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Enhancement of Vaccine Efficacy by Expression of a TLR5 Ligand in the Defined Live Attenuated *Francisella tularensis* subsp. *novicida* Strain U112 Δ iglB::fljB

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Abstract

Oral vaccination with the defined live attenuated *Francisella novicida* vaccine strain U112 Δ iglB has been demonstrated to induce protective immunity against pulmonary challenge with the highly human virulent *F. tularensis* strain SCHU S4. However, this vaccination regimen requires a booster dose in mice and exhibits 50% protective efficacy in the Fischer 344 rat model. To enhance the efficacy of this vaccine strain, we engineered U112 Δ iglB to express the *Salmonella typhimurium* FljB flagellin D1 domain, a TLR5 agonist. The U112 Δ iglB::fljB strain was highly attenuated for intracellular macrophage replication, and although the FljB protein was expressed within the cytosol, it exhibited TLR5 activation in a TLR5-expressing HEK cell line. Additionally, infection of splenocytes and lymphocytes with U112 Δ iglB::fljB induced significantly greater TNF- α production than infection with U112 Δ iglB. Oral vaccination with U112 Δ iglB::fljB also induced significantly greater protection than U112 Δ iglB against pulmonary SCHU S4 challenge in rats. The enhanced protection was accompanied by higher IgG2a production and serum-mediated reduction of *Francisella* infectivity. Thus, the U112 Δ iglB::fljB strain may serve as a potential vaccine candidate against pneumonic tularemia.

Keywords

Francisella tularensis; vaccine; TLR5; Fischer 344 rat; flagellin

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1. Introduction

Oral vaccination has been demonstrated as an efficacious route by which to induce immunity against a variety of agents, including *Francisella tularensis* [1-4]. Our laboratory previously has shown partial protection against pulmonary challenge with the highly human virulent SCHU S4 strain in both mice [5] and rats [4] following oral vaccination with the defined live attenuated *F. novicida* mutant U112 Δ iglB. This strain, created using a targeted mutagenesis approach [6], lacks the *Francisella* pathogenicity island (FPI) gene *iglB*, which comprises an important part of a Type VI secretion system in *F. tularensis* [7, 8]. U112 Δ iglB, like all FPI mutants, is highly attenuated both *in vitro* and *in vivo*, with defects in intracellular replication and decreased virulence in animals (pulmonary LD $> 10^7_{50}$ CFU in mice and rats compared to WT strain U112, which has a pulmonary LD < 10 CFU in mice and $\sim 5 \times 10^6_{50}$ CFU in rats). We have shown [5] that a prime-boost oral vaccination strategy with U112 Δ iglB induces 40% protection (against approx. 50 LD₅₀) in C57BL/6 mice, and a single immunizing dose is 50% protective (against approx. 25 LD₅₀) in Fischer 344 rats after pulmonary challenge with the highly human virulent *F. tularensis* SCHU S4 [4]. Protective immune responses were accompanied by antigen-specific IFN- γ production in the spleen, and potent antibody responses in mice (IgG1/IgG2a in sera, IgG1/IgG2a/IgA produced in lungs, and IgA in intestines) and rats (IgG2a in sera, IgG2a/IgA in lungs, and IgA in intestines).

Given that oral U112 Δ iglB vaccination was partially protective against SCHU S4 pulmonary challenge in the rat, we analyzed a means to further enhance the immunogenicity of this vaccine strain in this study. To this end, flagellin has been used successfully as an adjuvant against a variety of pathogens, including bacteria [9], viruses [10], and protozoans [11]. Flagellin promotes Th1-type immunity [12], and importantly, it has been shown that previous exposure to flagellin does not impair the ability of the host to induce robust immune responses when it is used as an adjuvant [13, 14]. Oral vaccination using a flagellin adjuvant has been reported to activate intestinal CD11c⁺ lamina propria dendritic cells (LPDCs), unconventional DCs which express TLR5 but not TLR4 [15]. TLR5 stimulation of LPDCs leads to subsequent production of acute pro-inflammatory cytokines, including TNF- α , IL-6 and IL-1 β , and induces local IgA production.

