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Vaccination with a live attenuated *Acinetobacter baumannii* deficient in thioredoxin provides protection against systemic *Acinetobacter* infection

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Abstract

Multi-drug resistant *Acinetobacter baumannii* (MDR-Ab), an opportunistic pathogen associated with nosocomial and combat related infections, has a high mortality due to its virulence and limited treatment options. Deletion of the thioredoxin gene (*trxA*) from a clinical isolate of MDR-Ab resulted in a 100-fold increase in 50% lethal dose (LD₅₀) in a systemic challenge murine model. Thus, we investigated the potential use of this attenuated strain as a live vaccine against MDR-Ab. Mice were vaccinated by subcutaneous (s.c.) injection of 2×10^5 CFU of the *trxA* mutant, boosted 14 days later with an equivalent inoculum, and then challenged 30 days post-vaccination by i.p. injection with 10 LD₅₀ of the wild type (WT) Ci79 strain. Efficacy of vaccination was evaluated by monitoring MDR-Ab specific antibody titers and cytokine production, observing pathology and organ burdens after WT challenge, and measuring levels of serum pentraxin-3, a molecular correlate of *A. baumannii* infection severity, before and after challenge. Mice vaccinated with the *trxA* mutant were fully protected against the lethal challenge of WT. However, little immunoglobulin class switching was observed with IgM predominating. Spleens harvested from vaccinated mice exhibited negligible levels of IL-4, IFN- γ and IL-17 production when stimulated with UV-inactivated WT Ci79. Importantly, tissues obtained from vaccinated mice displayed reduced pathology and organ burden compared to challenged non-vaccinated mice. Additionally, serum pentraxin-3 concentrations were not increased 24 hrs after challenge in vaccinated mice, correlating with reduction of WT MDR-Ab infection in *trxA* immunized mice. Furthermore, passive immunization with *trxA*-immune sera provided

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protection against lethal systemic Ci79 challenge. Collectively, the defined live attenuated *trxA* strain is a vaccine candidate against emerging MDR *Acinetobacter* infection.

Keywords

Acinetobacter baumannii; thioredoxin; pentraxin-3; vaccine

1. Introduction

Acinetobacter baumannii is a nonmotile, nonfermenting, Gram-negative bacterium that is pleomorphic, generally taking a rod shape in favorable conditions and a coccus shape in poor conditions, which could explain its ability to persist in a variety of environments [1]. Its ability to avoid desiccation and the increase in multi-drug resistance make this organism a very successful nosocomial pathogen [2]. Patients that are critically ill, especially those within intensive care units, are at the highest risk for *Acinetobacter* infection, which may include pneumonia, meningitis, septicemia, urinary tract and wound infections [3–5]. Nosocomial infections with *A. baumannii* increase hospitalization costs and have high mortality and morbidity rates [6], with reported rates as high as 52% and 10 – 35%, respectively [7].

A. baumannii also has emerged as an important MDR pathogen affecting the general population and military personnel, particularly in combat related wound infections in the Middle East [8]. A 2007 study of patients in Iraq showed that 36%, all of whom had trauma related wound injuries, were infected with *A. baumannii*, followed by *Escherichia coli* and *Pseudomonas aeruginosa*, both at 14% [8]. From 2003 to 2005, the incidences of MDR strains increased significantly in *A. baumannii* isolates obtained from injured soldiers, and is increasing each year due to the continued use of antibiotics to treat these injuries [9]. Drug resistance in *A. baumannii* occurs because it exhibits innate drug resistance mechanisms and a flexible genome that allows for the easy acquisition of drug resistance genes from their environment [10]. Treatment options are becoming progressively more limited. The carbapenems and colistin remain the most important antibiotics available to treat MDR *A. baumannii*, although there is now evidence of emerging carbapenem- and colistin (and other polymyxin-type) resistant strains [11, 12].

