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Host Immune Response and Acute Disease in a Zebrafish Model of *Francisella* Pathogenesis▽

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Members of the bacterial genus *Francisella* are highly virulent and infectious pathogens. New models to study *Francisella* pathogenesis in evolutionarily distinct species are needed to provide comparative insight, as the mechanisms of host resistance and pathogen virulence are not well understood. We took advantage of the recent discovery of a novel species of *Francisella* to establish a zebrafish/*Francisella* comparative model of pathogenesis and host immune response. Adult zebrafish were susceptible to acute *Francisella*-induced disease and suffered mortality in a dose-dependent manner. Using immunohistochemical analysis, we localized bacterial antigens primarily to lymphoid tissues and livers of zebrafish following infection by intraperitoneal injection, which corresponded to regions of local cellular necrosis. *Francisella* sp. bacteria replicated rapidly in these tissues beginning 12 h postinfection, and bacterial titers rose steadily, leveled off, and then decreased by 7 days postinfection. Zebrafish mounted a significant tissue-specific proinflammatory response to infection as measured by the upregulation of interleukin-1β (IL-1β), gamma interferon, and tumor necrosis factor alpha mRNA beginning by 6 h postinfection and persisting for up to 7 days postinfection. In addition, exposure of zebrafish to heat-killed bacteria demonstrated that the significant induction of IL-1β was highly specific to live bacteria. Taken together, the pathology and immune response to acute *Francisella* infection in zebrafish share many features with those in mammals, highlighting the usefulness of this new model system for addressing both general and specific questions about *Francisella* host-pathogen interactions via an evolutionary approach.

Members of the bacterial genus *Francisella*, a gram-negative coccobacillus, are found naturally in the environment and can survive in diverse animal hosts ranging from amoebas to humans (29). In mammals, *Francisella tularensis* is the causative agent of tularemia, a highly infectious zoonotic disease. The bacterium circulates naturally in lagomorphs and rodents, from which intermediate vectors, such as flies, ticks, and mosquitoes, can transmit the bacterium to humans (20). *Francisella* infection can also be acquired through ingestion of or contact with contaminated food, water, and aerosols. The high infectivity of *F. tularensis* has led to its development by the United States and Russia as a potential biowarfare agent (61). Consequently, the threat of an intentional malicious release of *F. tularensis* has generated considerable interest in basic research on virulence mechanisms and host immunity to *Francisella.* In addition, *Francisella* serves as an excellent model for studying the pathogenesis of intracellular bacterial infections (64).

The basis of the pathogenicity of *Francisella* is poorly understood, and advances in our understanding are hampered by the lack of knowledge of the precise mechanisms which underlie differences in virulence between the various strains of *F. tularensis* (21, 36). Furthermore, it is unclear what contribution host specificity plays in the immune response to *Francisella* infection, as different subspecies of *F. tularensis* vary widely in virulence between mice and humans (16, 21). Currently, mice are the most commonly utilized animal model for studies using LVS (a laboratory-attenuated strain of *F. tularensis*) or *F. novicida*, a subspecies of *F. tularensis* which is used primarily owing to its ease of genetic manipulation (21, 58). In vivo, the primary replication niche for *Francisella* is inside macrophages (3, 7). *Francisella* gains entry to macrophages via phagocytosis, where the bacteria arrest the maturation of the phagosome and escape to the cytosol to replicate (10, 13, 14). Hence, studies of *Francisella* typically are conducted using mouse and human macrophages, where there are major differences in the degrees to which inflammatory cytokines are elicited by either of the commonly used *Francisella* subspecies and by the more virulent type A *Francisella* pathogens (7–9, 30). Further complicating the matter, macrophages isolated from murine lung, rather than peripheral tissue, appear to use different mechanisms to combat *Francisella* infection (37, 56). Thus, it is difficult to determine the extent to which studies with different model systems and the use of different bacterial strains can apply to efforts to understand tularemia disease in humans (15). An additional comparative model system may help to clarify the general and unique aspects of the pathogenesis of *Francisella* in the context of interaction with different hosts.

Although studies with cell culture and model systems like *Drosophila melanogaster* and *Caenorhabditis elegans* can address fundamental questions about pathogenesis and host responses, the lack of complex immune systems typical of vertebrates in such models limits their relevance to mammalian in vivo disease processes (39). The zebrafish (*Danio rerio*) is well poised to fill an important gap between invertebrates and mice in studies of host-pathogen interactions. Unlike flies and worms, zebrafish share key immune effectors and pathways with mammals, having both B and T lymphocytes, antigen-

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presenting cells, phagocytic cells that produce reactive nitrogen and oxygen species, and elements of humoral immunity, including complement. Moreover, fish have orthologs of mammalian Toll-like receptors (TLRs) and Nod-like receptors, which, when engaged by microbial infection, cause the expression of specific cytokines and chemokines (42, 66). The similarities between fish and mammalian immune systems, coupled with practical advantages, such as a low cost for husbandry, a fully sequenced genome, and a growing collection of transgenic and knockout fish, have enabled numerous researchers to establish models of bacterial and viral infections in zebrafish (18, 35, 45, 57, 59, 63, 67).

Recently, an emerging virulent bacterial pathogen capable of infecting and causing systemic disease in a variety of fish species has been identified as a new species of *Francisella* (4, 41, 47, 48). Many aspects of the described pathology caused by this bacterium mimic tularemia in mammals (58, 61). Isolates of *Francisella* from fish are highly infectious and virulent, and infected fish develop skin lesions, swollen spleens and kidneys, and granulomas in internal organs. This discovery of *Francisella* isolates affecting fish provides an excellent opportunity to establish a new model system of *Francisella* infection in a vertebrate host.

Here, we establish a zebrafish/*Francisella* sp. model system and report on the transcriptional regulation of important innate immune effectors in response to infection. We adapted *Francisella* isolates from the hybrid striped bass (*Morone saxatilis*) for our investigation, as this host shares the similar optimal growth temperature of 25 to 29°C with zebrafish (47). Zebrafish infected with *Francisella* sp. undergo an acute disease process, succumbing to infection in a dose-dependent manner. Challenged fish elicit a robust proinflammatory immune response beginning as early as 6 h postinfection (p.i.) and persisting up to 1 week. These responses share many similarities with findings from studies of mice and humans (7, 9, 12, 15, 19, 22, 24, 40, 49, 65), suggesting that zebrafish are a useful and relevant comparative model system to study host-pathogen interactions during *Francisella* infection.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. Isolates of a *Francisella* species from hybrid striped bass (47) were used for this study. To confirm the bacterial identity of the *Francisella* sp. isolates, we isolated genomic DNA from cultures by using a QiaAMP DNA purification kit according to the manufacturer's instructions (Qiagen). The 16S rRNA gene was amplified using universal 16S bacterial primers 27F [5′-AGA GTT TGA TC(AC) TGG CTC AG] and 1492R [5′-GG(CT) TAC CTT GTT ACG ACT T] (62). PCR products were cloned into pTOPO (Invitrogen) for sequencing according to the manufacturer's instructions and sequenced using M13 forward and reverse primers. Sequencing was performed using an ABI Big Dye Terminator cycle sequencing kit v1.1 with an ABI Prism 3130 genetic analyzer. Bacteria were routinely cultured at 26°C on CHAH plates, consisting of cysteine heart agar (Difco) supplemented with 1% hemoglobin (Oxoid).

Generation of bacterial antisera. *Francisella* sp. colonies from CHAH plates were resuspended in sterile saline and used to inoculate heart infusion broth (Difco) supplemented with 0.2% cysteine and 2% glucose. After rotary incubation at 28°C for 48 h, an aliquot was removed and streaked onto CHAH plates to confirm purity. Bacteria were inactivated with 0.5% formaldehyde and held at room temperature for 7 days, after which an aliquot was streaked onto CHAH plates to confirm inactivation. Cells were washed two times with phosphate-buffered saline (PBS), and the cell suspension was adjusted to an optical density at 600 nm of 1.2. The bacterial cell suspension was combined with equal volumes of Freund's incomplete adjuvant and homogenized completely. Two female New Zealand White rabbits each received an initial subcutaneous injection at the shoulder region. This regimen was repeated three times at 2-week intervals. Seven days after the last immunization, rabbits were anesthetized, and blood was collected by exsanguination and allowed to clot overnight at 4°C. Serum was then harvested and stored at −20°C until required.