We sought to increase innate immune stimulation following oral delivery of U112 Δ iglB by addition of a non-functional, truncated version of the *Salmonella typhimurium* gene *fljB* [16]. The engineered U112 Δ iglB::fljB strain expresses the truncated fljB protein in the cytoplasm, activates TLR5, and increases TNF- α production. Oral U112 Δ iglB::fljB vaccination induces augmented protective immunity against heterologous pulmonary *F. tularensis* challenge in both mice and rats when compared with U112 Δ iglB. To this end, an U112 Δ iglB::fljB vaccination platform may be a viable strategy in the development of an efficacious vaccine against pulmonary tularemia.

2. Materials and methods

2.1. Animals

Four to six week old BALB/c mice and six to seven week old Fischer 344 rats were obtained from the National Cancer Institute (Frederick, MD). Animals were housed in ventilated cages at the AAALAC-accredited University of Texas at San Antonio vivarium and received food and water *ad libitum*. All work was done in accordance with the University of Texas at San Antonio Institutional Biosafety Committee (IBC) and Institutional Animal Care and Use Committee (IACUC), who approved these studies.

2.2. Bacteria

Francisella tularensis subspecies *novicida* was obtained from Francis Nano at the University of Victoria, Canada. The defined live attenuated mutant U112 Δ igIB was identical to that which previously has been described [4-6]. *F. tularensis* subspecies *tularensis* strain SCHU S4 was obtained from the Centers for Disease Control and Prevention, Atlanta, GA. All strains were grown at 37°C in tryptic soy broth or agar (TSB or TSA, obtained from BD Biosciences) supplemented with 0.1% (w/v) L-cysteine (Fisher Scientific). Dilution plating on this media was used to determine titers of all stocks.

2.3. Engineering of U112 Δ igIB::fljB strain

The *S. typhimurium* gene *fljB* D1 domain (with exclusion of D2 and D3 domains of the gene) [16, 17], was PCR amplified with the following primers: Flj-Nco: 5'-GCCCATGGCACAAGTAATCAACAC-3' and Flj-Xho: 5'-GCTCGAGTTAACGTAACAGAGACAGC-3' and then was cloned into pKEK1329, a plasmid containing the *F. novicida* GroE promoter, via Nco I and Xho I restriction sites. The pKEK1329-*fljB* was used to transform the U112 Δ igIB bacterium using the reported cryotransformation method [18]. One of the kanamycin resistant transformants, with confirmed the *fljB* gene presence (nucleotide sequencing) and protein expression (Western blot with specific antibody), was designated as U112 Δ igIB::fljB and used for this study.

2.4. Intramacrophage replication

J774 cells were seeded into 96 well plates at a density of 2×10^5 cells/well and infected for 2 hr with 100 MOI of U112, U112 Δ igIB, or U112 Δ igIB::fljB. Plates were washed and treated with medium containing gentamicin for 1 hr, then were washed again and incubated for 24 hr. At 3 and 24 hr post infection, cells were lysed with 0.2% deoxycholate solution and dilution plated onto supplemented TSA to enumerate intracellular bacteria.

2.5. TLR5 reporter assay

TLR5/NF- κ B/SEAPorterTM HEK 293 (IMGENEX), a cell line engineered to express human TLR5, was seeded into 96 well plates (2×10^5 cells/well) and allowed to adhere. Cells were then incubated with 10 MOI of bacteria (*F. novicida*: U112 Δ igIB or U112 Δ igIB::fljB, *S. typhimurium*: ATCC WT strain 14028 or Δ fljA as controls) or each respective bacterial lysate for 24 hr. Supernatants were collected after this incubation and assayed for alkaline phosphatase production as a measure of TLR5 activation.

2.6. Cellular activation assay

Naive F344 rats were sacrificed for collection of spleens and mesenteric lymph nodes. Single cell suspensions were prepared and seeded into 96 well plates (10^6 cells/well) for 2 hr to allow for adherence. Total cells, or adherent cells only, were then infected with 10 MOI of U112 Δ iglB or U112 Δ iglB::fljB. Supernatants were collected at 72 hr post-infection and assayed by ELISA for TNF- α production.

