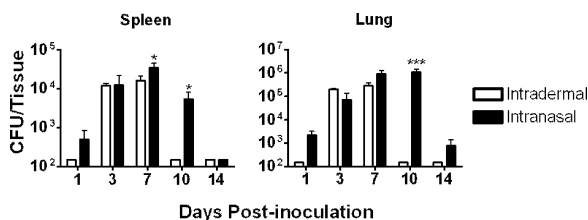

FIG. 2. Intranasal and intradermal infections have similar bacterial burdens in tissue. C57BL/6J mice were inoculated with *F. tularensis* LVS either intradermally (10^5 CFU) or intranasally (5×10^2 CFU). Spleen and lungs from each mouse were assessed for bacterial loads at indicated time points after infection. Asterisks denote statistical difference (∗, $P \leq 0.05$; ∗∗, $P \leq 0.01$; ∗∗∗, $P \leq 0.001$). Error bars represent standard errors of the means ($n = 8$). All comparisons are between routes of inoculation at each time point. Statistical analysis was done using Student's t test with log-transformed data. Data represented are a combination of two experiments with four mice per group for a total of eight mice per group per time point.
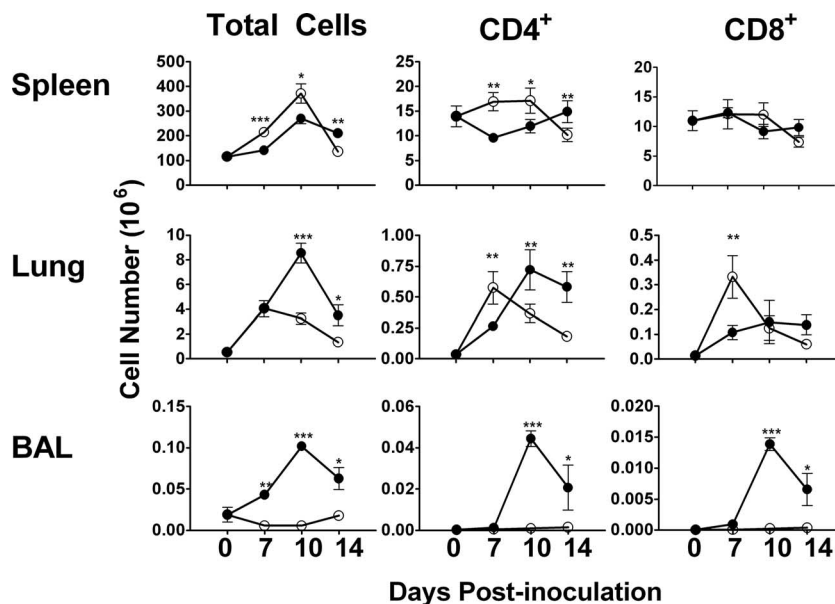
focus on the mechanism of effective immune responses rather than examining the failing immune response resulting in death.

Route of inoculation impacted composition of cells in lung and spleen. Since the rate of clearance but not the peak bacterial burden varied with the route of inoculation, we hypothesized that subsequent acute primary adaptive immune responses were different and were responsible for the difference in clearance rates. Furthermore, in other bacterial infections, the peak bacterial load, not the duration of infection, impacts the magnitude of the acute primary adaptive immune response (32, 46, 47). Initially we measured the total numbers of cells, CD4$^+$ T cells, and CD8$^+$ T cells in the lungs and spleens after both routes of infection. We intranasally or intradermally inoculated mice with *F. tularensis*. After inoculation, cells were collected from the airways, lungs, and spleens. Cells were counted and then stained for T-cell, B-cell, and APC markers. B-cell and APC cell markers were used to excluded these cells from T-cell analysis. Both routes of inoculation caused increased cellularity in the lung and spleen, while only the intranasal inoculation led to an increase in the number of cells in the BALF (Fig. 3). As expected with a site of inflammation, a variety of cell types increased in number. Both inoculation routes led to an increased number of B cells (CD19$^+$), macrophages (CD11b$^+$), DCs (CD11c$^+$), and NK cells (NK1.1 positive) in spleen and lung tissue (data not shown). Intranasally inoculated mice showed significant cellular infiltration into the bronchoalveolar space, including neutrophils, DCs, and macrophages, with the largest percentage being neutrophils (Table 1). Both routes of inoculation led to increased cellular inflammation in both the lung and spleen.