Zebrafish care and maintenance. EKW zebrafish were obtained from Ekkwill Waterlife Resources (Gibsonton, FL) and initially reared in 100-liter circular tanks, which received sand-filtered, UV-irradiated, fresh water in a flowthrough fashion (1 liter/min) with one air stone per tank under quarantine conditions. Average water quality parameters were as follows: temperature of 23°C, pH 7.5, total hardness of 51.3 mg/liter $CaCO_3$, alkalinity of 51.3 mg/liter $CaCO_3$, unionized ammonia at 0.0 mg/liter, and nitrite at 0.0 mg/liter. The EKW fish were spawned and specific-pathogen-free (SPF) fish were generated by bleaching fertilized eggs according to established protocols (70). Fish were then transferred to the WFRC's zebrafish suite. After static-configuration housing in incubators (set at 27°C) until 10 days posthatching, larval fish were moved to and reared in a commercial recirculating housing system (Aquatic Habitats, Apopka, FL). All fish were maintained at 27°C using a 14-h/10-h cycle of light and darkness at a maximum stocking density of 10 adult fish per liter of water. Average water quality parameters were as follows: pH 7.8, total hardness of 102.6 mg/liter $CaCO_3$, alkalinity of 51.3 mg/liter $CaCO_3$, un-ionized ammonia at <0.03 mg/liter, nitrite at 0.0 mg/liter, and nitrate at 0.0 mg/liter. Larval fish were fed live rotifers (*Brachionus plicatilis*) and shrimp larva diet (AP 100, 250, or 450; Zeigler Bros., Inc.) two to three times daily. Adult fish were fed twice daily with adult zebrafish complete diet, irradiated (Zeigler Bros., Inc.), and earthworm fish flake food (Aquatic Eco-Systems, Inc.). Adult SPF zebrafish used in experiments ranged in age from 4 months to 8 months. Prior to injection, zebrafish were anesthetized by immersion in 100 ng/ml of Tris-buffered MS-222 (Finquel; Argent Laboratories). Euthanasia of zebrafish consisted of immersion in Tris-buffered MS-222 at 300 ng/ml for at least 10 minutes following cessation of opercular movement. The protocols for experimental use of zebrafish for this study were approved by the Institutional Animal Care and Use Committee of the University of Washington (Seattle).

Infection of zebrafish. We initially tested three primary isolates of *Francisella* sp. that differed by only approximately 5 to 8% in virulence, using similar doses. Subsequent studies were done using the most virulent strain. *Francisella* sp. isolates were cultured from glycerol stocks on CHAH plates for 4 days at 26°C prior to infection. Bacteria were scraped into sterile PBS, vortexed, and diluted to specific optical density at 600 nm concentrations corresponding to approximate CFU/ml as previously determined. Bacterial cells diluted to the appropriate concentrations in PBS were used for injection of zebrafish. The final concentration of infectious bacteria was determined by plating serial dilutions of the injection stock on CHAH plates. Bacterial concentrations were also determined using a quantitative PCR (qPCR) assay for live *Francisella* sp. (see below). Specifically diluted samples of *Francisella* sp. were heat inactivated at 56°C in a water bath for 50 min; heat inactivation was confirmed by plating undiluted samples.

For intraperitoneal (i.p.) injections, anesthetized zebrafish were placed on a wet sponge and injected using a 30-gauge needle into the midline of the coelomic cavity, posterior to the pectoral fins. The correct positioning of the injection site was determined by injection of trypan blue as described by Neely et al. (45). By use of a repeat pipette equipped with a 30-gauge needle, fish were injected with a total of 20 μl of bacterial suspension, heat-killed bacteria, or PBS. Four to eight fish were injected per needle. Infected and control fish were held in static 3-liter tanks with lids, in groups of up to 12 fish per tank, at 24°C. A 100% water volume change occurred daily for each tank, by siphoning, and water was aerated continuously using air stones. Fish were fasted for 12 h prior to injection and for 24 h following injection, at which time once-daily feeding was resumed. All tanks were observed twice daily for signs of morbidity and mortality.

After euthanasia by 300 ng/ml MS-222, zebrafish tissues were sampled at 6 h, 12 h, 24 h, 48 h, 72 h, and 7 days following infection. Each sampling point consisted of eight control (PBS-injected) or *Francisella*-infected fish and five fish per time point for the fish challenged with heat-killed bacteria. Livers, spleens, kidneys, and gills were recovered individually from euthanized fish and snap-frozen in liquid nitrogen. Samples were stored at −80°C until RNA extraction.

Histology and IHC. Fish were euthanized with an overdose of Tris-buffered MS222, and 10 minutes after cessation of opercular movements, the body cavity was injected with Dietrich's solution (30% ethanol, 10% formalin, and 0.2% acetic acid) prior to immersion and fixation in Dietrich's solution. Entire fish, split lengthwise, were placed into standard tissue cassettes for routine overnight processing. After the tissue was embedded in paraffin, 4-μm-thick sagittal sections were cut and transferred to ProbeOnPlus glass slides (Thermo-Fisher Scientific, Inc.). Sections were deparaffinized, rehydrated through graded ethanols to deionized water, and then stained with Gill's hematoxylin and eosin

TABLE 1. qPCR primers and probes

Gene	Primer/probe	Sequence (5′–3′)	GenBank accession no.
ARP	ARP 67 Fwd	CTG CAA AGA TGC CCA GGG A	NM_131580
	ARP 187 Rev	TTG GAG CCG ACA TTG TCT GC	
	ARP Probe	TTC TGA AAA TCA TCC AAC TGC TGG ATG ACT **A**CC[a]	
IFN-γ	IFN-γ Fwd	CTT TCC AGG CAA GAG TGC AGA	NM_212864
	IFN-γ Rev	TCA GCT CAA ACA AAG CCT TTC G	
	IFN-γ Probe	ACC GCT ATG GGC GAT CAA GGA AAA CGA C	
TNF-α	TNF-α Fwd	TCG CAT TTC ACA AGG CAA TTT	NM_212859
	TNF-α Rev	GGC CTG GTC CTG GTC ATC TC	
	TNF-α Probe	AGG CTG CCA TCC ATT TAA CAG GTG GAT ACA A	
IL-1β	IL-1β Fwd	CAT CCA AGA GCG TGA AGT GAA	NM_212844
	IL-1β Rev	AAC ACA CAG GCT GAG CAG AAG T	
	IL-1β Probe	TCC ACA TTT GCA GGC CGT CAC C	
iNOS	iNOS Fwd	CCA GAG CCT TCG TCT GGA GA	NM_001104937
	iNOS Rev	TTA GAG CCT GGA CGA GCG TG	
	iNOS Probe	CCA GCC ATG AGG GAA AGA GAT GGC AG	
Francisella sp. 16S rRNA	FspQPCR Fwd	GCG AAG GCA ACA TTC TGG AT	DQ377177
	FspQPCR Rev	GCG TTG CAT CGA ATT AAA CCA	
	FspQPCR probe	AAT ACT GAC ACT GAG GGA CGA AAG CGT GG	

[a] There is a single-nucleotide polymorphism between the sequence for the EKW fish used in our experiments and the sequence in GenBank. The nucleotide is in bold; in the GenBank sequence, the A is a T.

(H&E) using standard protocols. Adjacent sections were used for immunohistochemistry (IHC), and all incubations were performed in a humidified chamber at room temperature using a biotin-free, polymer-based EnVision G2 System/AP detection kit (DakoCytomation). Serum-free protein block (DakoCytomation) was applied to sections, which were incubated for 10 min, and then drained off without washing. Rabbit antisera against *Francisella* sp. were diluted 1:20,000 in 50 mM Tris-buffered saline (TBS) (pH 7.4). Rabbit preimmune serum was diluted 1:5,000, also in TBS. Diluted antisera were applied to sections, incubated for 30 min, and then washed once in TBS with 0.05% Tween 20 (TBST) for 5 min. A polymer-conjugated anti-rabbit secondary antibody was applied, and the sections were incubated for 30 min, followed by two 5-min washes in TBST. Sections were then incubated with an alkaline phosphatase-labeled polymer for 30 min, followed by two 5-min washes in TBST. After incubation with Permanent Red chromogen for 10 min, sections were rinsed, counterstained with Mayer's hematoxylin for 5 min, and then immersed in 37 mM ammonia water. Slides were covered by a coverslip with Faramount aqueous mounting media (DakoCytomation). Two fish per time point were fixed. A blinded reviewer scored all H&E slides for pathology, and results were confirmed by a second reviewer and compared to results from IHC slides. Slides were examined by bright-field microscopy with a Zeiss Axiophot photomicroscope and images collected with an Evolution MP 5.0 charge-coupled-device digital camera (Media Cybernetics) and QCapture Pro software (QImaging Corp.).