2.7. Vaccination and challenge

Four to six week old BALB/c mice were orally vaccinated with 1000 CFU of U112 iglB or U112 iglB::fljB, or received PBS as a mock vaccination, then were rested for 30 days prior to intranasal challenge with 60,000 CFU LVS as previously described [5]; both vaccination and challenge were performed at the University of Texas at San Antonio BSL-2 animal vivarium. Seven to eight week old F344 rats were orally vaccinated with 10^7 CFU of U112 Δ iglB or U112 Δ iglB::fljB, or received PBS as a mock vaccination, following a previously described protocol [5]. Animals were rested for 30 days prior to intratracheal challenge with SCHU S4 (1×10^4 CFU, ~ 20 LD₅₀) in the University of Texas at San Antonio certified ABSL-3 as previously described [5, 19]. Animals were monitored daily post-challenge for morbidity and mortality.

2.8. Effects of antibody on *F. novicida* replication in naive BMDMs

F344 rats (n=6-8 per group) were orally vaccinated with 10^7 CFU of U112 Δ iglB or U112 Δ iglB::fljB, or were mock vaccinated with PBS. At day 21, animals were anesthetized with isoflurane and bled by cardiac puncture. Sera was prepared and stored at -20°C until use. Concurrently, naive F344 rats were sacrificed to collect femurs and prepare BMDMs using a previously described protocol [19]. BMDMs were seeded into 96-well plates (10^6 cells/well). Sera from individual animals were split in half and used either heat-inactivated (56°C for 30 min) or left untreated, then diluted 1:10 in DMEM + 10% FBS and mixed with 100 MOI of U112. Sera/bacterial mixtures were incubated at 37°C in a shaking incubator for 1 hr, then were added to BMDMs (50 μL /well) for assay of intracellular replication as described above (2.4).

2.9. Statistical analysis

The Kaplan-Meier test was used for analysis of survival experiments and 2 way ANOVA with Bonferroni post test for determination of differences in intramacrophage replication, as well as antibody titers and TNF- α - and ALP- production. Data are represented as mean $\pm$ standard deviation for each group.

3. Results

3.1. Generation of the U112 Δ iglB::fljB bacterial strain expressing a TLR5 activation ligand

To evaluate the potential enhancement of oral vaccine efficacy by TLR5 activation, we transformed U112 Δ iglB [4, 5], a previously characterized tularemia vaccine candidate, with a plasmid containing *S. typhimurium* gene *fljB*. Constitutive expression of the FljB D1 domain in the generated U112 Δ iglB::fljB was under the control of the *Francisella* heat-

shock protein groE gene promoter. To confirm the expression of FljB in U112 Δ iglB::fljB, we used anti-FljB antibody to detect the protein in the bacterial cytosolic and membrane fractions prepared according to our previous publication [20]. As shown in Fig. 1A, FljB was expressed in the U112 Δ iglB::fljB but undetectable in the parental U112 Δ iglB strain by Western blot analysis. Moreover, the expressed fljB protein was mainly localized to the cytosol of the U112 Δ iglB::fljB strain (Fig. 1A), in contrast to surface presence as in *Salmonella*, partially due to lack of flagellar assembly machinery in *Francisella* which is non-motile [21]. To confirm that expression of FljB in U112 Δ iglB::fljB can effectively activate TLR5 signaling, we used a TLR5 reporter cell line, TLR5/NF- κ B/SEAPorterTM HEK cells. WT *Salmonella typhimurium*, which naturally expresses flagellin, induced potent activation of the TLR5-NF- κ B pathway as demonstrated by the reporter alkaline phosphatase activity ($p < 0.001$ compared to all other groups in Fig. 1B). In contrast, the *S. typhimurium* mutant Δ fljA, used here as a negative control, was unable to activate TLR5, and thus induced negligible alkaline phosphatase production. In the same assay, TLR5 reporter cells infected with U112 Δ iglB::fljB bacteria or incubated with the lysate of the bacterium containing expressed FljB exhibited a significantly greater alkaline phosphatase activity than U112 Δ iglB ($p < 0.001$ for both cell and lysate), indicating increased levels of TLR5 activation. These analyses reveal that U112 Δ iglB::fljB expresses a functional TLR5 ligand.