The rise of MDR in *A. baumannii* makes it an increasing threat to military personnel who sustain injuries in the battlefield because of limited treatment options, and an ever increasing risk to the civilian population as well to the extent that the World Health Organization has recently listed it as the number one “Priority 1: Critical” pathogen for R&D of new antibiotics for treatment [13]. As such, development of preventive intervention by vaccination has become an important strategy to combat *Acinetobacter* infection. Indeed, several experimental vaccine candidates [14–18] have been evaluated in small vertebrate models with good protective efficacy suggesting vaccination against *Acinetobacter* may be achievable. These candidate vaccines include subunit antigens, such as OmpA (an outer membrane protein) and Ata (a membrane transporter), as well as inactivated whole cells (IWC), outer membrane complexes, and outer membrane vesicles.

An effective and licensable vaccine may require multivalent antigens with potent adjuvants or attenuated strains with multiple mutations (for reducing the chance of reversion to WT virulence). We have shown that one virulence factor of *A. baumannii* is the bacterial thioredoxin A gene (*TrxA*) product (Ketter *et al.*, in submission). Thioredoxins are small proteins containing a highly conserved active site with a redox-active disulfate [19]. Due to their low redoxpotential, thioredoxins are efficient thiol-disulfide reductants [20] and play an important role in redox regulation and oxidative stress defense [21]. Utilizing a tandem affinity purification (TAP) tagged *E. coli* TrxA, Kumar *et al.* [22] identified 80 TrxA associated proteins involving in distinct cellular processes that included transcription regulation, cell division, energy transduction, and several biosynthetic pathways. We observed that *A. baumannii* can evade host mucosal immune defenses by TrxA-mediated dissociation of secretory component from mucosal sIgA. We also generated a TrxA-null mutant (*trxA*) from the parental *A. baumannii* clinical isolate 79 (Ci79) by homologous recombination and demonstrated that TrxA is a key virulence factor involved in bacterial colonization of the gastrointestinal (GI) tract (Ketter *et al.*, in submission). Here, we report that TrxA can be a potential target for gene deletion in the vaccine development against systemic *Acinetobacter* infection.

2. Material and methods

2.1. Animals

All animal experiments were performed using four to six-week-old C57BL/6 mice purchased from Charles Rivers Laboratories (Frederick, MD). Animals were housed at the University of Texas in San Antonio AAALAC accredited animal facility and all experiments were performed in accordance with the guidelines set forth by the Institutional Animal Care and Use Committee (IACUC).

2.2. Bacteria

Acinetobacter baumannii clinical isolate 79 (Ci79) was obtained by the San Antonio Military Medical Center (SAMMC; Fort Sam Houston, San Antonio, TX) from injured military personnel and provided by Dr. James Jorgensen (University of Texas Health Science Center at San Antonio, San Antonio, TX). The *A. baumannii* Ci79 *trxA* strain was generated from the WT Ci79 strain (Ketter, et al, in submission). For all experiments, unless otherwise stated, bacteria were grown from frozen stocks on Luria-Bertani (LB) agar plates and incubated at 37° C for 24 hrs before being subcultured into LB broth. Overnight cultures were diluted 1:100 the following day and grown to an optical density for the specific concentration needed. Growth curves for strains Ci79 and *trxA* were previously generated for determination of accurate bacterial CFU/mL concentrations based on the optical density of log phase cultures measured at 600nm. Actual inoculums were determined by plating serial dilutions on LB agar plates.

2.3. Intraperitoneal and subcutaneous vaccinations

Immediately prior to vaccination, mice were anesthetized by isoflurane inhalation and 100µl volumes of bacteria (2×10^5 CFU/mouse, unless otherwise stated) in phosphate buffered saline (PBS) were administered either by intraperitoneal (i.p.) or subcutaneous (s.c.)

injection, followed with a booster 14 days later with an equivalent inoculum. Two weeks after the boost, mice were anesthetized and given a lethal dose of the WT Ci79 strain *via* i.p. injection. Mice were checked for morbidity and mortality for 21 days following the WT challenge.