FIG. 3. Inoculation route influences T-cell numbers in tissue. C57BL/6J mice were inoculated with *F. tularensis* LVS either intradermally (10^5 CFU) (○) or intranasally (5×10^2 CFU) (●). Cells were isolated from the airway, lungs, and spleen of each mouse at indicated time points after inoculation. Day zero represents mock-inoculated mice, used to determine basal numbers of cells in specific tissues. All comparisons are between routes of inoculation at each time point. Asterisks denote statistical difference (*, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P * 0.001$). Error bars represent standard errors of the means ($n = 8$). Data represented are a combined from two experiments with four mice per group for a total of eight mice per group per time point.

Intradermal inoculation led to an increase in the number of CD4^{+} T cells in the spleen at days 7 and 10 postinoculation compared to results with intranasal inoculation, which led to an increase in the number of CD4^{+} T cells late in infection (day 14) (Fig. 3). In the lung, intradermal inoculation led to a significant increase in the numbers of both CD4^{+} and CD8^{+} T cells at day 7 postinoculation, which returned to uninfected levels by day 14. In contrast, the intranasally inoculated mice had a delay in the increase of CD4^{+} T cells (not increasing until day 10), while there was no significant increase in the number of CD8^{+} T cells. Furthermore, only intranasal inoculation led to an increase of T cells in the bronchoalveolar space. Thus, the route of inoculation influenced the type, mag-

nitude, and kinetics of T-cell accumulation in the spleen and lung.

Intradermal but not intranasal inoculation induced a robust IFN-γ-mediated T-cell response. IFN-γ-mediated adaptive T-cell responses are important for resolving *F. tularensis* infection (4, 23, 41). The lung normally has an anti-inflammatory environment characterized by high levels of PGE$_2$ (45) which could interfere with the generation of IFN-γ-positive (IFN-γ^{+}) T cells. We hypothesized that intranasal inoculation fails to generate a robust IFN-γ[+] T-cell immune response. At various time points after inoculation, cells were collected from lungs and spleens. Leukocytes from these samples were stimulated with *F. tularensis*-infected splenocytes for 24 h. Cells

TABLE 1. Cellular infiltration into the bronchoalveolar space after intradermal or intranasal inoculation

Day[e]	Group[c]	Cell count[ab]					
		CD19^{+}	CD11c^{+}	CD11c^{+} CD11b^{low} GR1[mid]	CD11b^{+}	CD11b^{hi} GR1^{hi}	NK1.1^{+}
0	Control	18 ± 5	764 ± 213	11,000 ± 1,800	13 ± 3	152 ± 35	340 ± 262
7	ID	7 ± 7	875 ± 55	13,000 ± 2,300	19 ± 7	111 ± 26	1725 ± 620[d]
	IN	40 ± 10	2,291 ± 270[d]	7,600 ± 1,200	1,083 ± 147[d]	5,772 ± 808[d]	698 ± 69
10	ID	13 ± 1	996 ± 211	4,600 ± 770	16 ± 4	193 ± 27	376 ± 126
	IN	316 ± 107[d]	5,593 ± 665[d]	7,300 ± 130	1,870 ± 361[d]	8,937 ± 1375[d]	2,647 ± 253[d]
14	ID	17 ± 4	836 ± 103	4,300 ± 660	17 ± 2	240 ± 36	84 ± 18
	IN	9,644 ± 4,677[d]	3,334 ± 1,670	10,000 ± 4,400	2,351 ± 1,362[d]	3,452 ± 1,362[d]	950 ± 586