To evaluate U112 Δ iglB::fljB as a potential vaccine candidate, we first examined the virulence of this bacterium using a widely accepted intracellular replication assay [22, 23]. We found that, similar to U112 Δ iglB, U112 Δ iglB::fljB was taken into murine J774 macrophages in equivalent numbers, but replicated minimally (Fig. 2A). This was in contrast to WT *F. novicida* U112, which replicated robustly in J774 macrophages over the 24 hr time course ($p < 0.05$), suggesting that U112 Δ iglB::fljB may be as highly attenuated as its parental U112 Δ iglB mutant strain.

Given that U112 Δ iglB::fljB was deficient in intramacrophage replication, but oral administration of this vaccine strain induced TLR5 activation *in vitro* (Fig. 1B), we next assessed whether U112 Δ iglB::fljB induced the production of TNF- α , a surrogate marker for cellular activation [24, 25], more efficiently than U112 Δ iglB in an *ex vivo* assay using Fischer 344 rat tissues. Mesenteric lymph nodes and spleens were processed into single cell suspensions and exposed to live U112 Δ iglB or U112 Δ iglB::fljB for 72 hr. Then, supernatants were collected and assayed by ELISA for rat TNF- α production. As shown in Fig. 2B, exposure to both U112 Δ iglB::fljB and U112 Δ iglB induced significantly greater levels of TNF- α production than mock (PBS) exposure in both total and adherent splenocytes ($p < 0.001$). However, U112 Δ iglB::fljB-treated cells produced significantly greater TNF- α for total splenocytes (1167 ± 250 pg/mL) than U112 Δ iglB (773 ± 117 pg/mL, $p < 0.05$). Adherent splenocytes behaved similarly to total splenocytes, with U112 Δ iglB::fljB producing 752 ± 154 pg/mL, compared to U112 Δ iglB (656 ± 251 pg/mL) and mock treated-cells (77 ± 38 pg/mL). Mesenteric lymph node cells followed the same pattern with respect to TNF- α production. Total MLN cells treated with U112 Δ iglB::fljB produced 442 ± 109 pg/mL, U112 Δ iglB induced 363 ± 139 pg/mL and mock treatment induced minimal levels (64 ± 91 pg/mL) of TNF- α . For adherent MLN cells, only

U112 Δ igLB::fljB induced a significantly greater amount of TNF- α (459 ± 188 pg/mL) than to mock treated cells (89 ± 75 pg/mL), with U112 Δ igLB-infected adherent MLN cells producing 263 ± 63 pg/mL of TNF- α . Overall, these analyses revealed that U112 Δ igLB::fljB more effectively induces TNF- α production *ex vivo* than the parental U112 Δ igLB strain.

3.2. Protective efficacy of the vaccine strain against pneumonic tularemia was enhanced by expressing FljB

We previously have demonstrated a single oral dose of the parental vaccine strain U112 Δ igLB was 80% protective against intranasal LVS challenge in mice [3] and 50% protective against SCHU S4 challenge in F344 rats [4]. Since the parental U112 Δ igLB strain is normally quite efficacious against LVS challenge in mice, we increased the challenge dose of LVS from 60,000 CFU (approx. 10 LD₅₀) to 85,000 CFU (approx. 15 LD₅₀). At this challenge dose, oral vaccination with U112 Δ igLB::fljB induced significant levels of protection (83% survival; $p < 0.05$) compared to mock vaccination with PBS, but did not induce statistically different survival compared to immunization with the parental strain U112 Δ igLB (50%; Suppl. Fig. 1). We thus analyzed the vaccine efficacy of U112 Δ igLB::fljB in Fischer 344 rats, a secondary animal model which may more accurately reflect human susceptibility to tularemia [4, 19]. As shown in Fig. 3A, U112 Δ igLB::fljB was 87.5% effective against subsequent SCHU S4 intratracheal (i.t.) challenge (1×10^4 CFU, ~20 LD₅₀), as compared to U112 Δ igLB which was 25% effective ($p < 0.05$). The differences in mortality also were reflected in the morbidity of animals, as U112 Δ igLB::fljB vaccinated animals did not lose as much weight as U112 Δ igLB or mock vaccinated groups (Fig. 3B), and overall exhibited minimal signs of morbidity (hunched posture, ruffled fur, inactivity, porphyrin production around eyes) after bacterial challenge.