RNA preparation and cDNA synthesis. Tissue samples were homogenized in RLT (Qiagen) lysis buffer by use of lysing matrix D tubes (MP Biomedicals) in a Fastprep system (Bio101-ThermoSavant), followed by total RNA extraction from the homogenates using an RNeasy RNA extraction kit (Qiagen) with in-column DNA digestion according to the manufacturer's instructions. RNA was eluted in 40 μl of RNase-free water and quantified using a Nanodrop ND-1000 spectrophotometer (Thermo Scientific). Owing to low yields, zebrafish spleen and kidney RNA samples were concentrated using a speedvac prior to quantification. Samples were used immediately in cDNA synthesis reactions or stored for less than 1 week at −20°C before cDNA synthesis. Five hundred nanograms of total RNA was used in cDNA reactions for most samples; however, many spleen and kidney samples contained less than 500 ng of total RNA. In these cases, the entire sample was used in the cDNA synthesis reaction. cDNA was synthesized by incubation of RNA with 100 ng/μl of random hexamer primers (Promega) in a total volume of 10 μl at 68°C for 3 min, followed by 2 min at 4°C. Then, 10 μl of 2× reverse transcriptase buffer containing Moloney murine leukemia virus reaction buffer (Promega), 1 mM deoxynucleoside triphosphates (Promega), 10 U RNAsin (Promega), and 1 U Moloney murine leukemia virus reverse transcriptase (Promega) was added, and the reaction solution was mixed and incubated at 37°C for 1 h, followed by heat inactivation at 95°C for 5 min. Negative controls lacking reverse transcriptase or RNA were included for each group. The final cDNA reaction mixtures (20 μl) were diluted 1:5 with 80 μl of water and stored at −20°C until use in qPCRs.

Primer and probe design. Primers and probes were designed using ABI Primer Express software v1.5 from sequences deposited in GenBank (accession numbers are listed in Table 1). Amplicons were designed to span exon-exon boundaries to avoid amplifying genomic DNA. To confirm the specificity of each primer set, cDNA was amplified using a standard *Taq* polymerase PCR: 34 cycles of 94°C for 30 s, 57°C for 30 s, and 72°C for 1 min, followed by a final extension of 10 min at 72°C. Amplified products were checked for size by agarose gel electrophoresis and cloned into pTOPO (Invitrogen) according to the manufacturer's instructions. Selected clones were sequenced using M13 forward and reverse primers. Sequencing was performed using an ABI Big Dye Terminator cycle sequencing kit v1.1 with an ABI Prism 3130 genetic analyzer.

Quantitative real-time PCR. Quantitative real-time PCR assays were run using an ABI Prism 7900HT sequence detection system. Primers and gene-specific dual-labeled probes (5′FAM [5-carboxylfluoroscene] and 3′TAMRA [6-carboxytetramethylrhodamine]) were obtained from MWG-Biotech. Total reaction mixtures (12 μl) contained 5 μl of diluted cDNA samples, 6 μl of ABI universal PCR master mix, 917 nM each of forward and reverse primers, and 200 nM of the gene-specific probe. Standard cycling conditions were 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 61°C for 1 min. Samples were run in duplicate, and threshold values were averaged over the two samples.

PCR efficiency was determined separately for each primer/probe set. A standard cDNA template with relatively high levels of expression of target genes was made by pooling two zebrafish gill cDNA preparations. This pool was serially diluted sixfold before use in a standard qPCR with each individual primer/probe set. The threshold cycle (C_T) value in the qPCR was plotted against log-transformed cDNA input values to calculate the slope; efficiency was calculated using the equation $E = 10^{(-1/\text{slope})}$ (53). Efficiencies for our amplicons were calculated at the following values: for acidic ribosomal protein (ARP), 2.06; for gamma interferon (IFN-γ), 2.06; for interleukin-1β (IL-1β), 2.05; for inducible nitric oxide synthase (iNOS), 1.96; and for tumor necrosis factor alpha (TNF-α), 1.96.

ARP primers were used to normalize all samples to equivalent input cDNA amounts. C_T values for each reaction were reported by the ABI software. Relative expression levels were calculated using the Pfaffl method (53), with efficiency correction for each primer/probe set, using REST software (http://www.gene-quantification.de/rest.html) (54).

In order to quantify the bacterial load for each sampled tissue, a qPCR assay was developed based on the 16S rRNA sequence obtained from the specific

Francisella sp. isolates used in this study. qPCR was performed as described for the detection of zebrafish transcripts; primer and probe sequences are shown in Table 1. To correlate the detection of *Francisella* sp. 16S rRNA with bacterial burden and to ensure the specificity of the assay for *Francisella*, we performed side-by-side comparisons of plate bacterial counts and 16S rRNA levels as reported by threshold values in the qPCR assay. A range of set numbers of *Francisella* CFU as determined by plate counts was added to various uninfected zebrafish tissue samples. RNA was extracted and cDNA synthesized as described above. This experiment was repeated in triplicate, with various amounts of bacterial input. The equation derived from the direct correlation between plate counts and 16S rRNA detection ($r^2 = 0.92$) was used to calculate *Francisella* sp. burden in all sampled tissues (data not shown). The minimum number of reproducibly detectable bacteria was 40 CFU, as determined by plate counts. In all control samples tested, C_T values were greater than the threshold level of the assay, indicating that zebrafish or commensal bacterial cDNAs do not interfere with the specific detection of *Francisella* sp. 16S rRNA.

Statistical analysis. Kaplan-Meier curves were used to compare survival rates of zebrafish infected with different numbers of bacteria. Differences between doses were compared using a log rank test in SPSS version 11.5 (SPSS Inc., Chicago, IL). Nonparametric statistical significance of relative gene expression was calculated with REST software (54) by a pairwise fixed reallocation randomization test with 50,000 repeats. This calculation is based on the probability of observing ratios equal to or greater than no effect in randomly assigned control and challenged sample groups. The significance of differences in relative gene expression levels between fish challenged with heat-killed and live bacteria was calculated by a Student *t* test assuming equal variation. Correlations between bacterial load and relative gene expression were calculated by multiple stepwise linear regressions using SPSS software. For multiple regressions with four variables, gene expression and bacterial load data were pooled from individual liver, spleen, and kidney samples (equaling 24 total sample points) at each time point in order to achieve an appropriate sample size (46).

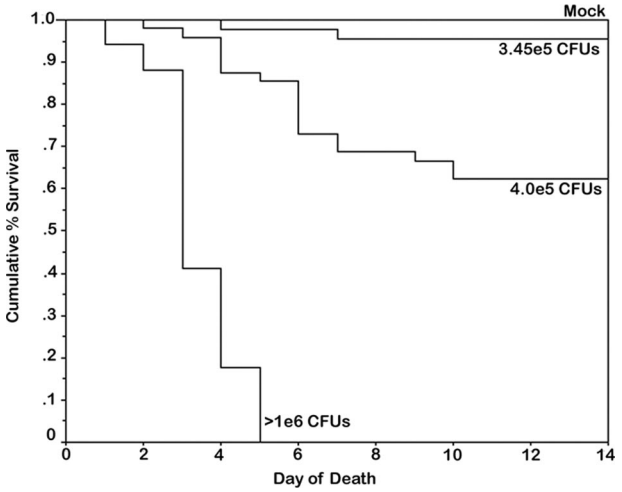

FIG. 1. Zebrafish succumb to *Francisella* sp. infection in a dose-dependent manner. Mortality data are from two independent experiments (25 fish per group in duplicate per experiment) and are representative of the four separate dose-response experiments conducted. Fish were in two or three separate tanks; no significant tank effects were observed, and data were pooled for analysis. Challenge doses in CFU are indicated. No mortality was observed for the control fish in any of the challenge experiments. Kaplan-Meier curves were calculated for these doses, each of which shows a significant difference from the PBS-injected control by the log rank test ($P < 0.001$).