2.4. Antibody titer

Serum antibody titers were determined using blood collected from vaccinated mice either 14 or 28 days post vaccination. Antibody ELISAs were performed on plates coated with UV-inactivated *A. baumannii* Ci79 or *trxA* using the protocol described previously [23]. Endpoint titers were calculated based on the method described by Frey et al, [24] using mock (PBS) vaccinated serum as a control to calculate cutoff values.

2.6. Splenocyte stimulation and cytokine ELISAs

Spleens from 3 *trxA* or PBS (mock) vaccinated mice, 2 weeks post boost, were pooled by group and processed as described previously [25]. Briefly, splenic single cells were seeded into 96 well plates at a concentration of 10^6 cells per well (in 200 μ l complete DMEM plus 10% FBS), and stimulated with either 10^6 CFU of UV-inactivated WT Ci79, 1 μ g/ml of concanavalin A (ConA), 1 μ g/ml bovine serum albumin (BSA), or media for 72 hrs. Plates were centrifuged to pellet cells, and the supernatants were collected to assess concentrations of the following cytokines using cytokine ELISA kits: IL-4 (BD OptEIA Mouse IL-4 ELISA set, BD Biosciences, San Diego, CA), IFN- γ (BD OptEIA Mouse IFN- γ ELISA set, BD Biosciences), and IL-17 (Affymetrix, Mouse IL-17 ELISA Ready-SET-Go!®, eBioscience, Inc, San Diego, CA).

2.7. Organ collection for tissue histology

Spleens and livers were collected from 3 PBS mock vaccinated and 3 *trxA* vaccinated mice at 24 hrs post challenge with WT Ci79 strain (5×10^6 CFU/mouse), as well as 3 naïve uninfected mice, in 10mL of 10% formalin, embedded in paraffin at the University of Texas Health Science Center San Antonio, and subsequently processed at the US Army Institute of Surgical Research. Liver and splenic sections were examined microscopically and scored by a veterinary pathologist (R.G.). Livers were scored as described by Dalle Luca, *et al*, [26], and based on the severity and extent of vascular congestion, hepatocellular necrosis, hepatocellular degeneration, polymorphonuclear inflammation, mononuclear inflammation, and hemorrhage. Spleen scores were based on the severity and extent of disruption, cell death and lymphocytolysis in the white pulp and congestion and hemorrhage in the red pulp. The pathologic severity and extent were either graded as none/minimal (0), minimal (1), mild (2), moderate (3), or severe (4).

2.8. Organ collection for bacterial burden

Spleens, livers and kidneys were collected 24 hrs post *A. baumannii* Ci79 challenge in 10mL sterile PBS, homogenized and dilution plated on LB agar containing chloramphenicol to enumerate bacterial burdens.

2.9. Serum pentraxin 3 concentrations

Sera were collected from *trxA* vaccinated and naïve mice 1 hr prior to i.p. challenge with 5×10^6 CFU/mouse of the WT and at 24 hrs following the challenge. Serum pentraxin 3 (PTX3) concentrations were determined by ELISA (PTX3 DuoSet® ELISA - R&D Systems, Minneapolis, MN).

2.10. Serum transfer

Sera were collected from 3 *trxA* i.p. vaccinated and 3 mock i.p. vaccinated mice one week post booster. For serum transfer, 2 groups of 6 mice received a 200µl bolus i.p. of either immune or mock serum diluted 1:5. Twelve hours later, the mice received another 200µl bolus of serum, followed one hour later with WT challenge i.p. of 5×10^6 CFU/mouse. Twelve hours post challenge, a final 200µl bolus of serum was administered. The mice were checked for morbidity and mortality for 21 days following the challenge.

2.11. Statistics

Statistical differences were assessed by one-way ANOVA with Holm-Sidak correction for multiple comparisons or Welch *t* test. Statistical differences in survival between challenge groups were determined by Mantel-Cox log rank test. All statistics were performed using GraphPad Prism statistical software.