3.3. U112 Δ igLB::fljB immune sera inhibited *Francisella* infectivity significantly greater than U112 Δ igLB immune sera

Orally vaccinated rats were bled via the tail vein on day 28 post-vaccination for sera, which were analyzed by ELISA to measure humoral immune responses. As shown in Figure 4, vaccinated rats induced significantly greater production of serum antibody to the respective vaccine strains than mock (PBS) vaccinated animals. Further isotype analysis revealed predominant IgG2a production (an indicator of Th1 immunity) following vaccination, with a significantly ($p < 0.05$) higher titer of 2494 ± 1005 for U112 Δ igLB::fljB-vaccinated rats compared to 832 ± 530 for U112 Δ igLB-vaccinated animals. There was negligible amounts of IgG1 isotypic antibody produced in all groups. We also assayed fecal supernatants for IgA production, but no significant differences were observed between U112 Δ igLB::fljB and U112 Δ igLB vaccinated animals (data not shown). To further examine the functional contribution of antibody to vaccine efficacy, an intramacrophage replication assay was performed in which rat BMDMs were infected with U112 that was pre-incubated for 1 hr with sera (heat-treated and untreated, 1:10 diluted in DMEM + 10% FBS) from rats previously orally vaccinated with PBS (i.e., normal rat serum, NRS), U112 Δ igLB or U112 Δ igLB::fljB. Uptake of U112 and intracellular replication were examined over a 24 hr time course (Fig. 5). At 3 hr after U112 inoculation, bacterial uptake in both the NRS and U112 Δ igLB::fljB serum pre-treated conditions were significantly decreased in heat-inactivated sera compared to non-inactivated sera ($p < 0.05$). Regardless of serum pre-

treatment, U112 Δ igLB::fljB conditions showed significantly lower bacterial loads at 24 hr than pre-treatment with NRS and serum from U112 Δ igLB vaccinated groups ($p < 0.01$ for untreated sera, $p < 0.001$ for heat-inactivated sera). Based on these results with U112, similar assays were performed with SCHU S4 (data not shown). No significant differences were observed in bacterial uptake or intramacrophage replication when SCHU S4 was pre-incubated with sera from animals orally vaccinated with PBS (NRS), U112 Δ igLB, or U112 Δ igLB::fljB; however, as shown previously [19], SCHU S4 does not replicate as efficiently as U112 within rat BMDMs.

In summary, antibody generated by oral U112 Δ igLB::fljB vaccination was able to significantly reduce intracellular replication of U112 bacteria within rat BMDMs over a 24 hr period. These results suggest that U112 Δ igLB::fljB vaccination may augment production of antibody which more effectively controls *F. novicida* U112 uptake and replication compared to that of the parental strain U112 Δ igLB.

3.4. U112 Δ igLB::fljB immune sera contributed to the vaccine protection against pneumonic tularemia

A previous report by Mara-Koosham *et al.* [26] demonstrated that passive immunization with LVS-immune serum protected rats against SCHU S4 i.t. challenge. However, the LD₅₀ of SCHU S4 in F344 rats receiving LVS-immune serum was 100-fold less than the LD₅₀ associated with LVS live cell vaccination [26]. To assess whether U112 Δ igLB- and U112 Δ igLB::fljB- immune sera also contribute to vaccination mediated protection, naïve F344 rats were intraperitoneally injected with 250 μ l of heat-inactivated sera from PBS, U112 Δ igLB or U112 Δ igLB::fljB orally vaccinated rats, at 24 hr prior to challenge as done by Mara-Koosham *et al.* [26]. The serum treated rats were then challenged i.t. with a small (1×10^3 , ~ 2 LD₅₀) but lethal dose of SCHU S4 with an intention to reduce the threshold required for detecting any immune serum-mediated protective effect similar to that used in the previous study [26]. As shown in Fig. 6A, both groups of rats that received immune sera had higher survival rates than the non-immune (PBS immunized) serum treated group, with the U112 Δ igLB::fljB immune serum treatment showing a significant difference ($p < 0.05$) compared to non-immune serum treatment. Additionally, the animals passively transferred with U112 Δ igLB::fljB immune serum had slightly higher, but significantly different ($p < 0.05$), total IgG and IgG2a titers to SCHU S4 compared to animals passively transferred with U112 Δ igLB immune serum (Fig. 6B). Collectively, these results suggested that U112 Δ igLB::fljB immune serum may contribute to the U112 Δ igLB::fljB vaccination-induced protective immunity against pulmonary Type A *Francisella* challenge.