RESULTS

***Francisella* sp. reproducibly infects and causes morbidity and mortality in zebrafish.** In order to determine the *Francisella* sp. infectious doses in zebrafish, SPF adult zebrafish were i.p. injected with different doses of *Francisella* or with PBS. We observed a dose-dependent mortality, with most deaths occurring between days 2 and 7 p.i. (Fig. 1). Fish injected with high concentrations of bacteria died more quickly than fish injected with intermediate or low concentrations of bacteria. Signs of disease included loss of appetite, darkening of the skin, increased respiration rates, listlessness, lethargy, and weak swimming. The coelomic cavity of moribund fish swelled substantially (ascites), and fish developed red or dark-brown lesions near the injection site. Zebrafish injected with PBS or with heat-inactivated bacteria developed no signs of disease and had no mortality.

***Francisella* sp. begins replicating in zebrafish 12 h p.i.** Since typical *Francisella* infections in mammals are characterized by the ability of bacteria to replicate within diverse tissues, we determined whether the fish-specific *Francisella* sp. has the same ability. *Francisella* species are fastidious organisms requiring rich media to culture, and commensal and environmental bacteria often interfere with the detection of *Francisella* in tissue samples (52). This was true for our zebrafish/*Francisella* sp. model as well; thus, we were not able to quantify bacterial load and spread by direct CFU counts from tissues. To circumvent this issue, we developed a qPCR assay for *Francisella* sp. 16S rRNA to follow bacterial spread and replication (15). An equation based on a direct correlation between bacterial plate counts and 16S rRNA contents as determined by qPCR was generated (data not shown). This equation was used to calculate a relative bacterial burden in total cDNA generated from zebrafish tissue samples. The kinetics of bacterial replication were examined in fish injected with 1.9×10^5 CFU. No detectable quantities of *Francisella* sp. bacteria were ever found in mock-infected control animal tissues by use of our qPCR assay.

In zebrafish challenged with live *Francisella* sp., bacteria were detected in all organs tested by 6 h p.i., but the cumulative number of bacteria detected represents only a small fraction of the number of bacteria initially injected. Thus, at 6 h the majority of the bacteria had not yet trafficked to the spleen, liver, or kidney or did not survive the initial phase of infection (Fig. 2). At 12 h p.i., the mean and median numbers of bacteria detected were less than those detected at 6 h p.i. in all organs, implying that some bacteria had died or moved from these tissues and that survivors had not yet begun to replicate (Fig. 2). By 24 h p.i., there were between 50- and 1,000-fold more bacteria detected in each organ, indicating that in zebrafish at 24°C, bacteria replicated between 12 and 24 h p.i. (Fig. 2). Alternatively, this could indicate that bacteria did not effectively traffic to the tested tissues until after 12 h p.i. The cumulative bacterial counts in the four tested tissues represented less than half the number of injected CFU. Overall, there was only a small degree of correlation between bacterial loads in different organs of the same individual; the animal with the highest number of bacteria in the liver and gill had only intermediate bacterial burden in the spleen and kidney, while an individual with higher counts in the spleen and kidney had intermediate levels of bacteria detected in the liver and gill.

The bacterial load detected in each tissue continued to increase between 24 and 72 h p.i., although at a lower rate than that observed between 12 and 24 h p.i. (Fig. 2). The number of

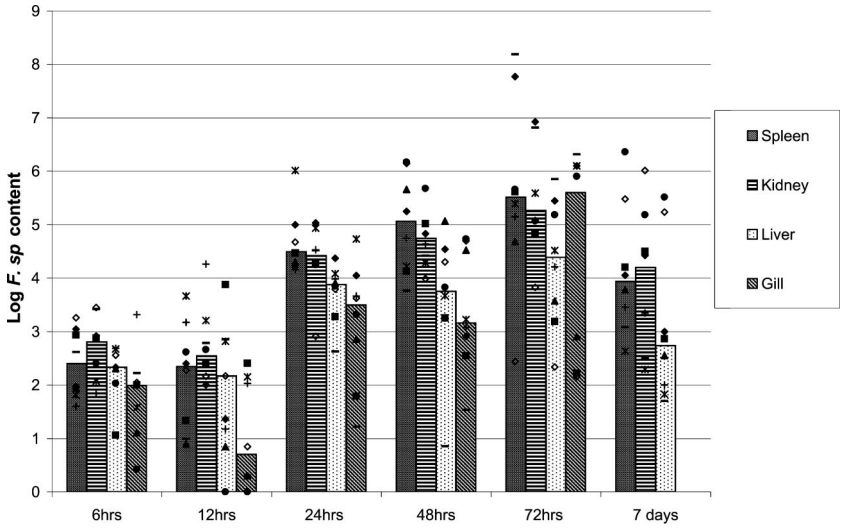

FIG. 2. Bacterial burdens in challenged zebrafish tissues. Fish were injected i.p. with $1.9 \times 10^5 \pm 0.24 \times 10^5$ CFU of *Francisella* sp (F. sp). The estimated cumulative mortality for this experiment was 30% after correcting for sampling. Bars represent median $\log_2$ CFU in individual tissues, as detected by qPCR for *Francisella* 16S rRNA, while symbols represent individual data points (eight individuals per point). Bacterial loads in the gill were not determined at the day 7 p.i. time point.

bacteria detected in the gill, a tissue to which bacteria must traffic through blood, increased markedly at 72 h p.i. By 7 days p.i., the overall number of bacteria had decreased, although all tested tissues from infected zebrafish were still colonized.

***Francisella* sp. colonizes and causes tissue damage in multiple organs.** Owing to the small size of zebrafish, an entire animal can be fixed, sectioned, and mounted onto one slide, allowing a histopathological evaluation of the progression of disease in a systemwide fashion. Serial sections of individual fish were alternately stained using H&E to examine pathological changes following infection or processed for IHC using *Francisella* sp.-specific antibodies, which allowed us to survey the types of tissues ultimately colonized. Histological examination of infected animals revealed the presence of *Francisella* sp. antigen and tissue damage in multiple organs as early as 48 h p.i. (Fig. 3 and data not shown). Infected animals had focal to diffuse areas of necrosis corresponding with the presence of bacterial antigen in the liver, spleen, pancreas, and kidneys. Entire kidneys were permeated with bacterial antigen by this time point (Fig. 3A and B). Spleens also contained bacterial antigen spread diffusely throughout the entire parenchyma (data not shown). As teleost fish species, including zebrafish, do not have lymph nodes and as mature lymphocytes interact in the kidney and spleen (23, 31), infected macrophages likely transit *Francisella* throughout the kidney and spleen. In contrast, the liver was colonized mainly in discrete spots at the capsule boundary and in surrounding connective and visceral adipose tissues (data not shown). IHC also detected the presence of bacteria in the gills of infected fish; however, there was no observable tissue damage on corresponding H&E-stained sections (Fig. 3C and D).

By 5 days p.i., focal regions of necrosis in the liver had become larger and more diffuse but were still associated with the periphery of the parenchyma. The regions of hepatic necrosis remained strongly correlated with the presence of bacterial antigen (Fig. 4A and B). In the kidney, discrete areas stained very densely by IHC, implying the aggregation of bacteria (Fig. 4C). These areas appeared to correspond with renal necrosis and early granuloma formation, visible by H&E staining (Fig. 4D). At this time point, granulomas are in the initial stages of development and are yet to be walled off from the surrounding renal tissue. The spleen is also densely and diffusely packed with bacterial antigen (Fig. 4E) and is severely necrotic and lacking normal parenchymal architecture (Fig. 4F). During histopathological evaluation, it was clear that the presence of *Francisella* sp. antigen correlated primarily with lymphoid tissue, the liver, and pancreatic tissue, as no other organs were clearly stained by IHC at the time points tested. No cellular necrosis or background IHC staining was observed in animals injected with PBS alone (Fig. 3F and data not shown), and preimmune antisera did not cause any IHC staining in infected animals (Fig. 3E).