3. Results

3.1. The *trxA* strain is attenuated in virulence

Previous work in our lab has shown that Ci79 injected i.p. has an LD₅₀ of 5×10^5 CFU/mL and causes severe sepsis and pathology in mice [27]. The *trxA* mutant strain, derived from Ci79 by deleting the gene encoding thioredoxin-A, exhibited notable phenotypic differences from the WT strain, including smaller colony sizes on LB agar plates and a slower *in vitro* growth rate. To determine whether TrxA contributes to bacterial virulence during infection, we challenged C57BL/6 mice (n=6 per group) i.p. with WT Ci79 strain (doses from 10^4 to 10^7 CFU) or *trxA* (from 10^5 to 10^9 CFU) and monitored the mice for survival for 4 weeks. The LD₅₀ of *trxA* was determined to be approximately 3.5×10^7 CFU/mouse, almost 100 fold higher than the LD₅₀ of the WT, indicating the *trxA* mutant strain was attenuated in virulence.

3.2. Survivors of primary systemic *trxA* challenge mounted protective immunity against secondary otherwise lethal Ci79 challenge

To assess whether protective immunity can be induced in *trxA* infected mice against WT MDR *A baumannii* infection, we injected mice i.p. with 2×10^5 CFU of *trxA* at two weeks apart and then two weeks later challenged these mice i.p. with WT at 2 and 10 LD₅₀ (1×10^6 and 5×10^6 CFU, respectively). Replication of the *trxA* vaccine strain in major organs (liver, spleen, and kidneys) after i.p. injection was minimal and, by 72 hrs, there was no detectable *trxA* in the blood and organs (data not shown). With consideration of plausible poor priming, a second dose was given to boost immunity. Mice vaccinated with *trxA* produced marked *anti-trxA* (end point titer mean=3340) as well as anti-WT Ci79

(mean=2560) antibody responses 14 days post vaccination (Fig. 1A). These antibody titers were elevated at day 28 (mean=6080 and 11520 for *anti-trxA* and anti-Ci79, respectively, Fig 1A). We also isotyped the serum antibody reactive with WT Ci79. Surprisingly, anti-Ci79 serum antibodies generated by *trxA* vaccinated mice exhibited little class switching to the IgG isotype, and IgM was the predominate antibody type present both at 14 and 28 days post vaccination (Fig. 1B). While the humoral response was robust in *trxA* vaccinated mice, cell-mediated immunity against *A. baumannii* was subdued as shown in splenocyte cytokine recall assays (Fig. 1C). Splenocytes, collected from *trxA* vaccinated and PBS mock treated mice at day 28 post-vaccination, were stimulated for 72 hrs with UV-inactivated Ci79 or other stimuli for positive (Con A) or negative (media and BSA) controls and concentrations of IL-4, IFN- γ , and IL-17 (surrogate/effector cytokines for bacterial-defensing Th2, Th1, and Th17 immunity, respectively) in the culture supernatants were measured. As shown in Fig. 1C, splenocytes from both PBS and *trxA* vaccinated mice were responsive to mitogen ConA stimulation resulting in high level IL-4, IFN- γ , and IL-17; however, there was minimal secretion of these cytokines by splenocytes that were stimulated with Ci79, even those from *trxA* vaccinated mice. These data suggest that T cell involvement may be limited in the protective immune response elicited by the *trxA* vaccine. While IL-17 induction was low, it was significantly higher in *trxA* than PBS primed splenocytes.