4. Discussion

Our laboratory has demonstrated protection with oral U112 Δ igLB vaccination against a subsequent pulmonary challenge with the highly human virulent strain SCHU S4 in both mice and rats [4, 5]. As this putative vaccine strain was demonstrated to be partially protective, we sought in this study to enhance the innate immune stimulation of U112 Δ igLB via the expression of a truncated version of the *S. typhimurium* fljB flagellin protein, a TLR5

agonist, and which also would generate potent Th1-type-mediated protective immunity against *F. tularensis* [27, 28].

We have demonstrated here that U112 Δ igLB::fljB may serve as a more effective vaccine platform than the parental mutant strain, U112 Δ igLB, as both mice and rats vaccinated with U112 Δ igLB::fljB exhibited increased survival ($p < 0.05$) and reduced morbidity following pulmonary *F. tularensis* challenge when compared to equivalent-dose vaccination with U112 Δ igLB. The enhanced protective response generated by U112 Δ igLB::fljB may be due to the augmented antibody responses produced by this vaccine when administered orally, as greater levels of Th1-driven IgG2a isotype were seen in sera compared to animals receiving an identical oral dose of U112 Δ igLB. Antibody generated by oral U112 Δ igLB::fljB immunization better facilitated the control of intracellular *F. tularensis* bacterial replication *in vitro* than antibody obtained after vaccination with the parental U112 Δ igLB strain. Additionally, passive immunization with U112 Δ igLB::fljB-immune serum increased survival rates against lethal SCHU S4 challenge. Although immune serum may contribute to vaccine induced protection, cell-mediated-immunity has been shown to play the major role in control *Francisella* infection. Antigen-specific IFN- γ production by rat splenocytes restimulated with the vaccine strain were robust (data not shown) and similar to our previous findings following oral vaccination with U112 Δ igLB [4]. However, reduction of *Francisella* replication at infection sites by T cell IFN- γ mediated phagocytic cell activation and bacterial killing remains to be examined in order to further evaluate the differences in U112 Δ igLB and U112 Δ igLB::fljB mediated protection. In addition, we have not assessed the copy numbers of the *fljB* containing plasmid in U112 Δ igLB::fljB and the stability of this plasmid following oral delivery. Whether an U112 Δ igLB with a stable chromosomal expression of fljB protein can further enhance the vaccine efficacy remains to be investigated.

Antibodies have been demonstrated to play an increasingly important role in protection against *F. tularensis* [29-31] and other facultative intracellular pathogens such as *Listeria monocytogenes*, *Salmonella* and *Ehrlichia* [32]. Passive transfer of *F. tularensis* specific antibody can provide protection against lethal pulmonary challenge in naive mice [28, 33, 34] or rats [26], as well as irradiated mice [35]. Interestingly, the protective antibody responses following vaccination with *F. novicida* or *novicida*-derived live attenuated mutants skew predominantly toward an IgG2a isotype in rats [4, 19] or a mixed IgG1/IgG2a isotype in mice [5, 28], demonstrating the importance of a Th1-driven immune response against this infection. Our results with oral U112 Δ igLB::fljB vaccination follow a similar pattern, with increased levels of IgG2a in immunized F344 rats (Fig. 4), which may more effectively control *F. tularensis* replication after pulmonary challenge (Fig. 3).

Enhanced antibody production and protection afforded by the U112 Δ igLB::fljB strain in our studies may be linked to the addition of the FljB protein. Flagellin has been successfully used as an adjuvant against a variety of pathogens, including bacteria [9, 14, 36], viruses [10], and protozoans [11]. While effective as an adjuvant in low doses, flagellin also has been shown to lead to an increase in T-cell dependent [14, 37] antibody production [14, 16, 38-40], specifically producing high titers of IgG1 and IgG2a that provide complete protection against a targeted pathogen. Our results corroborate these reports, as addition of

FljB to U112 Δ iglB increased Th1 antibody isotype production and afforded increased levels of protection in F344 rats against subsequent challenge with the highly human virulent SCHU S4.