***Francisella* sp. infection induces a potent proinflammatory gene response in zebrafish.** We chose five early time points during the infection process to assess the induction of inflammatory cytokines in our zebrafish/*Francisella* model. Specified tissues were isolated from eight individual infected and control fish at 6 h, 12 h, 24 h, 48 h, 72 h, and 7 days p.i. Differential expression of IFN-γ, TNF-α, IL-1β, and iNOS mRNA was measured by qPCR for each tissue and time point.

We observed two distinct waves of a proinflammatory immune response to infection (Fig. 5). Six hours p.i. may have been near the peak of an immediate-early response, as IFN-γ, TNF-α, and IL-1β were all significantly upregulated in the spleen and liver. By 12 h p.i., IFN-γ, TNF-α, and IL-1β were at levels lower than those at 6 h p.i. By 24 h p.i., which corresponds to a dramatic increase in the number of bacteria detected (Fig. 4), cytokine gene induction had again increased. In general, the spleen demonstrated the highest degree of transcriptional upregulation for the tested

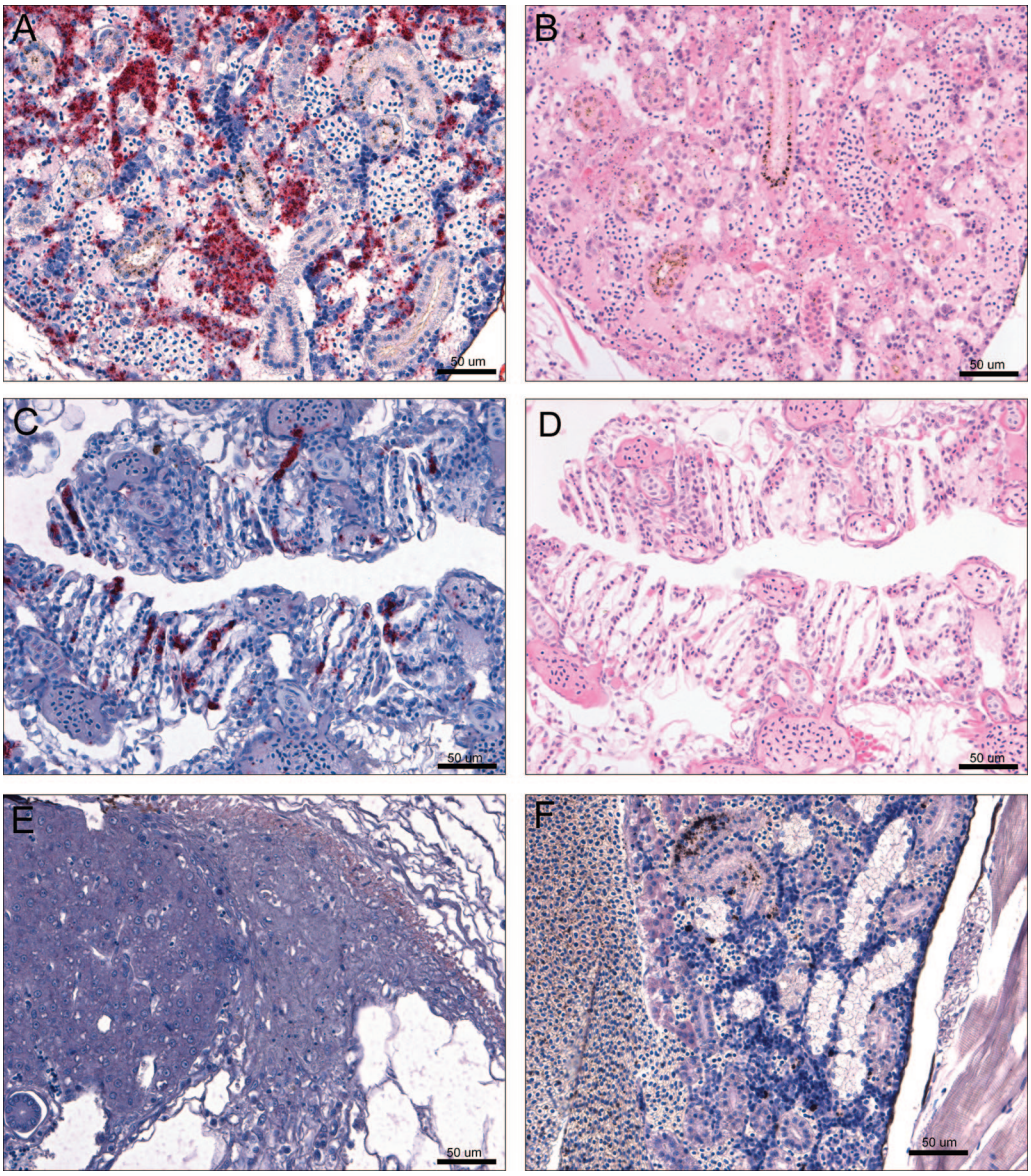

FIG. 3. Various zebrafish tissues are colonized and damaged by *Francisella* infection by 48 h p.i. Fish were infected as described in the legend for Fig. 2. Serial slides were stained with antiserum against *Francisella* (A, C, E, and F) or H&E (B and D), as described in Materials and Methods. Bacterial antigen is detected as dark-red staining. The presence of bacterial antigen throughout the renal interstitial parenchyma and focal necrosis is shown (A and B). Despite the presence of bacterial antigen in the gills of infected fish (C), there was no visible tissue damage (D). Antiserum used to stain slides is highly specific to *Francisella* sp.; no nonspecific background staining with preimmune sera (E) or against PBS-injected, uninfected animals (F) was observed.

genes, while the IL-1β gene was the only significantly upregulated gene in gills of infected fish at 6 and 24 h p.i.

In *Francisella* sp.-challenged zebrafish, the IL-1β gene was the most robustly upregulated gene we tested for the first 2 days following infection, and it remained upregulated in all tissues at all time points (Fig. 5). TNF-α was upregulated in a pattern very similar to that for IL-1β, although to a much lesser extent overall. IFN-γ was upregulated significantly in the spleens, livers, and kidneys of infected fish at most of the time points tested. While IL-1β induction was highest at earlier time points, the IFN-γ gene became the most highly upregulated gene tested at later time points.

There was no significant upregulation of iNOS in any tested tissues (Fig. 5). In fact, our data suggest a trend toward downregulation of iNOS during *Francisella* sp. infection, although only one point (gill at 12 h p.i.) reached statistical significance compared to the results for controls (Fig. 5).

We also tested the correlation between bacterial load and transcriptional regulation of IFN-γ, TNF-α, IL-1β, and iNOS in infected fish. Data from spleen, liver, and kidney were pooled (24 data points total) in order to perform stepwise multiple linear regression analyses. At 12 h p.i., bacterial load correlated with IFN-γ induction ($R^2 = 0.38$, $P < 0.005$). By 24 h p.i., this correlation was no longer significant, and instead