To evaluate whether *trxA* vaccination protects against *Acinetobacter* systemic infection, vaccinated or PBS treated mice were challenged i.p. with 2 or 10 LD₅₀ of WT Ci79 2 weeks after the final boost. While all PBS treated mice succumbed to both challenges within 72 hr (Fig. 2A), all *trxA* vaccinated mice were protected against the WT challenge even at the high 10 LD₅₀ dose. The protection was associated with rapid bacterial clearance as shown in Fig. 2B. The presence of Ci79 in the liver, spleen and kidney of mock (PBS) vaccinated WT challenged mice was evident at 24 hr post challenge; in contrast, Ci79 was not detectable in the organs of challenged *trxA* vaccinated mice (Fig. 2B) indicating that vaccination prevented dissemination and decreased organ burdens following lethal challenge. Our previous studies suggested that up-regulation of serum PTX 3 is an indicator of *A. baumannii* severe infection and sepsis [27]. PTX 3 is a protein involved in innate immunity that is stored and produced by neutrophils, macrophages, and myeloid dendritic cells in response to recognition of microbes and various inflammatory signals [28]. As expected, PTX 3 was minimally present in blood prior to Ci79 challenge, however, 24 hrs after challenge, serum PTX 3 concentrations were elevated to around 3000 ng/mL in the mock vaccinated mice, but did not rise above basal levels in the *trxA* vaccinated mice (Fig. 2C). These results imply that vaccination of mice with *trxA* prevents a severe infection and sepsis following the lethal *Acinetobacter* challenge.

We previously observed that infection with *A. baumannii* Ci79 causes severe pathology in the spleen and liver of mice 24 hrs post challenge [27]. To determine whether *trxA* vaccination could prevent organ pathology, we collected livers and spleens from mock and *trxA* vaccinated mice at 24 hrs post Ci79 challenge (10 LD₅₀). Tissues were embedded (paraffin), sectioned, stained (Hematoxylin and Eosin), and scored based on visible micropathology [27]. In the liver (Fig. 3, upper 2 panels), infected mock vaccinated mice exhibited infiltration of white blood cells (black arrows), especially neutrophils, in hepatic

veins and microvessels which in some cases was extensive, along with extensive vascular congestion (black arrowheads), hepatocellular necrosis and degeneration (pathology score = 8.3 ± 2.1). In contrast, WT challenged *trxA* vaccinated mice showed minimal pathology in the liver similar to unchallenged naïve mice (both pathology scores = 1.5 ± 0.5).

Additionally, marked differences in histopathology were observed in the spleens of WT challenged mock and *trxA* vaccinated mice. In spleen sections (Fig. 3 lower 2 panels), WT challenged mock vaccinated mice exhibited significantly more pathology (Score = 13 ± 2.1) than challenged *trxA* vaccinated (score = 4.5 ± 0.8) and naïve (score = 4.4 ± 0.3) mice with loss of white pulp structure, cell death and lymphocytolysis (white arrows), and red pulp congestion and hemorrhage (white arrowheads). Thus, there was little sign of pathology in WT challenged *trxA* vaccinated mice suggesting *trxA* vaccination not only prolonged survival but prevented the development of severe pathology in the spleen and liver following WT challenge.

3.3. Subcutaneous vaccination with *trxA* protected mice against systemic *Acinetobacter* infection

Since a lack of robust cell-mediated immune response was observed in mice vaccinated with *trxA* by the i.p. route, we investigated whether an alternative vaccination route would induce a similar host response while still providing protection. We vaccinated mice (n=10 per group) subcutaneously (s.c.) with *trxA* using the same dose (2×10^5 CFU/mL) and regimen as the i.p. vaccination experiments, with a PBS (mock) vaccinated group as a control. Similar to results of i.p. vaccination, immunization of mice with *trxA* by the s.c. route induced robust anti-Ci79 antibody production at days 14 and 28 post vaccination with predominant IgM isotype (Fig. 4A), and minimal cytokine induction in splenocyte recall assays (data not shown). Subcutaneous *trxA* vaccination also provided excellent protection (90% survival rate, Fig. 4B) against WT Ci79 challenge (10 LD₅₀, i.p.) compared to mock vaccination (10% survival).