[a] Values are means ± standard errors (eight samples per group).
[b] Cells were defined as B cells (CD19^{+}), DCs (CD11c^{+}), alveolar macrophages (CD11c^{+} CD11b^{low} GR1[mid]), macrophages (CD11b^{+} CD11c^{−}), neutrophils (CD11b^{hi} GR1^{hi}), or NK cells (NK1.1^{+}).
[c] ID, intradermally inoculated mice; IN, intranasally inoculated mice.
[d] Significant difference ($P \leq 0.05$) between results for routes of inoculation at each time point as calculated by Student's *t* test. Data represented are combined from two experiments with four mice per group for a total of eight mice per group per time point.
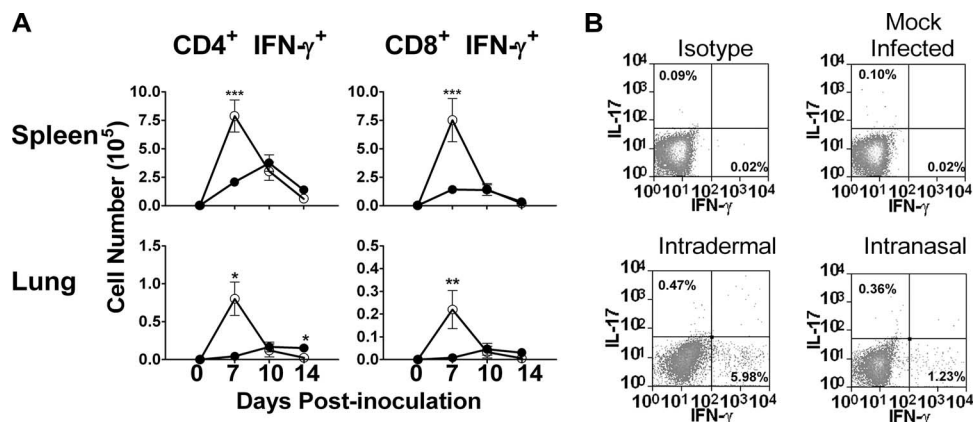[e] Day postinoculation. "0" indicates mock inoculation.

FIG. 4. Intradermal inoculation promotes an increased number of IFN-γ^+ T cells. C57BL/6J mice were inoculated with *F. tularensis* LVS either intradermally (10^5 CFU) ($\bigcirc$) or intranasally (5×10^2 CFU) ($\bullet$). Cells were isolated from the lungs and spleens of each mouse at indicated time points after inoculation. (A) T cells were then analyzed for total numbers of CD4$^+$ IFN-γ^+ or CD8$^+$ IFN-γ^+ cells. Day zero represents mock-inoculated mice, used to determine basal numbers of cells in specific tissues. All comparisons are between routes of inoculation at each time point. Asterisks denote statistical difference ($*$, $P \leq 0.05$; $**$, $P * 0.01$; $***$, $P * 0.001$). Error bars represent standard errors of the means ($n = 8$). Data represented are combined from two experiments with four mice per group for a total of eight mice per group per time point. (B) Representative dot plot histograms of CD4$^+$ T cells from the spleen on day 7 postinoculation.

were then stained for T-cell surface markers and intracellular IFN-γ. Bronchoalveolar lavage cells were not collected and stained for intracellular cytokines due to the low number of T cells recovered. Preliminary studies also examined NK cells; these cells did not contribute significantly to the IFN-γ response (data not shown). In fact, at a day-7 postinoculation, T cells made up more than 90% of the IFN-γ^+ cells, and by days 10 and 14 postinoculation, more than 95% of the IFN-γ^+ cells were T cells. We chose to do intracellular cytokine staining, versus accumulation of IFN-γ, since it allowed us to determine the total number of responding T cells in a given tissue, not just the general response. Figure 4A demonstrates representative staining profiles of CD4$^+$ T cells in spleen tissue for day 7 postinoculation mice. Percentages shown are percentages of CD4$^+$ T cells that were expressing either IFN-γ or IL-17. Similar responses were seen by CD8$^+$ T cells in the spleen and by CD4$^+$ and CD8$^+$ T cells in the lung. As can be seen with the dot plots, intradermal inoculation led to a more-vigorous IFN-γ^+ T-cell response than intranasal inoculation. By day 7 post-intradermal inoculation, there was a significant increase in the numbers of both CD4$^+$ and CD8$^+$ IFN-γ^+ T cells in the lung and spleen (Fig. 4B). In contrast, intranasal inoculation produced only a modest number of IFN-γ^+ T cells in either organ. There was no significant increase in IFN-γ^+ CD8$^+$ T cells in the lungs of intranasally inoculated mice. Thus, intradermal inoculation generated a robust IFN-γ^+ T-cell-mediated response not seen in intranasal inoculation, even in the lungs.