Additionally, complement also has been shown to facilitate opsonization of *F. tularensis* leading to enhanced phagocytosis by macrophages [41] and dendritic cells [42]. Specifically, C3 is deposited on the surface of *F. tularensis*, in a C1q-dependent manner [43]; both C1 and C3 are essential for complement-mediated phagocytosis of *F. tularensis* via complement receptor CR3 [42, 44, 45]. Our results correlate with these studies, as incubation of U112 with heat-treated rat sera (lacking complement proteins) significantly decreased the initial bacterial uptake at 3 hr compared to untreated sera containing complement factors (Fig. 5). However, the contribution of antigen-specific immunoglobulin (total and isotypes) in U112 Δ iglB::fljB and U112 Δ iglB immune sera to difference in inhibition of *Francisella* intramacrophage replication and disease prevention requires further study.

Although the expressed FljB is primarily localized to the cytosol of U112 Δ iglB::fljB, we have observed the activation of surface TLR5 signaling by this vaccine strain. This activation may be a result of FljB released from lysed bacteria in apoptotic phagocytic cells which consequently becomes accessible to the surface receptor. However, it has been shown that cytosolic flagellin can activate an intracellular pattern recognition protein, NLRC4 [46] (also known as IPAF), which belongs to a family of nucleotide-binding oligomerization domain receptors and contains a CARD (caspase recruitment domain) involved in intracellular signaling pathways of inflammation and apoptosis [47]. A study by Carvalho *et al.* [48] demonstrated the critical role of NLRC4 in protection against flagellated, but not non-flagellated, *Salmonella* challenge. The contribution of surface TLR5 and intracellular NLRC4 signaling to U112 Δ iglB::fljB-mediated protection against tularemia requires further investigation by using TLR5 and NLRC4 genetic knock-out mice.

In summary, we have demonstrated that U112 Δ iglB::fljB can serve as an effective vaccination platform against tularemia. Our studies further support the use of flagellin as an adjuvant, and provide increasing evidence that Th1-driven adjuvants may assist in providing enhanced protective against pulmonary tularemia.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

We generated a tularemia vaccine strain, U112 Δ iglB::fljB, expressing a TLR5 ligand.

Expression of flagellin (FljB) D1 domain in U112 Δ iglB enhances protection.

U112 Δ iglB::fljB immune sera mediated a greater reduction of *Francisella* infectivity.

Fig. 1.

Expression of FljB in the U112ΔiglB::fljB strain activates TLR5 signaling. (A) Total proteins of U112ΔiglB and U112ΔiglB::fljB, as well as cellular subfractions of U112ΔiglB::fljB, were separated in a 10% SDS-PAGE gel and stained with comassie blue. All protein samples also were subjected to Western blot analysis using anti-FljB serum. IM, inner membrane; OM, outer membrane. Predicted size of FljB is 27.9 kDa. (B) TLR5-transfected HEK cells were seeded in 96 well plates (2×10⁵ cells/well) and stimulated with 10 MOI of bacteria (*F. novicida*: U112ΔiglB or U112ΔiglB::fljB, *S. typhimurium*: ATCC WT strain 14028 or Δ*fljA* as controls) or each respective bacterial lysate for 24 hr. Supernatants were collected after this incubation and assayed for alkaline phosphatase (ALP) activity as a reporter of TLR5 activation. Difference in ALP activity is significant between U112ΔiglB and U112ΔiglB::fljB stimulation by both live bacteria and lysates (** $p < 0.01$). Representative of two independent experiments.

Fig. 2.