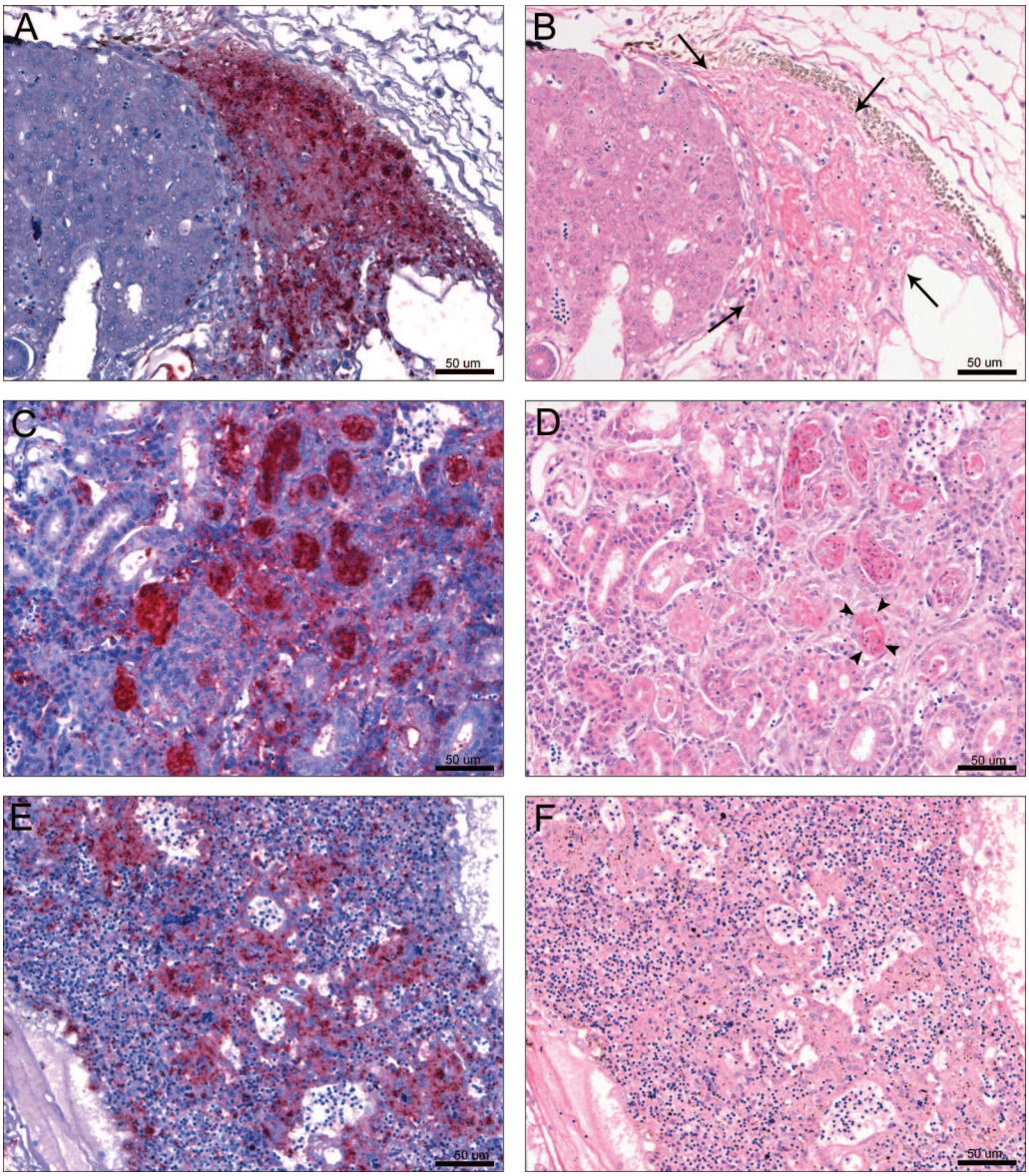

FIG. 4. Histopathology of zebrafish 5 days p.i. Fish were infected as described in the legend for Fig. 2. Serial slides were stained with antiserum against *Francisella* (left panels) or H&E (right panels), as described in Materials and Methods. Areas stained by IHC and focal, acute hepatic necrosis had become more diffuse by 5 days p.i. (A and B). Arrows surround zones of necrosis. In the kidney, bacterial antigen had begun to aggregate into discrete regions of the renal interstitium and proximal tubules (C). This is associated with corresponding multifocal to diffuse, subacute, interstitial, and proximal tubule renal necrosis with early granuloma formation (D). Arrowheads surround one example of an early-stage renal granuloma. Bacterial antigen is present throughout the spleen (E) and is associated with diffuse, subacute splenic necrosis (F).

IL-1β levels correlated with bacterial load ($R^2 = 0.61$, $P < 0.005$). At 48 h p.i., both TNF-α and IL-1β levels correlated with levels of bacteria in the tissues ($R^2 = 0.58$, $P < 0.005$); however, in contrast to the results at 24 h p.i., IL-1β was negatively correlated with bacterial load at this time point. Although the levels of induction of proinflammatory genes correlated with each other at later time points, there was no direct correlation with bacterial load.

Specificity of the inflammatory response to live bacteria. Differences in the cytokine responses to live versus heat-killed bacteria, as measured by gene induction, provide an opportunity to illuminate the interactions of *Francisella* with specific components of innate immunity. Live *Francisella* bacteria are able to escape the phagosome and replicate in macrophages, alerting the cytoplasmic microbial sensor system (69). Heat-killed bacteria are rendered replication incompetent and, in the case of *Francisella*, are unable to escape the phagosome postphagocytosis, while retaining some of the immunostimulatory properties of intact bacteria. To determine the extent to which proinflammatory cytokine gene induction was specific to bacteria able to escape from the phagosome, we injected zebrafish with heat-killed *Francisella* sp. and compared the induction of our panel of genes with that observed for zebrafish challenged with live bacteria. In animals injected with heat-

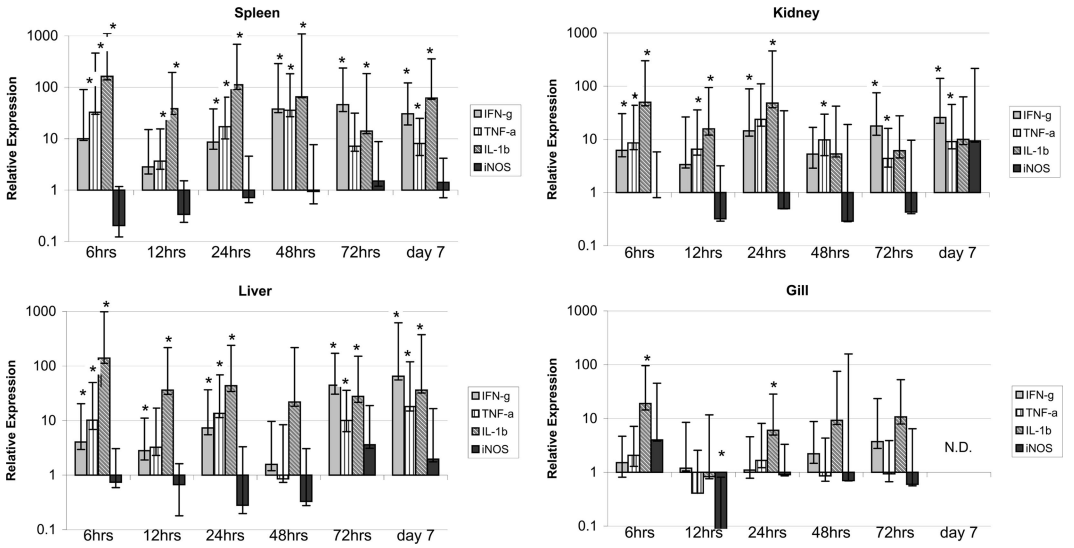

FIG. 5. Tissue-specific transcriptional responses to *Francisella* sp. infection. Eight individual fish were challenged with 1.9×10^5 CFU of *Francisella* sp., and RNA was isolated from tissues at the indicated time points. Fish were infected as described in the legend for Fig. 2. Bars represent the mean relative expression levels (eight individuals) compared to those for control (PBS-injected) animals (±standard errors), as determined by qPCR. Transcript levels are normalized to the expression of ARP. Significance was determined using a nonparametric fixed reallocation randomization test and is indicated with an asterisk ($P < 0.05$).

killed bacteria, only one sample (at 6 h p.i.) had detectable *Francisella* sp. at the threshold level of detection for our assay, corresponding to approximately 40 CFU of bacteria, indicating the specificity of our qPCR assay for live or very recently killed *Francisella* sp. bacteria.

In spleens and kidneys, the induction of IFN-γ occurred to similar extents in response to both heat-killed and live bacteria (Fig. 6). In these samples, although there was a trend toward higher upregulation of TNF-α in response to live bacteria, it was not statistically different from the data with heat-killed bacteria. The finding of nonspecificity of IFN-γ and TNF-α induction for live bacteria in these tissues is similar to the results of studies using mammalian dendritic cells and macrophages (15, 34, 65). In contrast, the transcriptional induction of IL-1β was significantly dependent on the presence of live *Francisella* (Fig. 6). In the spleens of fish challenged with heat-killed bacteria, at 6 h p.i. IL-1β was upregulated only 3.5-fold, compared to 162-fold upregulation in fish challenged with live bacteria.