Intranasal infection led to a Th17-cell response. Intranasally inoculated mice still cleared a low dose of *F. tularensis*, even without the generation of a robust IFN-γ-mediated T-cell response. Studies have demonstrated the importance of Th17-cell-mediated responses in clearance of *Klebsiella pneumoniae* and *Mycobacterium tuberculosis* from the lung (13, 19, 52). We hypothesized that Th17 T cells were being generated after intranasal but not intradermal inoculation with *F. tularensis*. At various time points after inoculation, cells were collected from

lungs and spleens. BALF was collected at the same time. Leukocytes from the spleen and lungs were stimulated with *F. tularensis*-infected splenocytes for 24 h. Cells were then stained for T-cell surface markers, intracellular IFN-γ and IL-17. The concentration of IL-17 was determined in BALF by ELISA.

There was a significant CD4$^+$ IL-17$^+$ IFN-γ^- T-cell (Th17) response in the lung after intranasal inoculation. By day 10, there was a significant increase in the number of Th17 cells in the lungs of intranasally inoculated mice (Fig. 5A) but not in those of intradermally inoculated mice. At day 10, 50% of the cytokine-positive CD4$^+$ T cells against *F. tularensis* were making IL-17, and these were the only cells making IL-17. As expected, these Th17 cells did not make IFN-γ, as seen in Fig. 5C. A small increase in Th17 cells was seen in spleen samples from both intradermally and intranasally inoculated mice, but there was no significant difference between intranasal and intradermal inoculation, and it is only a minor component overall (6% of *F. tularensis*-specific CD4$^+$ T cells) (data not shown). There was also a significant increase in the concentration of IL-17 within the BALF from intranasally inoculated mice that was absent for intradermally inoculated mice (Fig. 5B). Thus, T-cell responses in the lung after intranasal inoculation were a mix of both Th1- and Th17-mediated responses.

PGE$_2$ release after intranasal inoculation affected generation of IFN-γ^+ T-cell responses and clearance of *F. tularensis*. We previously demonstrated that *F. tularensis* infection of macrophages in vitro leads to the production of PGE$_2$ and dampens Th1-cell development (49). PGE$_2$ can also block an IFN-γ response and promote a Th17-cell response (14, 22, 39). We hypothesized that PGE$_2$ was affecting the acute adaptive immune response generated after intranasal but not intradermal inoculation. BALF collected at various time points after intranasal and intradermal inoculation was tested for the accumulation of PGE$_2$. Both routes led to a significant increase in the PGE$_2$ concentration in the BALF on day 7 postinoculation compared to results for mock-inoculated mice (day 0) (Fig. 6A). PGE$_2$ levels in the BALF of intradermally inoculated
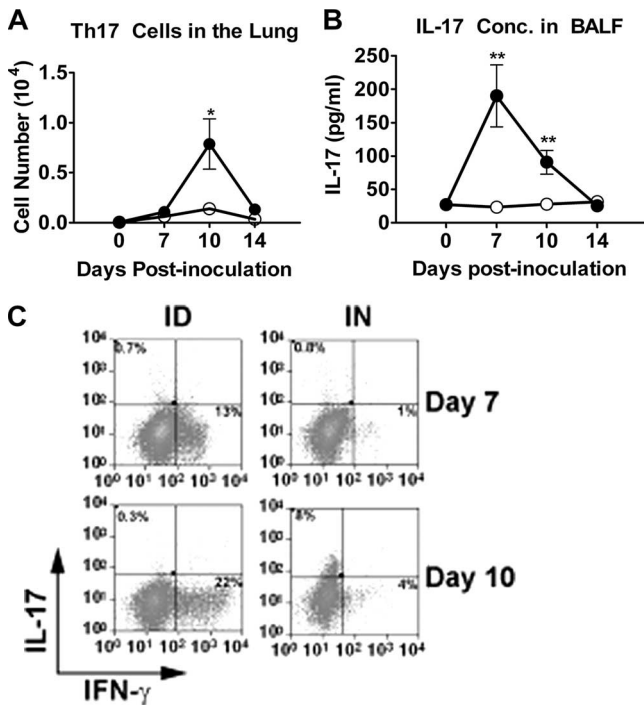
FIG. 5. Intranasally inoculated mice have increased number of Th17 cells and increased IL-17 in lung tissue. C57BL/6J mice were inoculated with *F. tularensis* LVS either intradermally (10^5 CFU) or intranasally (5×10^2 CFU). BALF and cells were isolated from the lungs of each mouse at indicated time points after inoculation. Day zero represents mock inoculation mice, used to determine basal numbers of cells in lung tissue and the concentration of IL-17 in BLF. (A) T cells were analyzed for total number of CD4$^+$ IL-17$^+$ cells. (B) BALF was analyzed for IL-17 by ELISA. All comparisons are between routes of inoculation at each time point. (C) Representative staining of CD4$^+$ T cells from the lungs of mice days 7 and 10 post-intranasal (IN) and intradermal (ID) inoculation. Asterisks denote statistical difference (**, $P \leq 0.01$). Error bars represent standard errors of the means ($n = 8$). Error bars on some samples are smaller than the symbols. Data represented are combined from two experiments with four mice per group for a total of eight mice per group per time point.