U112ΔiglB::fljB is attenuated for replication, but can activate splenocytes and lymph node cells. (A) J774 macrophages were seeded into 96-well plates (2×10^5 CFU/well) and were infected with 100 MOI of U112, U112ΔiglB, or U112ΔiglB::fljB. At 3 and 24 hr post-inoculation, viable bacteria within J774 cells were enumerated by dilution plating. *** $p < 0.001$. (B) Spleens and mesenteric lymph nodes were obtained from naive F344 rats, single cell suspensions were prepared and seeded into 96 well plates (10^6 cells/well) for 2 hr to allow for adherence. Total cells or adherent cells were mock treated with PBS or infected with 10 MOI of U112ΔiglB or U112ΔiglB::fljB (suspended in PBS), and supernatants collected 72 hr post-inoculation for measurement of TNF-α production by ELISA. Significant differences between bacterial and mock stimulation * $p < 0.05$, *** $p < 0.001$, and between U112ΔiglB and U112ΔiglB::fljB, # $p < 0.05$ are indicated. Both panels are representative of two independent experiments each.

Fig. 3.

Oral vaccination with U112 Δ iglB::fljB provides enhanced protection against pulmonary challenge with *F. tularensis* SCHU S4. Fischer 344 rats (n=6-8 per group) were orally vaccinated with 10^7 CFU of U112 Δ iglB or U112 Δ iglB::fljB, or received a mock vaccination of PBS. Animals were rested for 30 days prior to intratracheal challenge with 1×10^4 CFU SCHU S4 (~20 LD₅₀). All animals were monitored daily for 30 days for survival (A) and weight change (B). The difference in survival between U112 Δ iglB and U112 Δ iglB::fljB vaccinated groups was significant ($p < 0.05$). Representative of three independent experiments.

Fig. 4.

Humoral responses following oral vaccination with U112▲iglB and U112▲iglB::fljB. Fischer 344 rats (n=6 per group) were orally vaccinated with 10^7 CFU of U112▲iglB or U112▲iglB::fljB, or PBS as a mock vaccination, then were rested for 28 days. Sera antibody (total antibody, IgG1 and IgG2a) titers to UV-U112▲iglB (for PBS and U112▲iglB groups) and UV-U112▲iglB::fljB (for U112▲iglB::fljB group) were determined. Total Ig and IgG2a antibody titers were significantly elevated in both bacterial vaccinated rats compared to mock (PBS) vaccinated animals († $p < 0.001$). IgG2a titers between the two vaccinated groups were significantly different (* $p < 0.05$). Representative of three independent experiments.

Fig. 5.

U112 Δ iglB::fljB immune serum can control *F. novicida* U112 replication. Immune sera obtained from rats (n=6 per group) 28 days post vaccination with U112 Δ iglB, U112 Δ iglB::fljB or normal rat serum (NRS) from PBS immunized animals were heat-inactivated (HI) at 56°C for 30 min to inactivate complement or left untreated and incubated with U112 for 1 hr in a shaking incubator, then sera/bacterial mixtures were added to rat BMDMs for 2 hr. Afterwards, the BMDMs were washed, treated for 1 hr with gentamicin-containing media, then washed again and cultured in non-antibiotic containing medium. Cells were lysed with 0.2% deoxycholate solution at 3 and 24 hr post inoculation and dilution plated to enumerate intracellular U112. Bacterial numbers for each group are presented as box-whisker plots and are representative of two independent experiments. ** $p < 0.01$, *** $p < 0.001$. # $p < 0.05$, between respective untreated and heat-inactivated serum groups.

Fig. 6.

Passive transfer with U112ΔiglB::fljB immune serum protects against pulmonary SCHU S4 challenge. (A) Naïve F344 rats (n=6 per group) were i.p. injected with 250 μl heat-inactivated serum from PBS, U112ΔiglB, or U112ΔiglB::fljB immunized rats 24 hr prior to i.t. challenge with 1×10^3 CFU of SCHU S4. All animals were monitored daily for survival for 30 days. The difference in survival between U112ΔiglB::fljB and PBS groups was significant (* $p < 0.05$). (B) Antibody (total antibody and IgG2a) titers against SCHU S4 in sera used for adoptive transfer were determined. Total Ig and IgG2a antibody titers were significantly elevated in both immune sera (U112ΔiglB and U112ΔiglB::fljB) compared to non-immune serum († $p < 0.001$). Also total Ig and IgG2a titers between the two immune sera were significantly different (* $p < 0.05$). Representative of two independent experiments.