Despite the fact that there were similar median values of live *Francisella* bacteria in the livers, spleens, and kidneys of fish at 6 and 24 h p.i. (Fig. 4), transcriptional upregulation of IFN-γ, TNF-α, and IL-1β in the liver was remarkably and significantly dependent on the presence of live bacteria (Fig. 6). Killed bacteria may be taken up and circulated through the hematopoietic system, which includes the spleen and kidney, stimulating a cytokine response. These killed bacterial antigens may never reach the liver and are likely cleared in the spleen. Live bacteria, in contrast, may be able to transit actively to liver tissue via infected macrophages or circulation, where they cause proinflammatory cytokine induction and resultant tissue damage.

DISCUSSION

This study establishes a zebrafish model of *Francisella* infection. Despite recent interest prompted by the threat of bioweapon use, there remains no safe or widely effective treatment or vaccine against *Francisella*, underscoring the need for more basic research. Murine and other models of *Francisella* pathogenesis have been well studied for years; however, new advances in our general understanding of *Francisella* virulence are hindered by a lack of knowledge of which factors are specific to individual model systems. Thus, a new model of *Francisella* pathogenesis in an alternative vertebrate host should provide important comparative insight, revealing underlying conserved strategies of both bacterial virulence and host immune responses. In this study, we established that zebrafish are susceptible to a *Francisella*-induced disease that shares many features with tularemia in mammals. In our model, zebrafish induced a potent proinflammatory response to *Francisella* sp. bacteria, demonstrating the high degree of evolutionary conservation of innate immunity against this pathogen.

The complexity of different tissue environments and stages of host response during infection requires the consideration of the sum of the infectious process in order to truly understand pathogenesis. Live-animal models will be essential for addressing the next generation of questions regarding pathogens and their hosts. The advantages and relative cost effectiveness of zebrafish as models of infectious disease have led a few labs to begin studying bacterial infection in zebrafish. The Neely lab has pioneered the use of zebrafish to study streptococcal pathogenesis, highlighting the feasibility of large-scale in vivo screens with zebrafish and describing novel virulence factors of *Streptococcus pyogenes* (44, 55). Zebrafish have also proven to be an informative model for the study of mycobacterial pathogenesis, revealing insights into the roles of innate and adaptive immunity during the establishment of infection and the formation and maintenance of granulomas (18, 33). Some groups have begun to catalog the induction of innate immune effectors

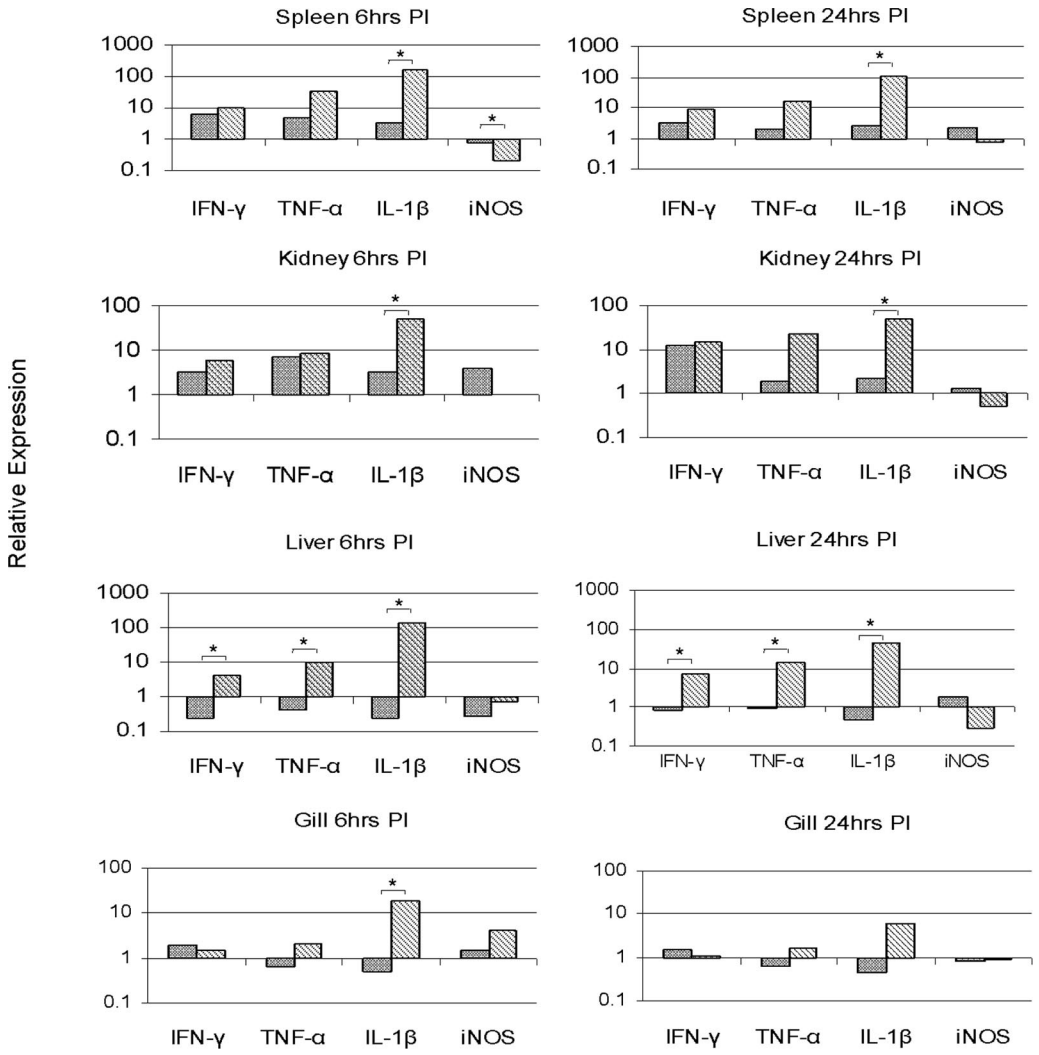

FIG. 6. Comparison of transcriptional responses to heat-killed and live bacteria. Six zebrafish were injected with $1.3 \times 10^5 \pm 0.2 \times 10^5$ CFU of heat-killed bacteria, and RNA was isolated from the listed tissues for evaluation of transcriptional responses at the indicated times. The dark-gray bars on the left are data from fish challenged with heat-killed bacteria, and the cross-hatched bars on the right are data from fish challenged with 1.92×10^5 CFU of live bacteria. Significant differences between the responses to heat-killed and live bacteria were calculated by a Student *t* test and are indicated with an asterisk ($P < 0.05$).

in zebrafish in response to bacterial infection as well (35, 43, 45, 57, 68). While there appear to be some fish-specific mechanisms of innate immunity, the acute-phase response to bacterial pathogens is largely conserved between zebrafish and mammals (35).

There are no described exotoxins produced by *Francisella*, but *Francisella* does possess a unique lipopolysaccharide structure that has very low endotoxicity or TLR4 stimulatory properties. These features are apparently shared with the fish-specific forms of *Francisella*, as they have a lipopolysaccharide structure that is almost identical to that of their mammalian counterparts (1, 11, 21, 28). The disease caused by *Francisella* is attributed largely to the ability of bacteria to proliferate extensively in numerous tissues and to cause damage resulting from subsequent overwhelming inflammatory responses (15, 16, 40). In zebrafish, following i.p. challenge, *Francisella* sp. bacterial antigens were observed in the spleens, kidneys, livers, and gills at all time points tested. As early as 48 h p.i. and throughout all time points examined, the spleens and kidneys of zebrafish contained numerous foci of IHC staining and corresponding cellular necrosis, spread diffusely throughout the entire tissue, while livers and gills of fish were more focally and peripherally stained (Fig. 3 and 4). Thus, the pattern of *Francisella* sp. antigen staining and subsequent necrosis during acute infections in zebrafish (primarily within fish lymphoid tissue and the liver) largely parallels the pattern observed in mammalian tularemia (6, 15). *Francisella* infection in other fish species has been reported to cause numerous granulomas in the spleen and kidney after much longer, chronic infections (47, 48). Although this study focused on an acute-infection model, we observed the beginning stages of granuloma formation in the kidney by 5 days p.i. (Fig. 4D). We did not observe granulomas forming in the spleen at 5 days p.i., as

the tissue was almost completely necrotic at this time point (Fig. 4F). Future research will address the pathological consequences of infection with lower doses and in chronic-infection models, where the spleen tissue may partially recover, thereby allowing granulomas to mature.