FIG. 6. PGE$_2$ delays the generation of IFN-γ^+ T cells and inhibits effective clearance of *F. tularensis* from the lung. C57BL/6J mice were inoculated with *F. tularensis* LVS either intradermally (10^5 CFU) or intranasally (5×10^2 CFU). (A) BALF was collected from all mice ($n = 8$) at indicated time points. BALF was analyzed for the concentration of PGE$_2$ using a standard ELISA. (B, C, D) C57BL/6J mice were inoculated with *F. tularensis* LVS intranasally (5×10^2 CFU). At days 5, 6, and 7 postinoculation, mice were treated with 1 mg/kg indomethacin in intraperitoneal injections. Untreated mice were injected with 1% DMSO. (B) On day 7, BALF was isolated from all mice and analyzed for the concentration of PGE$_2$ using a commercial ELISA. (C) On days 7, 10, and 14 postinoculation, cells were isolated from the lungs of all mice. T cells were then analyzed for IL-17 and IFN-γ. (D) On days 7, 10, and 14 postinoculation, the bacterial load was determined in the lungs of all mice. Panel A comparisons are between routes of inoculation at each given time point; panel B, C, and D comparisons are between treated mice and untreated mice. Asterisks denote statistical difference (*, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$). Error bars represent standard errors of the means ($n = 8$). Data represented are combined from two experiments with four mice per group for a total of eight mice per group per time point.

mice returned to control levels by day 10, while levels in the BALF of intranasally inoculated mice continued to increase from day 7 to day 14. Since we can detect PGE$_2$ in the BALF by day 7 postinoculation, it is likely that PGE$_2$ is functioning earlier and shaping the adaptive immune response in the lung. Thus, intranasal inoculation led to a prolonged elevation of PGE$_2$ in the BALF.

We hypothesized that indomethacin treatment of intranasally inoculated mice would lead to an increased IFN-γ^+ T-cell response and a decreased bacterial burden. Mice were either intranasally or intradermally inoculated with *F. tularensis* and then treated with indomethacin by intraperitoneal (i.p.) injection of 1 mg of indomethacin/kg of body weight in 1% dimethylsulfoxide (DMSO) on days 5, 6, and 7 postinoculation. Untreated mice were i.p. injected with 1% DMSO. To determine if indomethacin treatment was altering PGE$_2$ levels, BALF was collected at day 7. In preliminary studies, we demonstrated no difference in PGE$_2$ levels in the BALF of intranasally inoculated mice treated with i.p. injections of PBS or 1% DMSO (data not shown). Treatment of mice with indo-

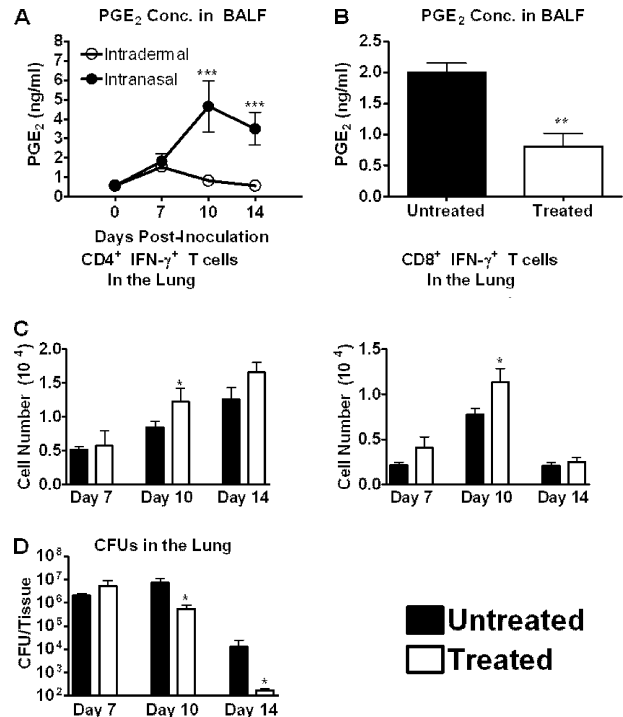methacin led to a significant decrease in the amount of PGE$_2$ in the BALF of intranasally inoculated mice (Fig. 6B). A similar response was seen with intradermally inoculated mice (data not shown).