3.4. Passive immune-serum transfer protects recipient naïve mice against systemic *Acinetobacter* infection

Since we observed that the host humoral defense against *Acinetobacter* infection appeared to be more critical than cell-mediated, we investigated the protective efficacy of immune sera induced by *trxA* vaccination. Each naïve mouse received 3 serum transfers (-25 and -13 hrs prior to and 12 hrs post WT Ci79 challenge) with sera collected from either PBS mock vaccinated or *trxA* vaccinated (immune serum, either heat-inactivated or untreated) mice. Naïve mice without serum transfer, but challenged with 10 LD₅₀ of Ci79, also were used as a control. As shown in Fig. 5A, 100% of mice that received sera from *trxA* vaccinated mice were able to survive a 10 LD₅₀ lethal challenge dose of WT, in contrast to 33% of mice that received sera from mock vaccinated mice and 16.7% of mice without serum transfer. There was no difference in survival between mice receiving heat-inactivated and untreated immune sera suggesting complement may not play an essential role in *trxA* mediated protection against WT Ci79 challenge. Collectively, these results strongly suggest that the antibody response is indispensable in the *trxA*-mediated protection of mice against a lethal challenge by *A. baumannii*.

Cross reactivity of *trxA* antisera against different clinical isolates—*Anti-Acinetobacter* antibody generated by *trxA* seems to be an important immune component for protection against *A. baumannii* infection. Thus, we investigated whether *trxA* (derived from Ci79 strain) vaccination induced antibodies that reacted with other clinical *Acinetobacter* isolates (Table 1). As shown in Fig. 5B, anti-*trxA* immune sera reacted with other MDR clinical isolates, particularly those (Ci79, Ci86, and Ci240) inducing high levels of PTX 3 (indicative of high virulence in our mouse model of *Acinetobacter* infection [27]).

4. Discussion

Multi-drug resistant *Acinetobacter baumannii* (MDR-Ab) is quickly becoming a significant worldwide concern for both civilian and military personnel. In 2013 the CDC classified MDR-Ab as one of the greatest emerging threats in the US due to the prevalence of multi-drug resistance emerging in the population [29], while the World Health Organization has recently listed it as the number one global “Priority 1: Critical” pathogen for R&D of new antibiotics for treatment [13]. Furthermore, with the continued use of antibiotics, multi-, extensively-, and pan-drug resistant strains have begun to emerge [30] making therapy difficult and necessitating the need for alternative treatment options. Numerous antibiotic alternatives have been described including blue-light therapy [31, 32], nanoparticle [33, 34] and vaccine [13–17] therapies. While all of these proposed therapies have shown promise in animal models, vaccination offers long term prevention and can be administered to individuals living in or visiting endemic areas. In this study, we demonstrated that *A. baumannii* lacking the *TrxA* gene was greatly reduced in virulence in mice challenged systemically. Additionally, we observed a robust protection against systemic WT *A. baumannii* infection *via* a predominantly antibody mediated immunity. Specifically, *trxA* vaccination stimulates a robust antibody response with a minimal T-cell mediated response irrespective of vaccination route. Although our studies indicated establishment of a protective memory response, antibody isotyping revealed nearly all antibody produced in response to *trxA* vaccination was of the IgM isotype. The protective role of antigen-specific IgM against bacterial infection has been documented. For example, del Barrio et.al. [35], showed a marked reduction of antigen-specific IgM in IL-1b^{-/-} compared to WT C57BL/6 mice, while total IgM levels remained the same at day 7 post *Francisella tularensis* (Ft) LVS strain challenge. Passive transfer of day 7 WT-immune serum, but not IL-1b^{-/-} or IgM-depleted WT immune-serum, protected the recipient mice against lethal LVS challenge suggesting protection was mainly mediated by Ft-specific IgM generated in an IL-1-dependent manner. Further studies revealed that B1a B cells were the major source of the anti-LVS LPS IgM which efficiently agglutinated the bacterium and promoted phagocytosis [35]. In an earlier study, immunization with purified Ft LPS caused expansion of B1a B cells producing a T-cell independent antibody response which provided long-term (at least 70 days) protection against lethal LVS challenge [36]. This long-term protection was correlated with a rapid increase of Ft-LPS B-1a cells in the peritoneal cavity which remained substantially above background for at least 2 months [36]. Furthermore, Yamamoto et.al. [37], demonstrated that a subset of peritoneal cavity originating B1a cells, which secreted higher affinity antigen-specific IgM, was essential for mediating early protection against pneumococci. These prior studies suggest a possible mechanism for protection against