Using a qPCR assay specific for *Francisella* sp., we observed the proliferation of bacteria in specific tissues concurrent with the individual host immune response. We observed a delay of approximately 12 h between the initial infection and the ability of *Francisella* bacteria to begin replicating to high numbers in zebrafish tissues. Contrary to one report from studies with mice, we found higher numbers of bacteria in the spleen and kidney than in the liver (15). However, despite the relatively low numbers of bacteria measured in the liver at 6 h p.i., there was a substantial and significant induction of IFN-γ, TNF-α, and IL-1β at this time point, supporting the importance of the liver as a site of induction for the proinflammatory response to *Francisella* infection in vertebrates (6, 15, 27).

A robust and early cytokine response to infection plays an important role in defense against *Francisella* in mammals (12). TNF-α has also been shown to play a central and complex role in the resolution of *Francisella* infection, being induced quickly in response to both LVS and more-virulent type A infections in mice (8, 17, 21, 22). Telepnev et al. found that in mammalian peripheral blood mononuclear cells challenged with attenuated *Francisella* LVS, TNF-α is initially upregulated, followed by downregulation a few hours later (65). This result requires live bacteria capable of escaping from the phagosome, implying that the downregulation is directly mediated by *Francisella*. However, in another study using a much lower multiplicity of infection to infect peripheral blood mononuclear cells, there was no downregulation of TNF-α. Rather, a slight delay in induction was observed (49). The ability to suppress or delay the activation of very specific immune responses may be an important virulence mechanism utilized by *Francisella* (21). We observed lower levels of TNF-α gene induction at 12 h p.i. than at 6 h p.i. However, after 12 h p.i. and concurrently with the rapid increase in bacterial levels, TNF-α was again upregulated to a highly significant degree. We also observed that in the spleens and kidneys of infected fish, the responses to heat-killed bacteria were not significantly different (Fig. 6), although live bacteria induced a trend toward higher TNF-α upregulation. This implies that, in our model, *Francisella* may not actively modulate TNF-α transcription, although we did not test the response to heat-killed bacteria at 12 h p.i. Whether or not the relative decrease in upregulation at 12 h p.i. is due to a virulence mechanism utilized by *Francisella* sp., bacteria nonetheless replicated to high levels despite the robust induction of TNF-α expression in all zebrafish tissues tested.

IFN-γ is produced in response to infection with *Francisella* in all vertebrate model systems (2, 6, 9, 12, 19, 22, 49, 51), and IFN-γ knockout mice and mice treated with anti-IFN-γ antibodies succumb to normally sublethal doses of *Francisella* (22, 32). In this study, although IFN-γ was upregulated at early time points, the most significant upregulation occurred during the later phases of acute infection: 72 h and 7 days p.i. We also observed that, in the spleen and kidney, heat-killed bacteria induced almost the same degree of upregulation of IFN-γ as live bacteria. Given that death following *Francisella* infection in zebrafish occurred primarily between days 3 and 8 (Fig. 1), IFN-γ upregulation may be a response to higher bacterial loads rather than a preemptive response that is able to significantly influence the outcome of infection. Concurrently with this idea, IFN-γ levels do not correlate with either death or survival of infected individuals in a mouse model of respiratory-induced tularemia (12). Further complicating the analysis of the role of IFN-γ during infection, *Francisella* bacteria may suppress IFN-γ-induced STAT1 expression and phosphorylation in phagocytes (50). The effects of this suppression may be broad, but one clear correlation is a reduction in the expression of iNOS in infected cells, resulting in decreased microbicidal activity. This may help to explain our observation of a lack of significant upregulation of iNOS at any time point, even given the high upregulation of both IFN-γ and TNF-α (Fig. 5), which are known to be potent stimulators of iNOS in mammals (5).

The exact role of iNOS in resolving acute *Francisella* infection is unclear. Although iNOS has been shown to be critical for killing intracellular *Francisella* by murine peripheral tissue macrophages (21, 37), alveolar macrophages from mice do not need nitric oxide species to kill intracellular *Francisella* (56). The role of iNOS and nitric oxide production in human macrophage responses to *Francisella* infection remains largely unexplored (21). While iNOS knockout mice have bacterial burdens in the liver similar to those of wild-type mice (6), during chronic infection they have a significantly reduced 50% lethal dose and exhibit severe liver pathology and disregulated cytokine profiles (38). These studies indicate that iNOS may not play a direct role in acute infection. However, beyond 1 week of infection, reactive nitrogen species produced by iNOS have important bactericidal and immunoregulatory roles in resolving *Francisella* infection (36). In our zebrafish model, there was no significant up- or downregulation of iNOS. As iNOS is normally transcribed at very low levels in zebrafish (data not shown), significant transcriptional downregulation is difficult to detect by measuring a decrease in transcript level relative to that for control animals. However, there is a trend toward a slight upregulation of iNOS in zebrafish challenged with heat-killed bacteria (Fig. 6), implying that the decrease in iNOS levels in response to live bacteria may be, in fact, a virulence mechanism utilized by *Francisella.* It is also possible that the time points chosen in this study were too early to observe transcriptional upregulation of iNOS. Future studies involving chronic-infection models should clarify the importance of iNOS in resolving *Francisella* infection in zebrafish.

In mammals, the production of IL-1β mRNA in response to *Francisella* infection is largely mediated by TLR2 engagement (34). However, after transcription, IL-1β undergoes another regulatory processing step before becoming biologically active. The cytokine is synthesized as a precursor (pro-IL-1), which must be processed by activated caspase-1 prior to secretion. Caspase-1 itself must be activated by autoprocessing in the cytoplasm of the cell. This occurs in phagocytes in response to many intracellular bacteria, including *Francisella* (26, 60, 69). In essence, the production of active IL-1β requires at least two signaling events: the transcriptional upregulation and synthesis of pro-IL-1β and the activation of caspase-1 in order to process pro-IL-1β. This may be a protective mechanism, as IL-1 family cytokines are potent initiators of inflammation and are often associated with cellular suicide, an important host defense mechanism (69). Thus, studies of mice have clarified the dif-

ferences between IL-1β transcriptional induction and processing and secretion in response to *Francisella* infection. In mice, the processing of pro-IL-1β and subsequent secretion of active cytokine require live bacteria capable of escaping from the phagosome into the cytosol (25, 34). High IL-1β levels are associated with survival in mice infected with *Francisella* (12). With zebrafish, we observed significant upregulation of IL-1β at all time points tested following live-*Francisella* sp. challenge (Fig. 5). This upregulation was to a level much higher than that observed using heat-killed bacteria, paralleling the response in mice, where there is also only a very modest upregulation of IL-1β in response to heat-killed bacteria (7, 34). Currently it is not known whether fish IL-1 family cytokines are processed by caspases in a manner analogous to that which occurs in mammals, and there are no tools available to measure biologically active IL-1β in zebrafish. Therefore, we hypothesize that the significant transcriptional upregulation of IL-1β that we observed results in active cytokine expression and consequent immune function, again signifying the considerable degree of conservation of a specific immune response to *Francisella* across vertebrate species.

In summary, this study establishes zebrafish as an important new model for addressing mechanisms of *Francisella* pathogenesis and the host immune response to *Francisella* infection in vertebrates. Preliminary sequencing studies reveal that the fish-specific isolates of *Francisella* encode gene products homologous to those associated with virulence in the *F. tularensis* pathogenicity island (M. Calcutt et al., personal observation). Accordingly, it is likely that the key pathways of virulence are conserved between the fish-specific pathogen and *F. tularensis*. We observed numerous similarities between zebrafish and mammals in the disease process and host immune response to *Francisella* infection, highlighting the utility of this model system as a comparative example of *Francisella* pathogenesis. Future studies will include the use of transgenic and knockout strains of zebrafish for assessing the roles of distinct cell populations and specific components of immunity during *Francisella* infection.

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