Mice were intranasally or intradermally inoculated and treated with indomethacin as described above. At days 7, 10, and 14 postinoculation, leukocytes from spleen and lung tissue were isolated and stimulated with *F. tularensis*-infected splenocytes for 24 h, after which the numbers of IFN-γ^+ cells, IL-17$^+$ cells, total cell populations, and bacteria were determined. The treatment of mice with indomethacin changed the kinetics of IFN-γ^+ T-cell accumulation but not the peak level. Indomethacin treatment of mice did not affect the dynamics of the responding cell populations, since there was no change in the numbers of CD19$^+$, CD11c$^+$, CD11b$^+$, TCRβ^+ CD4$^+$, or CD8$^+$ cells in indomethacin-treated mice versus untreated

mice (data not shown). Intranasally inoculated mice treated with indomethacin showed a significant increase in the numbers of both CD4$^+$ and CD8$^+$ IFN-γ-producing cells in the lungs by day 10 postinoculation (Fig. 6C). Importantly, at this time there was a 94% reduction in the absolute number of bacteria within the lung tissue of treated mice (Fig. 6D). By day 14 postinoculation, the number of IFN-γ^+ CD4$^+$ T cells for the untreated mice was equivalent to that for the treated mice; this also corresponds to increased clearance of organisms from the lungs of the untreated mice. By day 7 postinoculation, 90% of the IFN-γ^+ cells in the lungs were also TCRβ^+, with the other 10% not being any one dominant cell population, regardless of treatment. At days 10 and 14 postinoculation, more than 95% of the IFN-γ^+ cells were also TCRβ^+ (data not shown). There was no change in the number of Th17 cells for indomethacin-treated mice from that for untreated mice, suggesting that Th17 and Th1 development are independent. Treatment of intranasally inoculated mice with indomethacin had no effect on the number of organisms in the spleen or the number of IFN-γ^+ T cells, nor did treatment of intradermally inoculated mice with indomethacin have an effect on either the organism burden or the T-cell response (data not shown). Thus, intranasal infection of mice with *F. tularensis* led to a prolonged elevation of PGE$_2$ levels that altered the subsequent immune response and organism clearance.

DISCUSSION

F. tularensis is able to infect through both the skin and lungs, and the route has a dramatic impact on the disease course and disease outcome. Here we demonstrate that acute primary T-cell responses depend on the route of inoculation, since regardless of the route, similar peak bacteria burdens are seen in both the spleen and lung. Intradermal inoculation led to a dominant IFN-γ^+ T-cell response in both the lung and spleen. Intranasal inoculation, however, produced a lower number of IFN-γ^+ T cells and a significant increase in the number of Th17 cells. Interestingly, we see differences in the number of responding T cells, even though the peak bacterial burden is similar, which would suggest similar antigen loads for T-cell stimulation. In fact, with delayed clearance of organisms from the intranasally inoculated mice, we would expect increased numbers of IFN-γ^+ T cells. With other bacterial infection models, it is the peak bacterial load that determines the magnitude of the T-cell response (32, 46, 47), though this may differ in *F. tularensis* infections. Since there are fewer IFN-γ^+ T cells in intranasally inoculated mice, we believe it is the microenvironment of the initial T-cell activation that is shaping the subsequent T-cell response, not the size of the bacterial burden.

IFN-γ-mediated T-cell responses are an important arm of the immune response against *F. tularensis* (10, 11, 23). We found that intradermal inoculation led to a significant increase in the numbers of IFN-γ^+ CD4$^+$ and CD8$^+$ T cells 7 days postinoculation in both the lung and spleen. This response rapidly returned to control levels by day 14 postinoculation, and accordingly, bacteremia was resolved by day 14. In contrast, the accumulation of T cells in the spleen and lung after intranasal inoculation was delayed. Even when there was an increase in cell numbers, the numbers of IFN-γ^+ T cells in the

lung after intranasal inoculation was significantly lower than that after intradermal inoculation. Intranasally inoculated mice fail to generate the same robust IFN-γ^+ T-cell response observed in intradermally inoculated mice.