otherwise lethal WT *A. baumannii* via vaccination with the *trxA* mutant in our studies. However, actual proof of whether protection induced by *trxA* vaccination is primarily mediated through B1a B cell-generated antigen-specific IgM and the length of protection remains to be elucidated. Nevertheless, the protective immunity is clearly antibody mediated as evidenced by the ability to passively transfer protection to naïve mice using only serum from previously vaccinated mice. Additionally, we observed antibody cross-reactivity with other MDR-Ab isolates by ELISA using immune serum from *trxA* vaccinated mice suggesting that immunity provided by vaccination with this mutant may provide protection against multiple *A. baumannii* clinical isolates.

In the development of an attenuated live bacterial vaccine for human use, it is highly desirable to construct the strain with multiple gene deletions to prevent spontaneous virulence reversion. Results from this study clearly demonstrate that TrxA of *A. baumannii* is a virulence factor and a target gene suitable for future “multi-gene deletion” vaccine development against this emerging MDR bacterium. It also should be noted that we tested vaccine efficacy using a sepsis model; however, pneumonia is another leading cause of mortality produced by *Acinetobacter* infection [38]. Thus, additional evaluation of candidate vaccines using animal models of pulmonary infection [39] are important towards success in vaccine development for this major MDR pathogen.

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Highlights

- A *trxA* mutant of *Acinetobacter baumannii* was evaluated as a putative vaccine
- The *trxA* attenuated vaccine is protective against *Acinetobacter baumannii* sepsis
- *trxA* vaccination reduces organ bacterial burden and prevents pathology
- Passive vaccination with *trxA*-immune sera modulates *Acinetobacter* sepsis

Fig. 1.

trxA vaccination induces robust humoral responses but minimal splenic cell-mediated immunity. C57BL6 mice ($n = 13$) were injected i.p. with 2×10^5 CFU of trxA and received a booster injection 2 weeks later. Mice were then rested for 2 weeks before challenge with *A. baumannii* WT Ci79. For assessing humoral responses, bloods ($n = 10$) were collected on days 14 and 28 after initial vaccination and serum antibody reactivity against UV-inactivated trxA and Ci79 were determined. Endpoint titers were calculated and presented as a box-and-whisker plot for total anti- trxA and Ci79 antibodies (A) and anti-Ci79 isotypes (B). For assessing cell-mediated immune responses (C), spleens ($n = 3$) were collected 14 days after the booster and single spleenocytes were made and stimulated with UV inactivated Ci79, ConA (mitogen, positive control), BSA (unrelated antigen control) or unstimulated (media) for 72 hrs. Levels of cytokines IL-4, IFN- γ and IL-17 produced by spleenocytes were measured in culture supernatants by ELISA. * $p < 0.05$. (Representative of two independent experiments).

Fig. 2.

trxA vaccinated mice are protected against systemic WT challenge. C57BL/6 mice ($n = 10$) received a primary and a booster dose of *trxA* (2×10^5 CFU) or PBS alone (mock control) at 2 weeks apart via i.p. injection. Mice were rested for 2 weeks and then challenged (i.p.) with either 2 or 10 LD₅₀ of the WT Ci79 strain. (A) Survival of the mice was monitored for 4 weeks. * $p < 0.05$ (Mantel-Cox log rank test), comparing WT challenged *trxA* vaccinated to PBS treated groups. (Representative of three independent experiments). In addition, (B) spleens, livers, and kidneys were collected from *trxA* or mock vaccinated mice ($n=3$ per

group) 24 hrs after i.p. WT Ci79 challenge (10 LD₅₀). Tissues were homogenized and bacteria were enumerated by dilution plating on LB agar containing chloramphenicol. (C) Sera were collected from mice 1 hour before and 24 hrs after Ci79 challenge and PTX 3 concentrations were assessed using a PTX 3 ELISA kit. (Representative of two independent experiments).