Intranasally inoculated mice clear *F. tularensis* without a robust IFN-γ-mediated response. This is not surprising, since IL-12p35$^{-/-}$ mice clear *F. tularensis* and generate long-term immunity, even in the presence of little to no IFN-γ (9). IL-12p35$^{-/-}$ mice still produce the IL-12p40 subunit, which combines with the IL-23p19 subunit to make functional IL-23 (30), an important mediator in stabilizing IL-17 expression in T cells (16), consistent with the IL-17 responses we detected. This would suggest that IL-17 can at least partially substitute for IFN-γ in controlling *F. tularensis.* Th17 cells aid in the recruitment of neutrophils by stimulating epithelial cells to secrete granulocyte-macrophage colony-stimulating factor (21, 53). Along with T-cell responses, we saw a significant neutrophil infiltrate in the bronchoalveolar space of intranasally inoculated mice. Interestingly, human neutrophils are unable to kill *F. tularensis* (27). Other reports suggest that neutrophils are important for intradermal infections (2, 40); however, these studies are difficult to interpret, since they never determine which cells are depleted by the RB6-8C5 antibody treatment. This antibody, which depletes GR-1 cells, can deplete a variety of cells, including DCs, macrophages, and lymphocytes (7). Furthermore, a variety of macrophages express GR-1. In fact, one study demonstrates that in the liver, the major cell group infected with *F. tularensis* is GR-1$^+$ macrophages (33). Thus, making unequivocal conclusions about neutrophils is impossible. Further studies need to be done to clarify the role of neutrophils and IL-17 in tularemia. Since in humans neutrophils seem unlikely to kill *F. tularensis*, it would suggest that the neutrophil response seen in intranasal infection, along with IL-17, would be ill equipped to deal with a large dose of *F. tularensis*.

Prostaglandins are known to be powerful mediators of immune responses. The immunoregulatory properties of PGE$_2$ are the best characterized of those of the known prostaglandins. We have previously demonstrated the importance of PGE$_2$ in in vitro *F. tularensis* infection of macrophages (49). We have also demonstrated in our laboratory that both *Francisella novicida* strain U112 and *F. tularensis* subsp. *tularensis* strain Schu S4 induce PGE$_2$ production from bone marrow-derived macrophages (data not shown). It has also been clearly defined that PGE$_2$ is a major contributor to the microenvironment of the lung (45). Intranasal inoculation led to a significant increase in the amounts of PGE$_2$ in the lung. Due to the multiple receptors that PGE$_2$ can bind, it has both inflammatory and anti-inflammatory effects on immune cells (28). It is well established that in both humans and mice, PGE$_2$ leads to increased cAMP levels within T cells, which blocks IFN-γ and IL-2 production and subsequent T-cell proliferation and promotes the production of the Th2 cytokine IL-5 (1, 14, 22). This has also been documented in an in vivo setting (54). We demonstrate that pharmacological inhibition of PGE$_2$ production led to increased IFN-γ^+ T-cell numbers and a decreased bacterial burden in intranasally inoculated mice earlier than in untreated mice. Indomethacin treatment had no effect on intradermally inoculated mice (data not shown), suggesting that PGE$_2$ is more important in altering T-cell responses in the

lung. We specifically waited until day 5 postinoculation to treat mice with indomethacin to allow innate events to function normally in the context of PGE_2-mediated signaling but decrease during activation of adaptive T-cell responses. It is possible that the effect of decreased PGE_2 is not directly affecting the T-cell response but rather is affecting some unknown function. The simplest explanation, however, is that PGE_2 is directly blocking the generation of IFN-γ^+ T cells and the inhibition of PGE_2 production alleviates this inhibition. Regardless of the mechanism, inhibition of PGE_2 production in mice leads to both increased IFN-γ^+ T cells and a decreased bacterial burden, demonstrating that PGE_2 is detrimental to the host in clearance of *F. tularensis*. Further studies on the in vivo role of PGE_2 during *F. tularensis* infection are ongoing in our laboratory.

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