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Fig. 3.

Vaccination with *trxA* reduced organ pathology following WT Ci79 challenge.

Representative pathology (H&E staining) associated with *A. baumannii* challenge of mock and *trxA* mutant vaccinated mice. A. Liver 100X: Infected mock vaccinated animals show inflammatory infiltrates (arrows) and congestion of vessels (arrowheads), whereas livers from infected *trxA* mutant vaccinated animals were similar to naïve mice; inserts show hepatic veins. B. Liver: Marked inflammatory infiltrates (arrows) and congestion (arrowheads) in hepatic veins and microvessels (MV) of infected mock mice (400X). In contrast, similar histological features (100X) without significant pathology were observed

with both naïve and infected *trxA* vaccinated mice. C. Spleen 40X: Disruption of the white pulp (WP) in infected mock vaccinated animals; similar appearance of naïve and infected *trxA* vaccinated mouse spleens. D. Spleen 100X: Greater lymphocyte depletion, or clear areas indicative of cell death, (white arrows) in the WP and hemorrhage in the red pulp (white arrowheads) of infected mock, compared to *trxA* vaccinated animals; inserts 200X. (Representative of groups of 3 mice from two independent experiments).

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Fig. 4.

Subcutaneous immunization with *trxA* also induces a robust humoral response and protects mice against a lethal WT Ci79 challenge. C57BL6 mice (n=10 per group) were injected s.c. with 2×10^5 CFU of *trxA* and received a booster injection 2 weeks later. Mice were then rested for 2 weeks before challenge with *A. baumannii* Ci79 strain. (A) Bloods were collected on days 14 and 28 after initial vaccination and serum antibody reactivity against UV-inactivated *trxA* and WT Ci79 were determined. Endpoint titers were calculated and presented as a box-and-whisker plot for total and IgM anti-Ci79 antibodies. (B) Mice were

monitored for mortality for 4 weeks. The difference in survival between WT challenged trxA and mock vaccinated mice is significant ($p < 0.05$, Mantel-Cox log rank test). (Representative of two independent experiments).

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Fig. 5.

Passive transfer of *trxA*-immune serum protects mice against systemic WT *A. baumannii* Ci79 challenge. (A) Naïve mice (n=6 per group) were i.p. injected with serum (heat-inactivated or untreated) from *trxA* or mock vaccinated mice at -25 and -13 hrs prior to and +12 hrs post Ci79 (10 LD₅₀) i.p. challenge. Naïve mice without serum transfer were used as a control. All animals were monitored daily for survival for 4 weeks. **p* < 0.05 (Mantel-Cox log rank test), comparing *trxA* immune serum transfer group to PBS none-immune serum and no serum transfer treated groups. (B) *trxA* antisera cross reacts with

various MDR *A baumannii* clinical isolates. Sera from *trxA* immunized mice (n =6) were serially diluted and reacted with various clinical isolates. Endpoint total antibody titers were calculated and presented as individual dots for each serum. (Representative of two independent experiments).

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Table 1*Acinetobacter* strains used in this study

Isolate	Origin ^a	Source	PTX3 induction ^b	High molecular weight (>200 kDa) polysaccharide ^c
Ci28	OIF	Blood	+	+++
Ci77	OIF	Superficial wound	+	+++
Ci79	OIF	Respiratory	+++	+
Ci86	OIF	Superficial wound	+++	+
Ci240	OEF	Respiratory	+++	+

^a OIF, Operation Iraqi Freedom (Iraq); OEF, Operation Enduring Freedom (Afghanistan).

^b PTX3 production in J774 macrophage cells at 24 hrs post bacterial challenge (+, less than 250 pg/ml; +++, greater than 750 pg/ml PTX3).

^c Assessed by glyco-stain of whole bacterial SDS extract on SDS-polyacrylamide gels (+, minimal glyco-stain; +++ very strong glyco-stain).